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Investigations into soil microbiome stability in the face of agriculture and climate change related perturbations

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Doctor of Philosophy

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Declaration of Authorship

I, Camilla Thorn, declare that this thesis entitled:

Investigations into soil microbiome stability in the face of agriculture and climate change related perturbations

and the work presented in it are my own and have been generated by me as a result of my own original research.

I confirm that:

- ➤ This work was done wholly or mainly while in candidature for a research degree at this University
- ➤ Where I have consulted the published work of others this is always clearly attributed
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- ➤ With the exception of such quotations, this thesis is my own work
- ➤ I have acknowledged all main sources of help
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Signed:

Date: 31st of October 2017

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Abstract

Soils and the vital ecosystem services performed by their indigenous microbiota are under increasing pressure from anthropogenic stresses and climate change. In an effort to understand how the provision of soil ecosystem services will evolve with such pressures, significant research has been undertaken into the stability of microbial community functioning and composition, in terms of resistance and resilience. The overall aim of this thesis was to contribute to such knowledge through investigation of the responses of soil microbial communities subjected to a variety of perturbations relevant in the context of agriculture and climate change. These include lime addition, slurry application and flooding, which were chosen to include representative disturbances commonly encountered by agrarian soil ecosystems. As metaproteomics represents a valuable tool for linking microbial phylogeny and function, the first step was to develop a method allowing for the co-extraction of DNA, RNA and proteins from soil samples. We then employed culture-independent techniques to investigate the microbial community composition in the face of the three perturbations. In addition, we assessed the functioning of the microbial communities by assessing rates of litter decomposition, potential nitrification and potential denitrification. While the lime product did not perform as hoped in terms of pH alteration, microbial communities responded strongly to the development of the barley plants with which they were associated. Slurry induced significant functional and compositional alterations in the microbial assemblage; while some functions were transiently affected, others remained altered 140 days post-application. While the soil microbiome was sensitive to flooding, it appeared remarkably resilient in terms of functioning. When the flooding perturbation was compounded by a preceding slurry

application, improvements in resistance were seen in functional assays. In closing, a co-extraction method was developed which is easily employed for a number of sample types including soil. Additionally, we addressed certain hypotheses regarding microbial community stability while contributing to advancing knowledge in the field.

--------- Chapter I ---------

Introduction

1.1 Soil microbial communities and ecosystem services

Soils are home to a diverse range of microorganisms, with estimates of species richness ranging from $4 \cdot 10^3$ (Schloss & Handelsman 2006) to $2 \cdot 10^5$ (Roesch *et al.* 2007) per gram of soil. These complex microbial communities play a central role in global soil ecosystems and thus contribute to the sustained functioning of our planet (Barrios 2007; Wall *et al.* 2012). Due to the benefits humanity derives from the provision of such ecosystems functions, they are collectively referred to as ecosystems services (Daily 1997). A primary soil ecosystem service is geochemical cycling, of which the microbial consortia of the soil are major drivers, due to their role in the decomposition of organic matter, in addition to the elemental transformations they perform (Coleman *et al.* 2004). Indeed, biological transformations of the major elements, including carbon, nitrogen, sulphur, oxygen and hydrogen are principally mediated by microbes (Falkowski *et al.* 2008). As well as the production of extracellular enzymes for the decomposition of larger organic molecules (Sinsabaugh 1994), soil microbes also secrete extracellular polysaccharides which can promote the formation of soil aggregates (Holden 2011) whose stability is further enhanced by fungal hyphae (Tisdall & Oades 1982). This serves to improve soil structure thereby enhancing moisture retention (Augé *et al.* 2001; Hamblin 1986) in addition to contributing to carbon sequestration (Trivedi *et al.* 2017). These processes, among a plethora of others, are then linked to the productivity of the crops we rely on for food, energy and fuel, thereby providing yet another ecosystems service (van der Heijden *et al.* 2008). Such processes include the symbiotic relationships of mycorrhizal fungi, which can form associations with a diverse range of terrestrial plants including, for example, around 80 % of angiosperms (Harrison 2005), where they aid in water and nutrient acquisition (Dighton 2016). Also well-characterised are the symbiotic interactions

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between rhizobia, who fix atmospheric nitrogen into plant available forms for their leguminous hosts, in return for photosynthetically derived carbon sources (Spaink 2000). Non-symbiotic microbes also aid in plant nutrient acquisition through many of the paths associated with biogeochemical cycling. For example, complex, organic forms of nitrogen which are unavailable for direct plant uptake are converted by the process of mineralization to inorganic forms of ammonium, which in turn are converted to plant available nitrates via the process of nitrification (Hart *et al.* 1994; Schmidt 1982). Additionally, plants have been shown capable of recruiting specific microbial groups from the bulk soil in order to enhance defence from pathogens, as reviewed by Berendsen *et al.* (2012). These processes of symbiosis and defence from pathogens both involve complex modulations of plant immunity by the soil microbiota, thus creating a delicate balance between the plant hosts and their associated microflora (Zamioudis & Pieterse 2012). Indeed, the relationship between plants and their microbiota is frequently paralleled with that of humans and their microbiome (Hacquard & Schadt 2015; Herrera Paredes & Lebeis 2016; Zamioudis & Pieterse 2012) which has led to the emergence of a tenet whereby plants and their associated microbes can be considered collectively, as a holobiont (Vandenkoornhuyse *et al.* 2015; Sánchez-Cañizares *et al.* 2017).

All global ecosystems are under increasing pressure from the continued expansion of the human population (Richardson & Poloczanska 2008), with soil ecosystems impacted by intensification of agriculture (Giller *et al.* 1997; Trivedi *et al.* 2016), which exacerbates soil erosion (Pimentel 2006), and pollution-associated deposition of nitrogen (Adams 2003), among others (Turbé *et al.* 2010). Soil ecosystems are also subject to current and continuing climatic shifts such as changing moisture regimes, temperatures and atmospheric CO_2 levels (Classen *et al.* 2015; Walther *et al.* 2002).

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Pressure from anthropogenic activity, resulting in loss of biodiversity, and the potential impacts this has on ecosystem functioning have been well characterised in the plant and animal context, as reviewed by Hooper *et al.* (2005) and Cardinale *et al.* (2012). However, despite comprising a significant proportion of the Earth's biomass (Whitman *et al.* 1998) and contributing significantly to ecosystems services (Barrios 2007), the implication of such stresses on prokaryotic communities is less well characterised. This, however, is no mean feat.

The functioning of soil, and indeed other microbial communities is the consequence of a multitude of interactions which collectively contribute to ecosystems functioning (Prosser 2012). Investigating and interpreting these interactions is a complex undertaking, partly as a result of insufficient techniques or approaches (Abreu & Taga 2016), but also due to the remarkable diversity of the microbes inhabiting the soil (Allison & Martiny 2008). The activity and community composition of this microbial consortium is in turn inextricably linked to environmental variables (Graham *et al.* 2016), most of which alter seasonally (Lv *et al.* 2016). As well as such quantifiable, deterministic variables, microbial communities are also subject to stochastic change (Graham & Stegen 2017; Stegen *et al.* 2012). Together these serve to complicate modelling these microbial communities for their inclusion in global ecosystems models, the overall aim of which is to assess their vulnerability in the face of anthropogenically exacerbated climate change (Allison & Martiny 2008). Exploring microbial community stability, in terms of resistance (whether they can withstand a disturbance) and resilience (their recovery from a disturbance), is a frame-work by which to investigate how communities are affected by a range of perturbations (Griffiths & Philippot 2013).

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1.2 Soil microbiome responses to disturbances

Diverse research has been undertaken into the stability of soil microbial communities in the face of a range of disturbances including, but not limited to, drought (de Vries *et al.* 2012), drying and re-wetting (Barnard *et al.* 2013), chemical contaminants (Girvan *et al.* 2005), heat (Kuan *et al.* 2007), salinity (Berga *et al.* 2012), fire (Lee *et al.* 2017) and flooding (González Macé *et al.* 2016). Comparing a 'perturbed' sample to a 'control' (unperturbed) sample allows for the impact of a disturbance to be assessed in terms of the resistance and/or resilience of functional traits and microbial community structure (Wallenstein & Hall 2012). In other words, we can begin to try and unpick how disturbance-related changes in soil processes might be related to changes in microbial community composition or richness, if at all (Nemergut *et al.* 2014; Prosser 2012). Given the large diversity of the soil microbial assemblage, investigating such compositional changes can be somewhat streamlined by classifying microorganisms into 'ecologically meaningful' phylotypes (Fierer 2017). For example, Fierer *et al.* (2007) demonstrated that certain bacterial phylotypes responded in a consistent manner to increasing labile carbon inputs. Some phylotypes responded positively and others negatively, thus allowing for their classification into the K- and r-strategist nomenclature introduced by MacArthur & Wilson (1967) or, perhaps more appropriately for microorganisms, as oligotrophs or copiotrophs (Koch 2001). This approach was further supported by Ramirez *et al.* (2012) who demonstrated that certain bacterial phylotypes responded to the addition of inorganic forms of nitrogen in a consistent manner, across a range of 28 different soils. Similar trends were seen in further experiments based at 25 field sites distributed throughout the African, Australian, European and North American continents, allowing the binning of further bacterial groups into the oligotroph or copiotroph categories. Among the phenotypic

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traits associated with oligotrophy are slower growth rates (Schut *et al.* 1997), which have frequently been associated with reduced copy numbers of ribosomal RNAs (Klappenbach *et al.* 2000; Vieira-Silva & Rocha 2010). Thus, information such as the mean rRNA copy number of a particular taxonomic group, as provided by curated databases such as the rrnaDB (Stoddard *et al.* 2015), can be used as a further tool to investigate microbial phylotypes as classified in terms of their growth rates and efficiencies (Roller *et al.* 2016). As well as these rRNA 'related' traits, there are a diverse range of functional traits by which microbial ecosystems can be investigated (Green *et al.* 2008). Such traits can include the activity of specific enzymes associated with nutrient cycling, for example phosphatases (Margalef *et al.* 2017), cellulases (Chaer *et al.* 2009), xylanases and proteases (Kandeler *et al.* 1999). Or they can include assessing a range of enzymes which collectively facilitate conversion of one product to another, for example the conversion of organic, nitrogen containing compounds to inorganic nitrogen forms via mineralisation (Griffiths *et al.* 2000) or the conversion of ammonium to more plant-available forms via ammonia oxidation (Pett-Ridge *et al.* 2013) and nitrite oxidation (Le Roux *et al.* 2016). While the aforementioned traits have direct impacts upon ecosystems processes, there are also characteristics that are likely equally important in a more indirect manner, as they confer a competitive advantage under certain conditions. For example, traits that provide advantages in the highly competitive rhizosphere, such as antibiotic production (Wiener 1996), or those conferring improved survival or nutrient acquisition under varying moisture conditions such as motility (Hibbing *et al.* 2010) and biofilm formation (Lennon & Lehmkuhl 2016). While many of these traits can be assessed experimentally (as detailed within each of the referenced articles), the introduction of 'trait' databases is allowing for the assessment of genomic (Weimann *et al.* 2016) and metagenomic, or even 16S rRNA

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survey, datasets (Barberán *et al.* 2017) in terms of phylogenetically conserved traits (Goberna & Verdú 2016; Martiny *et al.* 2015). Although of course such tools only assess the potential, not the actuated trait.

Wallenstein and Hall (2012) proposed that it is through the investigation of traits in response to perturbations that we can begin to understand how changes in climate might alter microbially-driven processes of geochemical cycling. The article of de Vries and Shade (2013) further consolidates these ideas into hypotheses regarding the stability of microbial communities in the face of perturbations associated with climate change. First, the authors hypothesise that microbial community stability will be affected by the proportion of K- to r-strategists, with improved resistance associated with increased ratios of resource-efficient, oligotrophs to more inefficient copiotrophs, while the inverse ratio would allow for more effective resilience, thank to enhanced growth rates of copiotrophs (de Vries & Shade 2013). An additional hypothesis from their article mirrors that of Griffiths and Philippot (2013) and Wallenstein and Hall (2012), where it is proposed that the resilience of a microbial community after a perturbation will be affected by resource availability, with resilience being constrained in ecosystems were labile resources are rare. This has been demonstrated in the context of plants, whereby improved resilience following a disturbance was seen in soils with plant cover versus those without, likely a consequence of plant exudates improving resources availability (de Vries *et al.* 2012). Orwin & Wardle (2005) observed a similar trend of increased resilience in planted systems, however they also noted that different microbial functions responded in varying ways to their treatments. This has been observed in a number of experiments, and is likely partly a consequence of the variety of microbes performing a particular function, and thus of microbial diversity (Loreau *et al.* 2002). Litter decomposition is often regarded as a 'broad scale' function

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as it is a process performed by a diverse range of microbiota (Andrén *et al.* 1995). It has been proposed that more specialised microbial functions (less redundant across taxa) are impacted to a greater degree than 'broad scale' functions (more redundant across taxa) by disturbances (Griffiths *et al.* 2000), and that biodiversity is therefore important for ecosystem function and stability. This has been tested in a number of experiments where soil microbial diversity was manipulated for example by fumigation (Griffiths *et al.* 2000) or using the dilution to extinction approach (Wertz *et al.* 2006) and mostly demonstrated that indeed specialised functions were impacted to a greater extent than broad scale functions. Examples include reduced rates of nitrite oxidation versus stable rates of denitrification (Wertz *et al.* 2007) and compromised rates of xenobiotic degradation versus stable rates of litter decomposition (Girvan *et al.* 2005). In fact, Delgado-Baquerizo *et al.* (2016), in an effort to determine the relationship between microbial diversity and ecosystem functioning, observed reductions in both broad and fine scale functions in seven different freshwater ecosystems after reducing microbial species richness (but accounting for different population sizes), thereby calling into question the role of functional redundancy in these systems. In soil microcosms there have also been contrasting results, where Philippot *et al.* (2013) demonstrated that reduced diversity resulted in significantly lower rates of denitrification, which was somewhat ameliorated by the incorporation of litter. Therein the authors note that responses to diversity loss are likely affected by microbial community evenness as well as composition (Wittebolle *et al.* 2009), and that the responses further be modified by resource availability (Philippot *et al.* 2013).

1.3 Soil microbiome responses to disturbances: soil management practices

As noted by many of the referenced articles, agrarian soils in particular are under increasing pressure from intensified agricultural practices (Trivedi *et al.* 2016) as well as climatic changes (Classen *et al.* 2015). The detrimental effects of conventional agriculture upon soil biological quality can be a result of any number of practices including but not limited to tillage, inorganic fertilizer and pesticide application (Dick 1992). Roesch *et al.* (2007) demonstrated that despite harbouring more cells, agricultural soils had reduced number of bacterial phyla present, compared to a forest soil, and the consequences of these in terms of ecosystems services warrants further investigation.

Much of the soil throughout Ireland, due to its acidic nature, requires amendment with lime or other alkali products in order to provide soil at an optimum pH for crops. Sub-optimal pH has severe implications for crops including slow growth, limited productivity and increased susceptibility to disease (Moore *et al.* 1998). Much of this is a consequence of limited plant availability of essential nutrients such nitrogen, phosphorous, potassium and sulphur whose availability is typically optimal at neutral pH (Truog 1947). Additionally, acidic soils allow for the solubilisation of certain elements, in particular aluminium, which then poses toxicity problems for plants growing in these soils (Sumner *et al.* 1991). Plants range in their sensitivities to pH, which equates to an optimum pH range within which the maximum crop yields can be achieved (Fageria *et al.* 1990; Goulding 2016).

As in the plant kingdom, soil pH has significant effects on the soil microbiota, and thus on ecosystems processes. For example, acidic soils often limit biological processes such as nitrogen fixation, with root nodulation significantly hampered in soils with a

pH below 5.5 (Bordeleau & Prévost 1994). Booth *et al.* (2005) demonstrated that nitrification rates on the other hand, do not appear to correlate with soil pH. Despite this, ammonia oxidisers, even those isolated from acidic soils, were for some time unculturable in media with a pH lower than 6.5 (Prosser 1990). Using culture-independent techniques, Nicol *et al.* (2008) revealed that archaeal and bacterial groups driving ammonia oxidation respond contrastingly to soil pH, with ammonia oxidising bacteria (AOB) being more suited to neutral or alkali pH, while their archaeal counterparts appear more adapted to performing ammonia oxidation in acidic soils. The isolation of an acidophilic ammonia oxidising archaea (AOA) that was culturable at pH 4, seemed to support this finding and represented the first ammonia oxidiser cultivable at acidic pH (Lehtovirta-Morley *et al.* 2011). Further work into the niche specialisation of AOA and AOB suggests that in soils with a pH below 5.5, AOA do indeed seem to drive nitrification, however, aside from this, ratios of AOA to AOB cannot be explained simply by pH alone (Prosser & Nicol 2012) as they are a product of interacting factors including ammonium concentration (Verhamme *et al.* 2011), temperature (Tourna *et al.* 2008) and soil type (Chen *et al.* 2010). As well as microbial species performing specialised functions, broader taxonomic guilds are also influenced by pH, for example, the ratio of fungi to bacteria has been shown to increase with decreasing pH (Bååth & Anderson 2003). In addition, bacterial diversity and abundance have been shown to increase with increasing pH (Rousk & Jones 2010). Large scale biogeographical surveys have also identified pH as having a strong influence on the total microbial community composition across different soils (Griffiths *et al.* 2011; Lauber *et al.* 2009). These studies both found increased relative abundances of Acidobacteria at lower pH, where this phylotype constituted as much as 80 % of the communities in certain soils at pH 4 (Lauber *et al.* 2009). The

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Acidobacteria are resource-efficient (Fierer *et al.* 2007) and slow growing, with a mean rRNA copy number of 1.3 (Stoddard *et al.* 2015). Thus, when we consider the hypotheses regarding increased resistance associated with higher oligotrophs to copiotrophs ratio (de Vries & Shade 2013), the prevalence of Acidobacteria at low pH, has potential consequences for the stability of ecosystems processes following a disturbance in highly acidic soils.

In order to alter soil pH to be within the optimum range of the crop of interest, liming is performed, and this is traditionally achieved through the application of milled, mined rock rich in calcium carbonate, principally limestone (Truog 1947). With time, the milled granules weather and release calcium carbonate which helps to increase the pH of soils (Peters *et al.* 1996). In terms of lime application and its effects upon soil microbial communities, previous studies have demonstrated the resulting rise in pH caused increased soil bacterial biomass and activity (using respiration rates as proxy) (Fuentes *et al.* 2006; Kennedy *et al.* 2004; Neale *et al.* 1997). Additionally, lime application appeared to decrease species richness, when assessed using fingerprinting methods (TRFLP) (Kennedy *et al.* 2004). When the same experimental procedure was employed to investigate effects upon the fungal community however, there were no significant effects (Kennedy *et al.* 2005), and this corroborated other research where lime addition altered bacterial but not fungal communities when investigated using PLFA (Treonis *et al.* 2004). Change in pH as a result of liming has also been shown to affect ecosystems processes. For example, after increasing soil pH from 3.5 to 6.5 using lime, Neale *et al.* (1997) observed an immediate positive effect on nitrogen mineralization rates, which persisted for roughly 30 days, at which stage nitrification rates increased. When smaller alterations in pH are investigated, the effects appear to be less defined, for example Rooney *et al.* (2010) observed no change in the

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community structure of ammonia oxidising bacteria after lime application, perhaps as a consequence of the relatively small increase in pH from 4.2 to 4.9. Thus, it would appear that liming indeed effects microbial community structure, however a deeper characterisation of these compositional changes, using culture-independent techniques, are required to investigate how such changes might relate to plant productivity and ecosystems processes.

In addition to amendments for improving soil pH, agricultural soils are also frequently subject to inputs including inorganic forms of nitrogen, phosphorous and potassium, used to increase crop productivity. Organic amendments such as animal and plant wastes have been identified as more sustainable means of enhancing soil nutrient status, as reviewed by Diacono & Montemurro (2010). Such wastes typically increase soil organic matter content which in turn has a positive influence on water holding capacity and infiltration (Haynes & Naidu 1998). This is especially important for improving sustainability as conventionally farmed soils often have severely reduced soil organic matter content, typically resulting from the removal of crop residues (Komatsuzaki & Ohta 2007). The use of wastes, in particular animal faecal waste, on agricultural soil also represents a way of recovering valuable products, such as nitrogen and phosphorous, which would otherwise be lost from the value chain (Scholz *et al.* 2014). Therefore, land spreading represents a solution for the disposal of at least a portion of such wastes (Polprasert & Koottatep 2017), which are produced in huge quantities, for example, in Ireland approximately 30 million tons of cattle slurry are produced annually (Singh *et al.* 2010). Such amendments are not without their issues and care should be taken to categorise waste prior to soil application in order to avoid over-supply of nutrients and/or introduce pathogenic microbes to the soil (Cameron *et al.* 1997). Organic amendments also affect the soil microbiota, either through direct

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effects, such inputs of labile carbon forms, or indirect effects upon soil physico-chemical parameters (Drinkwater *et al.* 1995). Much research has demonstrated an overall improvement in the biological quality of soils that have been organically amended (Thiele-Bruhn *et al.* 2012; van Eekeren *et al.* 2009). For example, the addition of organic amendments has been shown to more than double microbial biomass carbon (Bastida *et al.* 2008) and biomass nitrogen (Marschner *et al.* 2003) relative to the un-amended control. These studies also both demonstrated increased enzyme activity in the organically amended soils. In addition to these assays, Bastida *et al.* (2008) and Marschner *et al.* (2003) also investigated the effects upon the microbial community structure using PLFA and observed increased ratios of Gram-positive to negative bacteria. In a similar study, when soils were amended with pig slurry, (Hammesfahr *et al.* 2008) observed the opposite trend, whereby Gram-negative bacteria were more abundant. As such amendments affect not only microbial community composition but also nutrient availability, which is proposed to affect resilience. Thus, amendments can then be considered for their effects upon microbial community stability when exposed to additional stress (de Vries & Shade 2013; Griffiths & Philippot 2013). The effect of organic amendments on microbial stability in the face of a drought event was investigated by Bastida *et al.* (2017), who concluded that, relative to the untreated control, the application of sludge or composted sludge increased the resistance of the microbial community. More specifically, increased microbial biomass and enzyme activities were seen despite 45 days of drought, at 20 % WHC, compared to un-amended controls at both 20 % and 60 % WHC, suggesting improved resistance as a result of the amendment (Bastida *et al.* 2017). A similar experiment by Hueso *et al.* (2012) revealed that manure amendment conferred larger microbial biomass during a subsequent drought stress. Additionally, the changes in

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microbial community composition were more pronounced in un-amended than manure amended soils (Hueso *et al.* 2012). With regards to increased precipitation, resource availability resulting from amendment with urea, potash and sulphur, also appeared to confer some resistance, in terms of enzyme activities and potential nitrification rates (Mentzer *et al.* 2006).

1.4 Soil microbiome responses to disturbances: climate change

One of the primary consequences of climate change in North Western Europe is increased incidences of heavy precipitation (European Environmental Agency 2017) and the Environmental Protection Agency has identified flooding as having an increasingly significant impact on Ireland (Desmond *et al.* 2017). When soils are subjected to waterlogged conditions, significant alterations in physico-chemical parameters result due to increasingly reducing conditions (in terms of redox potential) (de Campos *et al.* 2009; Patrick & Reddy 1976). Upon waterlogging, the first in a series of events leading to the lower redox state is the rapid consumption of the available oxygen by the resident aerobic, microflora (Reddy *et al.* 1984). Other compounds must then be used as electron acceptors and the first of these are typically nitrates, followed in order, by iron, manganese, sulphur and organic substances resulting in the depletion of dissolved oxygen within 2 to 36 hours (de Campos *et al.* 2009; Ponnamperuma 1972). The resulting redox conditions typically lead to changes in pH, where a trend toward neutrality is seen, and also to changes in cation exchange capacity (Kirk 2004; Ponnamperuma 1972) which has implications for nutrient availability within the soil (Hazelton & Murphy 2007). In the absence of oxygen, organic matter decomposition, including nitrogen mineralisation (Drury *et al.* 2003),

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proceeds at a much slower rate (Meurant 1984). While this may result in slower release of CO_2 via decomposition, methane production on the other hand is significantly increased in flooded soils (Davidson *et al* 2006; Frank *et al.* 2015). Additionally, rates of nitrification are constrained by the low oxygen availability, typically resulting in an accumulation of ammonium in flooded soils (Patrick & Reddy 1976). This process can still however proceed at a much-reduced rate thanks to oxygen diffusing from the water surface (Reddy *et al.* 1976), and indeed many nitrifying bacteria being capable of surviving in low oxygen conditions (Ward 2013). The anoxic conditions of flooded soils are typically associated with increased rates of denitrification, where nitrates are used as electron acceptors, resulting in the production of dinitrogen gas, or if the reaction is incomplete then to nitrous oxide (Knowles 1982) (Knowles 1982). These oxygen limited conditions also favour the pathway of dissimilatory nitrate reduction to ammonium (DNRA), resulting in competition for nitrates (Rubol *et al.* 2013; Sgouridis *et al.* 2011; Tiedje 1988).

As expected due the physico-chemical chemical alterations, and the favouring of different metabolic strategies, flooding has frequently been shown to be accompanied by changes in the soil microbial community. Using PLFA, Mentzer *et al.* (2006) observed improved survival of Gram-positive versus Gram-negative bacteria during flooding. Unger *et al.* (2009) also employed PLFA to investigate soil microbial community response to flooding and observed a similar trend of Gram-positive bacteria however only in soils exposed to moving floodwaters, not in static floodwaters. As Gram-positive bacteria have been proposed as typically more K-strategic due to their slower growth (de Vries & Shade 2013), this could have implications for the resilience of these soil microbiota after flood waters have abated. In other words, resilience will be slower in these systems due to the predominance of

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slower growing phylotypes (de Vries & Shade 2013). Most other research in the vein of altered precipitation regimes on the soil microbiota typically focuses on shorter fluxes for example in terms of carbon dioxide fluxes (Barnard *et al.* 2013) and methane fluxes (Ferré *et al.* 2012) from rice paddies. A significant amount of research has also been performed to investigate responses of the soil microbiota to drying-rewetting cycles, for example by Barnard *et al.* (2013); Placella *et al.* (2012) and Evans & Wallenstein (2014). While these works may not be directly relevant to the effects of long term flooding, many suggest a strong role for the precipitation history upon the microbial response (Evans & Wallenstein 2012). Meanwhile, characterisation of microbial communities along a moisture gradient, by Lennon *et al.* (2012) suggests a strong influence of phylogeny and that the trait of moisture adaptation is typically conserved at a relatively high phylogenetic order, namely phylum. To date, however, this has not been investigated in the context of flooding and finer taxonomic resolution of the responses of microbial communities to extended waterlogging is needed in order to test existing hypotheses. These include hypotheses relating to i) improved resilience when resource availability is non-limiting (Griffiths & Philippot 2013; Wallenstein & Hall 2012) ii) alterations in resistance and resilience as a function of the ratio of copiotrophs to oligotrophs (de Vries & Shade 2013) and iii) the response of microbial communities to alterations in soil moisture status is phylogenetically conserved at a high order (Lennon *et al.* 2012). In order to thoroughly investigate microbiome stability, many of the recent studies mentioned herein employed cutting-edge technologies, presented briefly below, to allow for *in situ* assessments of microbial community composition and functioning.

1.5 Molecular toolbox for investigating soil microbiome responses to changes in environmental conditions

Initially, the field of microbial ecology relied heavily on culture-based techniques for assessing the soil microbiota, a process hampered by difficulties in culturing the vast majority of soil microflora (Staley & Konopka 1985). While there have been a number of innovations for increasing the 'culturability' of soil microbes, as detailed by Epstein (2013), it has been the introduction of culture-independent techniques which has truly revolutionised soil microbial ecology (Cardenas & Tiedje 2008; Metzker 2010). Typically, this involves the use of one or more biomolecules, DNA, RNA, proteins and/or metabolites, extracted from a mixed microbial community. A suite of meta-omics tools can then be employed using the biomolecules, as reviewed by Abram (2015), allowing for a systems biology based experimental approach. These include metagenomics using DNA (Thomas *et al.* 2012), which provides insights into microbial potential, metatranscriptomics with RNA (Moran *et al.* 2013) and metaproteomics with proteins (Siggins *et al.* 2012) inferring microbial function, and metabolomics (Patti *et al.* 2012) informing on microbial activity, as reviewed in the relevant citations. DNA and/or RNA can also be used for taxonomic surveys using 16S or 18S rRNA gene profiling of prokaryotes and eukaryotes respectively (Huse *et al.* 2008). Metaproteomics holds enormous potential for answering questions regarding the role of different microbial groups in ecosystems processes through its ability to inform on phylogeny and function (Maron *et al.* 2006).

Recent advances in high throughput sequencing (HTS) technology accompanied by increasingly competitive prices have vastly improved accessibility to this suite of omics tools, which can now be used in conjunction. When resulting data are then integrated, it provides a more holistic view of microbial structure and function in the

system (Franzosa *et al.* 2015). Indeed, the findings from omics-centric studies have allowed for unique insights into the physiology of previously uncharacterised microbes, which has in turn informed on optimal growth conditions and so permitted their cultivation, as reviewed by Gutleben *et al.* (2017). For example, efforts to culture the obligate, intracellular pathogen *Coxiella burneti* typically have not been successful, primarily as a result of insufficient protein synthesis to permit replication (Omsland *et al.* 2009). However, using genomic and metabolic information, Omsland *et al.* (2009) were able to identify a number of amino acid auxotrophies, and thus supplement the media accordingly, which, in conjunction with microaerophilic conditions allowed for host-free cultivation of *C. burneti* for the first time.

From a data analysis point of view, data integration is also of benefit as it provides more robust biological interpretation; for example, metagenomics data can inform on more accurate assembly of metatranscriptomes (Gilbert *et al.* 2008; Ju & Zhang, 2015) and improved identification rate of metaproteomes (Cantarel *et al.* 2011). As such, it is desired that two or more of these tools are employed simultaneously, using biomolecules extracted from any range of environmental sample. With regards to the extraction of one type of biomolecules including DNA, RNA or protein, protocols and commercial kits abound for all sample types. However, an inherent feature of mixed microbial communities is their heterogeneity, which can impose problems when obtaining biomolecules from separate samples. This is exemplified to the extreme in the case of soil, where spatial heterogeneity occurs at sub-millimetre distances (Raynaud & Nunan 2014). Thus, as already well detailed by Roume *et al.* (2013), if different meta-omics tools are to be integrated to provide meaningful data, DNA, RNA, proteins and/or metabolites must be co-extracted from the same sample. In order to achieve this, a robust co-extraction method must be employed allowing

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biomolecules of interest to be sequentially removed from the same sample. Additionally, the sample should be handled in such a way that each isolation step does not introduce further bias. As yet, we unaware of such a method for soil and as such believe that this represents a significant research gap and tackling it will allow for the integrated use of -omics tools. This, in conjunction with well researched hypotheses will help to further field of soil microbial ecology (Jansson & Prosser 2013).

1.6   Thesis overview

In an effort to further knowledge in the field of microbial community stability in the face of perturbations, while answering some recent hypotheses in this area (de Vries & Shade 2013), we aimed to tackle the following knowledge gaps:

1. Appropriate biomolecule (DNA, RNA and proteins) co-extraction method for soil samples

2. Effect of liming on microbial community composition and transcriptionally active taxa using 16S rRNA sequencing

3. Effect of slurry amendment on the soil microbiome stability upon flooding deploying a rigorous experimental set-up to investigate ecosystem functioning and microbial community composition

The stresses investigated in this work, i.e., lime and slurry amendments and flooding, were chosen as they not only represent some of the more relevant disturbances for our geographic region, but are also relevant for many global agricultural systems.

1.7   References

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--------- Chapter II --------

A robust, cost-effective method for the co-extraction of DNA, RNA and proteins from soil and other complex, environmental samples

2.1   Introduction

The growing field of microbial community analysis involves the study of microbial populations using culture independent techniques, which necessitates the investigation of DNA, RNA and/or proteins from a variety of environments, subsequent to their extraction. In highly heterogenous samples including soil, such biomolecules should ideally be co-extracted from the same biological sample. Moreover, as good experimental design involves the use of replicated sampling regimes the use of commercial kits can become prohibitive. DNA/RNA and protein co-extraction kits are available for a variety of sample types but at present no kit or appropriate method are available for soil. Thus, we aimed to develop a simple method for the co-extraction of DNA, RNA and proteins from soil samples. The proposed protocol is based on the phenol-chloroform method of Griffiths *et al.* (2000), but is scaled up and modified to allow for the co-extraction of DNA, RNA and proteins from soil in sufficient yields to allow downstream analyses using meta-omics tools. A similar adaptation has been published by Gunnigle *et al.* (2014) using different bead-beating times to isolate nucleic acid and protein fractions. Feinstein *et al.* (2009), demonstrated that DNA continues to be isolated from consecutive bead beating regimes, due to lysis of more resilient microbial cells. Thus, if we are to use metagenomic information to guide protein annotations, this bias must be reduced by preventing sample interference between biomolecule isolation steps.

We therefore propose a phenol-chloroform based method for DNA, RNA and protein extraction from soil summarised in Figure 2.1, with adaptations for cattle slurry, anaerobic sludge granules and bacterial isolate culture samples. As a proof of

applicability, biomolecules co-extracted from soil samples were analysed using 16S

rRNA sequencing of DNA and cDNA, as well as metaproteomics.
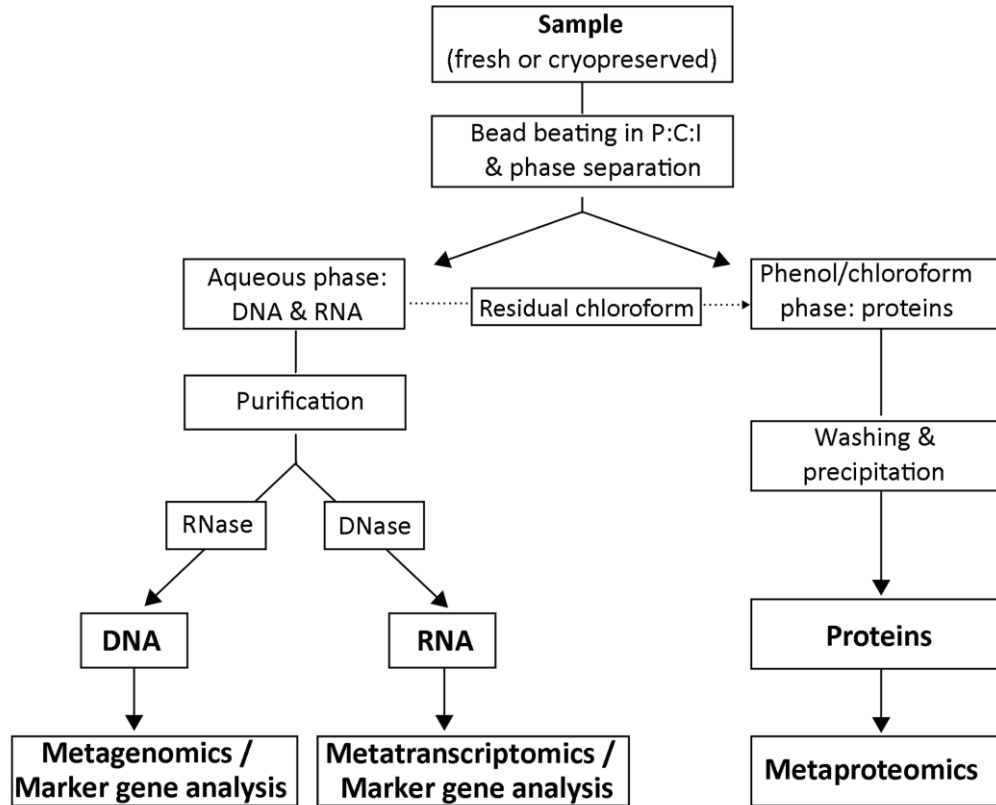
Figure 2.1. Schematic of DNA, RNA and protein co-extraction. Briefly, samples first undergo bead beating in extraction buffer (CTAB in potassium phosphate) and P:C:I (as per Griffiths *et al.* (2000). Phases are separated for isolation of nucleic acids from proteins. Each fraction is purified using washing steps and a spin-column for nucleic acids, and combined precipitation and washing steps (Heyer *et al.* 2013), for proteins. Residual chloroform was only retained for soil samples (represented by dashed line).

2.2   Materials and Methods

2.2.1 Sample preparation

2.2.1.1   *Soil*

Eight soils were used in the present study to develop and validate the co-extraction

method. These were selected from a collection of thirty-five Irish soils, compiled by

McDonald *et al.* (2014). Each consisted of a composite sample taken from up to 10 cm depth, which was homogenised and sieved at 6.3 mm. An aliquot of each soil type was stored at 4 °C while the remainder was used for in depth soil characterisation, as described in detail by McDonald and colleagues (2014) and summarised in Table 2.1. Two days prior to extraction, soil samples were placed at room temperature to increase microbial activity. Aliquots of 2 g dry weight equivalent of soil were placed in 5 ml tubes for co-extraction.

Table 2.1. Texture & chemistry of the eight soil types used in this study. The first column details the soil identification number used throughout this text while the second column details the original soil identification numbers used in the work of McDonald *et al.* (2014) for the purposes for cross referencing.

Soil ID	Original soil ID	Clay (%)	Silt (%)	Sand (%)	pH	SOM [1] (g/kg)	TC [2] (g/kg)	TN [3] (g/kg)
1	2	28.1	45.3	26.6	5.2	201	100	7.8
2	8	11.1	20.6	68.2	5.1	93.5	43.6	4.3
3	14	30.7	37.4	31.9	6.2	136.5	57.2	5.1
4	16	16.7	38.7	44.6	6	83	40.5	3.9
5	21	9.5	15.3	75.2	5.2	61	24.5	2.4
6	28	19.4	33.5	47.2	6.6	94.5	40.4	3.9
7	29	35.1	42.3	22.7	5.2	175	80.8	6.7
8	34	32.8	46.9	20.3	5.7	107.5	47.9	5.1

[1] SOM = soil organic matter; [2] TC = total carbon; [3] TN = total nitrogen.

Due to the charged nature of many particles within soil samples, pre-treatments are often required to improve yields *via* desorption. After trialling three pre-treatments mentioned in the literature, casein (Wang *et al.* 2012), salmon sperm DNA (Paulin *et al.* 2013) and an amino acid mix (Nicora *et al.* 2013), the amino acid mix was identified as the most successful (data not shown). Each of the eight soil types was

tested to determine if the pre-treatment could improve yields of DNA, RNA and/or protein. The amino acid desorption pre-treatment was prepared from a solution of three polar positive amino acids, namely arginine, histidine and lysine. The three amino acids were added to MilliQ water in a 1:1:1 ratio at 7 % w/v for each (Nicora *et al.* 2013), and the pH was adjusted to that of co-extraction buffer (pH 8.0) before autoclaving. Efforts to use amino acids directly in the co-extraction buffer were not successful, thus on the day of extraction, the amino acid solution was mixed with extraction buffer (1:2) to form an amino acid modified version of the co-extraction buffer of Griffiths *et al.* (2000). Tests were conducted to ensure improvements in yield were due to amino acid addition and not dilution of the extraction buffer by addition of MilliQ water (data not shown). To determine suitability of this pre-treatment for each soil type, biomolecule co-extraction was performed in triplicate for the following two treatments: 1) unmodified co-extraction buffer - here on referred to as 'untreated'; 2) co-extraction buffer + amino acid solution ('aa treated').

2.2.1.2 Granular Sludge.

Granular sludge samples were collected from a full-scale, mesophilic anaerobic digester treating wastewater at Carbery bioethanol production facility (Cork, Ireland). Granules were cryogenically ground using a pestle and mortar in the presence of liquid nitrogen. Aliquots of 0.25 g were placed in 2 ml screw cap tubes for bio-molecule co-extraction.

2.2.1.3 Cattle slurry

A sample of cattle slurry (7.5 % dry matter) from a local dairy farm was collected and stored at 15 °C for one week. On the day of extraction, replicate 0.5 g subsamples were removed and placed in 2 ml screw-cap tubes.

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2.2.1.4   Pure culture

An environmental isolate of *Escherichia coli* (isolate 3; Brennan *et al.* 2010) was grown anaerobically, shaking at 37 °C, in 20 ml of Luria-Bertani broth. Cells were grown to mid-exponential phase (OD 0.6) and collected by centrifugation at 3 000 *g* for 8 minutes. The resulting cell pellets were re-suspended in 500 µl of co-extraction buffer and transferred to a 2 ml screw cap tube for bio-molecule co-extraction.

2.2.2   DNA, RNA and protein co-extraction

For preparation of solutions, nuclease-free water and tubes were used, and all glassware was baked overnight at 180 °C. Additionally, samples were kept on ice during all intermediary steps to maintain RNA integrity. Unless otherwise stated, all centrifugation steps were performed at 16 000 *g* and 4 °C.

2.2.2.1   Soil co-extraction method

A 5 % hexadecyltrimethyl-ammonium bromide (CTAB) co-extraction buffer was prepared by mixing an equal volume of 10 % CTAB solution prepared in 0.7 M NaCl, with 0.24 M potassium phosphate buffer at pH 8 (Griffiths *et al.* 2000). Equal volumes (1.25 ml) of co-extraction buffer (+/- pre-treatment) and phenol:chloroform:isoamyl alcohol (P:C:I, 25:24:1) (pH 8) were added to 5 ml tubes containing 2 g (dry weight) soil and a 2 g mix of 0.1 mm and 0.5 mm zirconia beads. Cell lysis was achieved by vortexing at high speed for 2.5 minutes. Centrifugation for 10 minutes was employed for phase separation. The upper aqueous phase was transferred to a clean tube prior to nucleic acid purification, while the lower phase and remaining tube contents were set aside, on ice, for subsequent protein isolation from the phenol phase.

Nucleic acid isolation: Two volumes of chloroform:isoamyl alcohol (24:1) (Amresco) were added to the upper aqueous phase and mixed. This serves to remove any residual

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phenol and/or proteins from the aqueous phase. After 5 minutes centrifugation, the upper phase was transferred to a fresh tube, combined with two volumes of a 30 % polyethylene glycol (PEG 6000) solution in 1.6 M NaCl, and placed on ice for two hours for nucleic acid precipitation. In an effort to maximise protein recovery, the remaining, lower chloroform phase from each sample was kept aside to be added to the phenol phase during the protein isolation steps. Precipitated nucleic acids were collected *via* centrifugation for 20 minutes and the resulting supernatant was gently removed. The pellets were washed in a 70 % ethanol solution and samples were centrifuged for 25 minutes. Ethanol was poured off and tubes left open near a flame for 10 minutes to remove any residual ethanol. Nucleic acid pellets were re-suspended in 100 µl nuclease-free water from which 5 µl was run on a 1 % agarose gel to check DNA and RNA integrity. Nanodrop ratios were used to assess contamination of nucleic acids with protein (260/280 ratio) or aromatic moieties such as phenol (260/230 ratio) (Gallagher *et al.* 2008). Typically, a 280/260 ratio of 1.8 to 2 indicates nucleic acids free of proteins, while a 260/230 of the same or higher indicates a pure, contaminant free sample (Desjardins & Conklin 2010). Nucleic acid concentrations were determined using the broad range Qubit® dsDNA or RNA assay kit, as appropriate, with the Qubit™ 2.0 Fluorometer (Life Technologies) as per the manufacturer's instructions.

Protein isolation*:* To improve protein retrieval from soil samples, chloroform:isoamyl alcohol (24:1) remaining from the nucleic acid washing step (as described above), was added to the corresponding phenol phase containing tube, along with 500 µl extra P:C:I (25:24:1) and contents were gently mixed with a sterile pipette tip. Samples were briefly re-centrifuged (2 minutes) to separate the phenol/chloroform phase which was then removed to a sterile 15 ml tube. This step was not required for other sample types,

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which proceeded directly to phenol phase removal after phase separation. For all sample types, following phenol removal, an equal volume of 1 M sucrose solution, in MilliQ water, was added and samples were mixed and centrifuged for 10 minutes at 12 000 g for phase separation. The lower phase was removed (avoiding remaining soil debris and beads), placed into a fresh 15 ml tube along with 4 volumes of ice cold 0.1 M ammonium acetate in methanol and incubated overnight at -20 °C for protein precipitation (Heyer *et al.* 2013). Following the overnight incubation, precipitated proteins were collected by centrifugation at 12 000 g for 10 minutes at 4 °C, the supernatants removed and four volumes of 0.1 M ice cold ammonium acetate were added. Samples were then left for 2 to 18 hours at -20 °C to re-precipitate. Protein samples were then successively washed, following the method of Heyer *et al.* (2013) where all centrifugation steps were performed at 12 000 g for 10 minutes at 4 °C. Briefly, precipitated proteins collected by centrifugation, were washed successively in 80 % freezer-chilled acetone and 70 % freezer-chilled ethanol with 15 minute incubations at -20 °C after addition of each solution. The samples were then centrifuged, and the supernatants removed. This was repeated a second time to give a total of four washes. Finally, the resulting protein pellets were dried in a laminar flow hood, ensuring they did not become too dry thereby impeding re-suspension. Protein pellets were solubilised in 30 µl urea buffer (7 M urea, 2 M thiourea and 0.01 g/mL dithiothreitol). For protein quantification, duplicate 1 in 10 dilutions of each sample were analysed using the Amido black assay (Schaffner & Weissmann 1973). The remaining 25 µl of neat sample were combined with 10 µl of 2 x Laemmli sample buffer and separated using 1D SDS-PAGE (12 % bis-acrylamide) as detailed by Laemmli (1970).

In order to extract from different samples types, slight modifications were applied, namely to initial sample weight or volume and extraction buffer formulation. These changes are summarised in Table 2.2 and detailed below, in sections 2.2.2.2, 2.2.2.3 and 2.2.2.4.

Table 2.2. Summary of modifications to DNA, RNA and protein co-extraction protocol for different sample types.

Sample type	Sample vol / weight	Co-extraction buffer
Soil	2 g (dw)	5 % CTAB buffer/PO_4
Cattle slurry	0.5 g	5 % CTAB buffer/PO_4
Granular Sludge	0.25 g	1 % CTAB buffer/PO_4
Pure Culture (OD 0.6)	20 ml	2 % CTAB buffer/PO_4

OD = optical density; dw = dry weight; vol = volume before centrifuging

2.2.2.2 Granular sludge modifications

Co-extractions were performed in 2 ml screw cap tubes containing 1 g of the zirconia bead mix and 0.25 g of the cryogenically pulverised granular sludge, to which 400 µl of 1 % CTAB co-extraction buffer (equal volumes of 2 % CTAB in 0.7 M NaCl and combined on the day of extraction with an equal volume of freshly prepared 0.24 M potassium phosphate buffer at pH 8) were added along with 800 µl of P:C:I (25:24:1). Bead beating, phase separation and chloroform washing were undertaken as for soil, with volumes altered accordingly, with respect to ratio. Nucleic acids were precipitated and washed as above, then re-suspended in 100 µl of nuclease-free water. The phenol phase was removed to a fresh 2 ml tube and underwent protein precipitation and washing steps as detailed above, with volumes of washing solutions changed to

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maintain ratios used in soil method. Protein pellets were re-suspended in 30 µl urea buffer and stored at 4 °C until further analysis.

2.2.2.3 Cattle slurry modifications

A 5 % CTAB buffer solution was added to the slurry, which then underwent all steps as for granular sludge. The resulting nucleic acid pellet required purification immediately following elution in 60 µl nuclease-free water, using the OneStep PCR inhibitor removal kit from Zymo. Protein pellets were re-suspended in 30 µl urea buffer and stored at 4 °C until quantification and other downstream analyses.

2.2.2.4 Single bacterial isolate modifications

Cell pellets were re-suspended in 500 µl of 2 % CTAB co-extraction buffer (4 % CTAB in a 0.7 M NaCl solution, combined with an equal volume of fresh 0.24 M potassium phosphate buffer at pH 8). The same modifications as for granular sludge were then applied.

2.2.3 Downstream analysis of DNA and RNA

2.2.3.1 Sample purification and cDNA generation

Any samples containing evidence of humic acid contamination (as indicated by a yellow-tinted nucleic acid pellet) were run through a OneStep™ PCR Inhibitor Removal column (Zymo) before downstream analyses. Aliquots of DNA and RNA were split into two fractions of equal volumes. RNA was DNase treated with Turbo DNase (Ambion) following the manufacturer's instructions. Complete removal of genomic DNA was verified by performing PCR with universal bacterial primers

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515F/806R (Caporaso *et al.* 2011) and DNase-treated RNA as template, where the absence of product (at a series of dilutions) indicated complete DNA removal. RNA concentrations were normalised, and generation of cDNA was performed using SuperScript® III Reverse Transcriptase (Invitrogen), following the manufacturer's recommended guidelines.

2.2.3.2 Amplification of 16S rRNA genes from all soil types

The universal bacterial/archaeal primers 515F/806R (Caporaso *et al.* 2011) were used to amplify the V4 region of the 16S rRNA from both DNA and cDNA samples in order to verify their suitability for downstream analyses. Each PCR amplification contained 2 U of BioTaq (BioLine); 1 x NH_4 Reaction Buffer (NEB); 200 µM of dNTPs (BioLine); 10 µg BSA (NEB); 2 mM $MgCl_2$; 0.4 µM of each 515f and 806r with 11 µg of genomic DNA or cDNA as appropriate. Negative and positive controls were included as standard and cycle conditions consisted of an initial denaturation of 1 minute at 95 °C followed by 30 cycles of 95 °C for 15 seconds, 55 °C for 15 seconds and 72 °C for 20 seconds. After a final annealing step of 72 °C for five minutes, 8 µl of each PCR product was visualised on a 1 % agarose gel stained with SybrSafe DNA stain (Invitrogen).

To demonstrate that biomolecules obtained using this method are compatible with downstream analyses, one soil type was chosen for 16S rRNA profiling (from DNA and cDNA samples) and metaproteomics. As PCR amplification of Soil 2 proved somewhat problematic and as such required some optimisation (addition of BSA and increased $MgCl_2$ concentration), molecular extracts from this soil were selected for further analysis in order to provide a robust validation of our co-extraction method.

2.2.3.3 16S rRNA amplicon library preparation

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Barcoded, universal bacterial archaeal primers 515F/806R (Caporaso *et al.* 2011) were used to amplify the V4 region of 16S rRNA genes from DNA and corresponding transcripts from cDNA from triplicate biomolecule extractions from Soil 2. Each PCR amplification contained 1 x Q5 Reaction Buffer (NEB); 0.4 U Q5 High-Fidelity DNA Polymerase; 200 µM of dNTPs; 0.4 µM of each primer and 11 µg of genomic DNA or cDNA as appropriate. Negative and positive controls were systematically included, and samples amplified in triplicate, to reduce PCR based bias (Goodrich *et al.* 2014). PCR conditions were as follows: 30 seconds hot start at 98 °C followed by 25 cycles of 10 seconds denaturation at 96 °C; 30 seconds annealing at 50 °C and 30 seconds elongation at 72 °C, followed by a final extension of 2 minutes at 72 °C. Triplicate amplicons per sample were pooled and run on a 1.5 % agarose gel. The correct size amplicons (~300 bp) were excised and purified (Promega SV Gel Wizard) following the recommended guidelines for gel extraction. Purified amplicons were eluted in nuclease-free water before quantification using the High Sensitivity dsDNA quantification kit (Qubit). Amplicons from each sample were normalised to equimolar concentrations and pooled before analysis on the Illumina MiSeq platform (Research & Testing Laboratory, Texas, USA).

2.2.4 Downstream analysis of proteins for metaproteomic analysis

SDS gel chunks corresponding to the three soil replicates analysed for 16S rRNA profiling (two chunks per lane) were subjected to in-gel tryptic digestion. Nanoflow liquid chromatography-electrospray ionization tandem mass spectrometry (nLC-ESI-MS/MS) was performed by BSRC Mass Spectrometry and Proteomics Facility (University of Saint Andrews, UK), using a Q-Star XL tandem mass spectrometer (Applied Biosystems) as described in detail by Abram *et al.* (2011).

2.2.5 Analysis of 16S rRNA and metaproteomic datasets

2.2.5.1 *Processing of 16S rRNA data from DNA and cDNA fractions*

Processing of paired-end 16S rRNA amplicon reads was undertaken using Mothur software version 1.36.1 (Schloss *et al.* 2009) and by following the MiSeq 16S rRNA amplicon analysis procedure detailed by Kozich *et al.* (2013). Briefly, sequences were filtered to remove those containing ambiguous bases, homo-polymers exceeding 8 base pairs or erroneous sequence lengths (min 75 – max 275 bp). The SILVA database (v119) was used for alignment of sequences to the V4 region corresponding to primers 515F/806R. Sequencing errors were further reduced by pre-clustering and chimeras removed using the UCHIME algorithm integrated in Mothur (Edgar *et al.* 2011). Sequences were classified using Bayesian analysis of k-mers at an 80 % bootstrap cut-off (Wang *et al.* 2007) against the Ribosomal Database Project's v.9 reference file and sequences from non-target lineages were removed. Finally, sequences were clustered into operational taxonomic units (OTUs) by grouping them according to their taxonomy at a 3 % dissimilarity level.

2.2.5.2 *OTU based analysis of DNA and cDNA fractions*

Alpha and beta diversity metrics were calculated using Mothur (Schloss *et al.* 2009) and the statistical programme R (R Core Team 2017). Sample coverage, using Good's estimator of coverage was estimated for each sample, using the formula:

1

$$Coverage = 1 - \left(\frac{n}{N}\right)$$

Where:

44

n = the number of OTUS represented by one sequence (singleton) and
N = the total number of OTUs in that sample (Good, 1953).

Rarefaction curves were constructed using Mothur's randomisation procedure in conjunction with a 're-sampling without replacement' approach. Species richness (S), was determined for each sample, based on the number of observed OTUs. Alpha diversity was calculated using the reciprocal of Simpson's diversity estimator (D), which calculates the probability of sampling the sample OTU twice when randomly choosing two sequences from a sample (Simpson 1949). The following equation was used:

2

$$D = \sum_{i=1}^{S} \frac{n_i(n_i - 1)}{N(N - 1)}$$

Where:
D = Simpson's diversity indexs
S = the number of observed OTUs
n_i = the number of individuals in the i th OTU (i.e. the OTU of interest)
N = the total number of individuals in the community

The Inverse Simpson's index (D⁻¹) was therefore:

3

$$D^{-1} = \frac{1}{D}$$

Simpson's community evenness (E), to determine numerical equitability of the OTUs within each sample, was calculated using the following formula:

4

$$E = \frac{D^{-1}}{S}$$

Where:
45

E = community evenness
D^{-1} = inverse Simpson's diversity index
S = the number of observed OTUs

2.2.5.3   Protein identification

The MetaProteomeAnalyser (MPA) software, developed by Muth *et al.* (2015) was

used for both identification of mass spectra and protein assignment. Database searches

were run using the X!Tandem (version ALANINE (2017.02.01) and OMSSA (version

2.1.8) algorithms integrated in MPA, and spectra were identified against the

UniProt/SwissProt (release 2016_10) database. Search parameters included: trypsin as

the default cleavage enzyme; maximum allowed number of missed cleavages set to

one; a parent-ion mass tolerance of 10 ppm and a fragment-ion mass tolerance of 0.5

Da. Target decoy searching was performed as standard. Protein hits were grouped into

meta-proteins using the peptide rule (at least one peptide in common) in order to reduce

data redundancy (false-discovery rate of < 1 %). Annotation was achieved *via* a

number of external sources, including NCBI, UniProt and KEGG as detailed by Muth

et al. (2015). Through taxonomic assignment of peptides, those belonging to

contaminants were removed (e.g. keratin and trypsin) and UniProt ontologies,

integrated in MPA, were used to group meta-proteins into molecular functions and

cellular components.

2.2.6   Data representation and statistical analysis

Correlation matrices of biomolecule yield and purity *vs* soil physicochemical properties were constructed using the Hmisc package (Harrel & Dupont 2017) in the statistical program R (R Core Team 2017). For comparisons of mean biomolecule yields, data were first tested for equality of variance, using an F test for two-sample variances, before performing the appropriate two tailed t-test. The resulting *p* values were collated and adjusted for multiple comparisons using the false discovery rate (FDR) method of Benjamini and Hochberg (1995) as implemented in the p.adjust function of R. Bar plots of yield improvements were constructed in the open access plotting package SciDavis (Standish *et al.* 2007). Bar plots of 16S rRNA relative abundances and meta-protein ontologies were graphically represented in R using the packages ggplot2 (Wickham 2009) and phyloseq (McMurdie & Holmes 2013). Taxonomic assignment and KEGG orthologies of meta-proteins, as well as 16S rRNA composition were plotted using Krona plots *via* the Excel template provided by Ondov *et al.* (2011).

2.3   Results

2.3.1   DNA, RNA and protein yields from diverse soil types

DNA, RNA and proteins were co-extracted from eight soils with a range of clay contents, pH and other physicochemical characteristics known to affect extraction yields (Table 2.1). DNA, RNA and proteins were co-extracted from eight soils with a range of clay contents, pH and other physicochemical characteristics known to affect extraction yields.

To determine which desorption pre-treatment, if any, was appropriate for each of the eight soils, extractions were performed in triplicate on a) untreated samples (no

desorption pre-treatment applied) b) amino acid pre-treated samples c) casein pre-treated samples (Figure 2.2). While the casein pre-treatment was shown to be very effective in improving nucleic acid yields from a number of problematic soils (data not shown), in these soils, it only served to improve the RNA yield in soil 5 (Figure 2.2). With regards to protein extraction however, it proved an unfeasible pre-treatment, firstly because any increases in protein yield could not accurately be determined due to the co-quantification of casein within the extracted soil proteins. Secondly, while proteins identified by mass spectrometry as casein / casein derived, could have been removed from the results database prior to further analyses, there existed the potential that the soil-derived proteins, typically present in very low abundances, would have their signal supressed by the highly abundant casein during mass spectrometry (Guerrier *et al.* 2008). Thus, only untreated and amino acid pre-treated samples were further analysed.
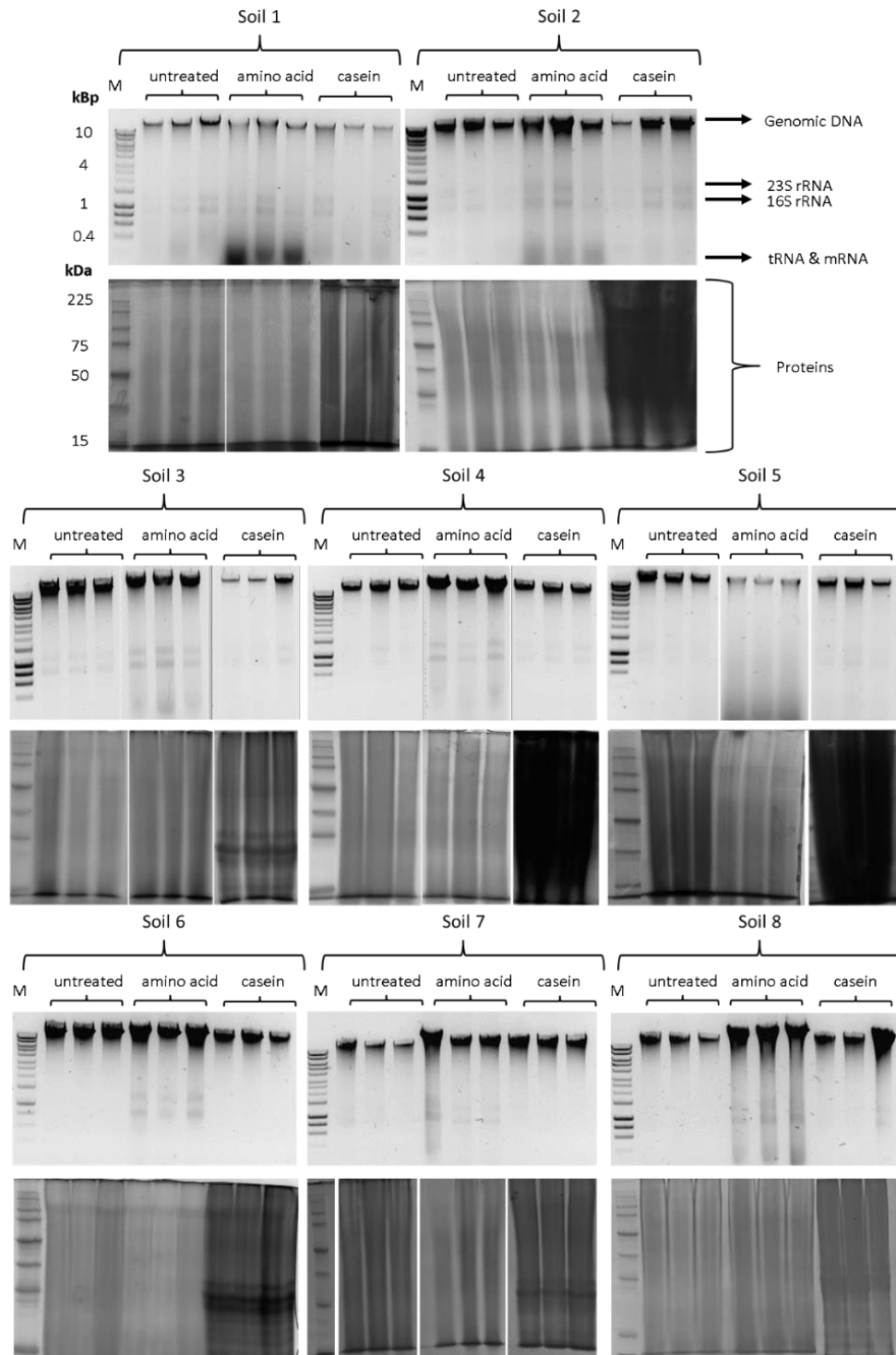
Figure 2.2. Nucleic acid and protein extracts obtained from the eight soils with and without pre-treatments. For each soil, extractions were performed in triplicate for a) no pre-treatment b) amino acid pre-treatment c) casein pre-treatment. DNA and RNA run on 1 % agarose gels are presented in the top panel for each soil, and proteins run on 12 % bis-acrylamide SDS page gels are presented below. M= marker, where Hyperladder I was used for nucleic acid gels and Promega's Broad Range protein marker for protein gels.

The use of an amino acid desorption pre-treatment increased the yield of at least one biomolecule of interest in six of the eight soils (Figure 2.3). Although this modification was originally employed by Nicora *et al.* (2013) to improve protein yields, in the soils we tested, it principally served to increase nucleic acid yields. However, in soil 5, the buffer had the opposite effect, whereby it significantly reduced the RNA yields. This could have been a result of the low clay content (just 9.5 %) of this soil. Indeed, the desorption buffer is proposed to bind principally to charged clay particles, as such, perhaps the amino acids were in excess and interfered with RNA extraction in some way.
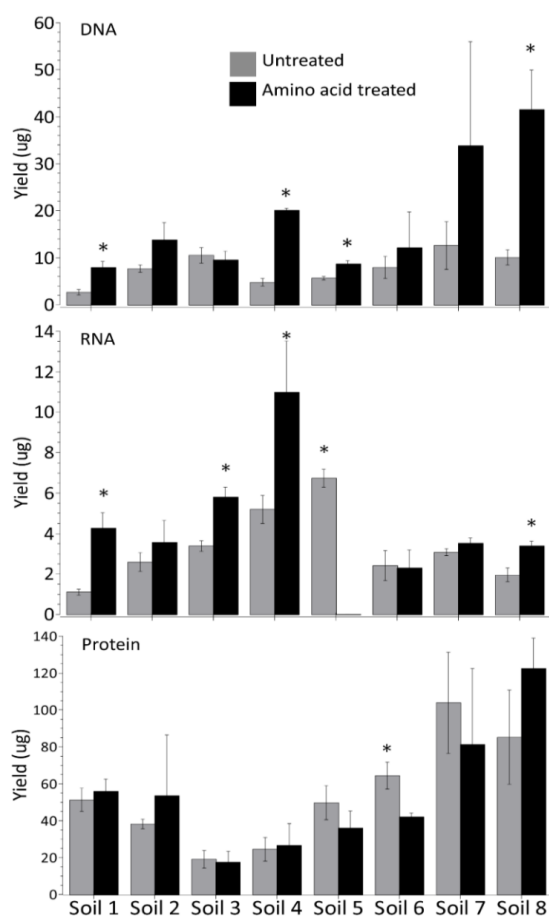
Figure 2.3: Comparison of DNA, RNA and protein yields, in total µg per extraction, from all 8 soils, with (black) and without (grey) pre-treatment desorption buffer. Error bars show standard deviation (n=3). Statistically significant differences in mean yield between pre- and untreated samples are represented by asterisks denoting FDR-adjusted p values < 0.05 (determined by two tailed t-tests, subsequent to testing for equality of variance).

Both yields and quality scores for nucleic acids, showed significant variation between soils, with technical replicates showing minor variation (Table 2.3). Nucleic acid yields were highest in Soil 4, with DNA concentration yields of 28.6 µg (± 0.20) and RNA yields of 7.1 µg (± 1.68) per extraction, being at least a factor of two higher than any other soil. The yield of proteins however was not highest for this soil. Indeed, protein yields did not follow the same trends seen for nucleic acid yields, implying the extraction of nucleic acid and protein fractions are perhaps differentially affected by soil physiochemical properties. Although nucleic acids were generally of suitable purity to carry out downstream analyses, as shown by the nanodrop ratios (Table 2.3), Soil 2 required purification. Therefore, for the purposes of consistency, nucleic acids from all soils were run through a OneStep PCR inhibitor remover (Zymo) prior to downstream analyses. In general, this served to improve the 280/260 ratios to ~1.8 and 260/230 ratios to ~1.6 (data not shown).

Table 2.3. Yields per extraction of DNA, RNA and protein, as well as quality ratios, from the eight soils tested, as assessed prior to purification.

Soil	DNA yield µg [1]	RNA yield µg [2]	A260/A280 [3]	A260/A230 [3]	Protein yield µg [4]
Soil 1	7.99 ± 1.21	4.27 ± 0.75	1.69 ± 0.02	0.82 ± 0.06	96.63 ± 4.33
Soil 2	13.80 ± 3.70	3.56 ± 1.10	1.58 ± 0.02	0.79 ± 0.01	54.68 ± 6.76
Soil 3	11.37 ± 1.85	2.85 ± 0.48	1.74 ± 0.05	1.11 ± 0.11	96.00 ± 8.90
Soil 4	28.60 ± 0.20	7.07 ± 1.68	1.68 ± 0.07	0.98 ± 0.03	82.36 ± 11.08
Soil 5	5.27 ± 1.36	2.56 ± 0.38	1.87 ± 0.17	1.32 ± 0.35	67.01 ± 12.56
Soil 6	14.17 ± 4.80	3.47 ± 1.30	1.79 ± 0.03	1.23 ± 0.10	51.54 ± 18.07
Soil 7	11.40 ± 3.18	3.40 ± 0.70	1.71 ± 0.05	1.06 ± 0.06	68.76 ± 20.34
Soil 8	12.13 ± 0.50	3.43 ± 0.18	1.78 ± 0.03	1.07 ± 0.09	87.64 ± 15.85

1 DNA yields were determined using the broad range, ds DNA Qubit (Invitrogen); 2 RNA yields were established using the broad range, RNA Qubit (Invitrogen); 3 Nucleic acid absorbance ratios, for assessing nucleic acid purity, were determined using a NanoDrop spectrophotometer. 4 The Amido Black assay was employed for protein quantification (Standard deviation of mean; n = 3).

A Pearson's correlation matrix was used to determine if extract yields, or nucleic acid purity, varied consistently as a function of any of the soil physiochemical properties (Table 2.4). This analysis revealed no significant correlations between extract properties and soil properties, implying that perhaps it is an interaction of soil properties (for example clay content and pH) that affects extraction success. Some statistically significant intra-group correlations were detected, as expected, in particular within the group of soil physicochemical properties (Table 2.4). As expected, DNA yields were correlated with RNA yields ($R^2 = 0.88$), however no statistically significant correlation existed between nucleic acid yields and protein yields. This further demonstrates how the physicochemical properties of these eight soils influenced the extraction of these two fractions in different ways.

Table 2.4. Pearson's correlation matrix of DNA, RNA and protein yields and nucleic purity (280/260 and 260/230 Nanodrop ratios) with soil physiochemical characteristics. Statistically significant correlations are represented in bold.

	DNA	RNA	280/260	260/230	Protein	Clay	Silt	Sand	pH	SOM	TC
RNA	0.88 [a]										
280/260	-0.41	-0.42									
260/230	-0.24	-0.41	0.91 [a]								
Protein	-0.01	0.20	0.02	-0.23							
Clay	-0.15	-0.13	0.02	-0.09	0.57						
Silt	0.21	0.32	-0.12	-0.27	0.63	0.86 [a]					
Sand	-0.05	-0.11	0.06	0.20	-0.62	-0.96 [a]	-0.97 [a]				
pH	0.42	0.17	0.27	0.42	0.00	0.10	0.23	-0.18			
SOM	-0.29	-0.06	-0.30	-0.46	0.49	0.75 [b]	0.67	-0.73 [b]	-0.26		
TC	-0.24	0.04	-0.36	-0.54	0.49	0.68	0.66	-0.70 [b]	-0.31	0.99 [a]	
TN	-0.19	0.04	-0.40	-0.58	0.50	0.76 [b]	0.74 [b]	-0.78 [b]	-0.27	0.98 [a]	0.98 [a]

a = statistically significant correlation of $p < 0.01$; b = statistically significant correlation of $p < 0.05$

2.3.2 Biomolecule yields from granular sludge, cattle slurry and pure culture

With the implementation of small adaptations, biomolecules were also co-extracted from a diverse range of complex, mixed microbial communities as well as from pure culture (Figure 2.4). Assessment of nucleic acid integrity *via* visualisation on agarose gels revealed intact genomic DNA and RNA for all samples (Figure 2.4).
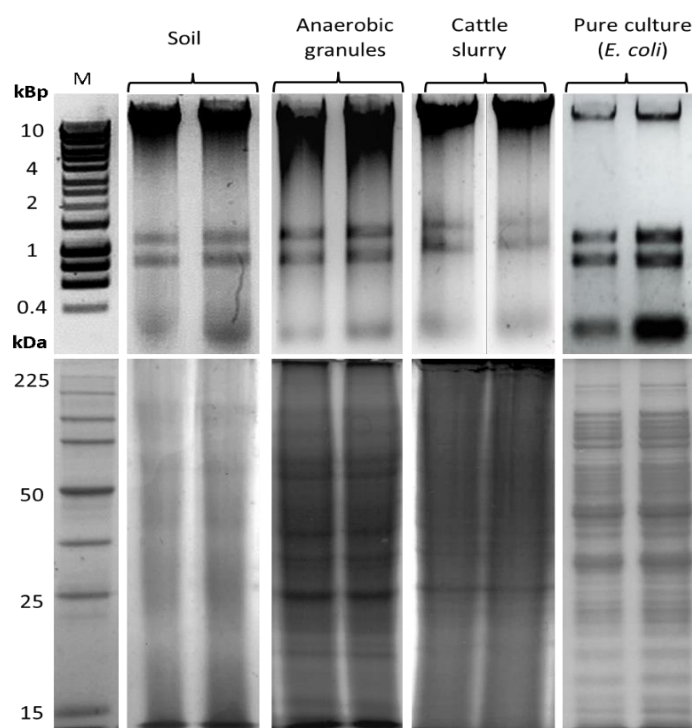
Figure 2.4. Representative agarose gels and their corresponding SDS-page gel images from the three biomolecules recovered from a diverse range of sample types. Technical duplicates samples are shown for each sample type. For all samples, excluding cattle slurry, raw nucleic acid extracts are shown. Purification using the OneStep kit (Zymo) was necessary for cattle slurry samples prior to imaging. The soil sample shown is Soil 2.

Nucleic acid purity of extracts from granular sludge and pure culture was high (Table 2.5). However, the nucleic pellet acquired from slurry samples was dark in colour, therefore the corresponding DNA and RNA fractions required purification (Zymo OneStep PCR inhibitor removal column), after which the extracts' quality and quantity

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were sufficient for downstream analyses (Table 2.5). It should also be noted that this co-extraction method was successfully employed by other members of the research group to obtain nucleic acids and proteins from biomass obtained from both digestate and leachate fractions of a bioreactor treating food waste, as well as from a Gram positive culture of *Streptococcus pyogenes*. This data, taken from the manuscript version of this work, can be seen in Appendix I.

Table 2.5. Yields per extraction of DNA, RNA and protein from all sample types tested, as well as quality ratios. Data shown are prior to any purification.

Sample type	DNA yield µg [1]	RNA yield µg [2]	A260/A280 [3]	A260/A230 [3]	Protein yield µg [4]
Anaerobic granules	18.03 ± 5.05	11.31 ± 2.10	1.93 ± 0.01	1.97 ± 0.03	142.53 ± 13.42
Cattle slurry	9.93 ± 0.72	3.99 ± 0.23	1.81 ± 0.01	1.22 ± 0.10	140.70 ± 2.52
Pure culture	3.14 ± 0.57	31.12 ± 0.57	1.95 ± 0.10	1.98 ± 0.05	113.95 ± 25.01

[1] DNA yields were determined using the broad range, ds DNA Qubit (Invitrogen); [2] RNA yields were established using the broad range, RNA Qubit (Invitrogen); [3] Nucleic acid absorbance ratios, for assessment of nucleic acid purity were determined using a NanoDrop spectrophotometer. [4] The Amido Black assay was employed for protein quantification (Standard deviation of mean; n = 3).

When considering the recommended concentrations for commonly used library preparation kits, yields of nucleic acids from all sample types (Figure 2.5) were shown to be sufficient to allow for metagenomics (amplification free, at > 250 ng DNA per sample for Illumina Nextera™ kit) and metatranscriptomics (between 2 µg and 10 µg for MICROBExpress™ Kit for mRNA enrichment) (Figure 2.5). In addition, protein yields were comparable to recent published metaproteomics studies for complex systems such as sediments (Moore *et al.* 2014) and soil (Bastida *et al.* 2014), where co-extraction was not performed.
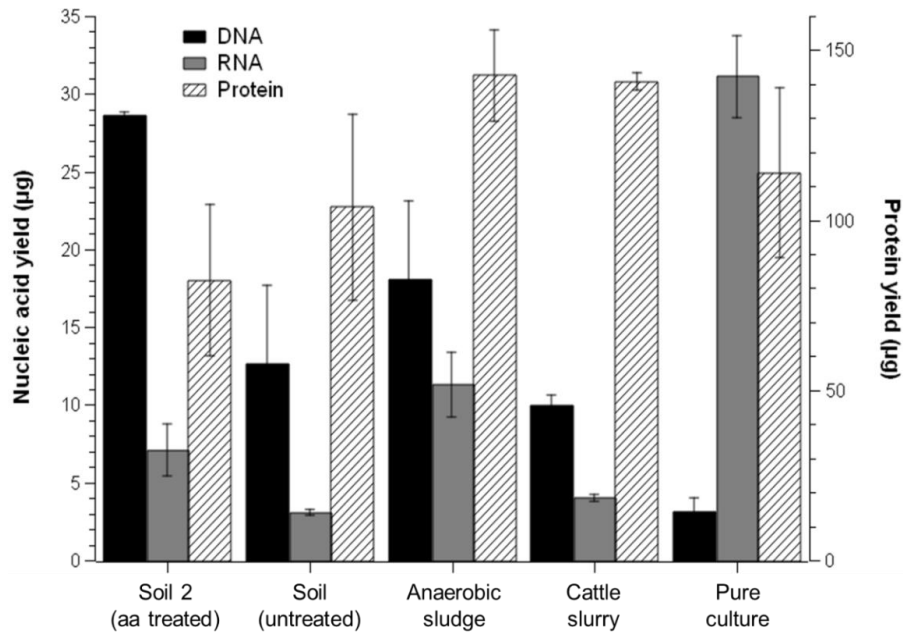
Figure 2.5: Total yields of DNA (black), RNA (grey) and proteins (shaded) per extraction, from all sample types tested. 'Aa treated' = amino acid pre-treatment applied; 'untreated' = no pre-treatment applied. Error bars show standard deviation of the mean where n = 3.

2.3.3 Downstream analyses of soil-extracted bio-molecules for microbial community analysis

To validate the suitability of soil nucleic acid fractions for downstream analyses, 16S rRNA genes from all eight soils were successfully PCR amplified using the universal primers 515F/806R (Caporaso *et al.* 2011) from both DNA and cDNA (Figure 2.6). As a proof of concept, three biological replicates from one soil type (Soil 2) underwent in-depth microbial community analysis. This soil was chosen as it was more problematic that other soil samples during PCR amplification, thus presenting a more robust test of the success of downstream analyses.
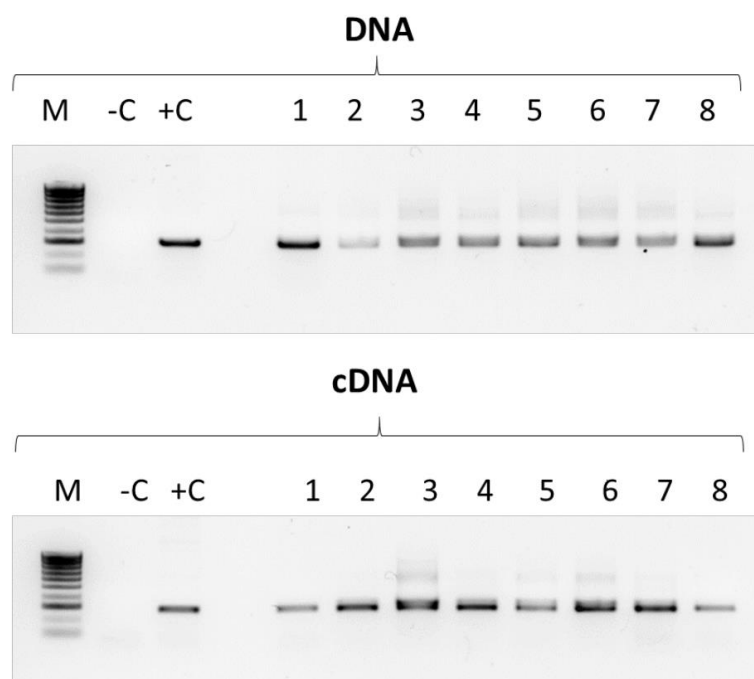
Figure 2.6. PCR products from amplification of 16S rRNA from DNA and cDNA fractions of the eight soils tested. M = marker (Hyperladder I); -C = negative control (no template); + C = positive control (*E. coli* genomic DNA as template)

2.3.3.1 16S rRNA gene sequencing

Sequences of 16S rRNA genes obtained from the six resulting samples (3 technical replicates for DNA and 3 for cDNA) consisted of a total number of 178798 raw reads, of which 93727 and 85071 corresponded to DNA and cDNA samples respectively. After quality filtering, sequences were clustered into OTUs and using subsampled datasets, 12 % of OTUs were shared between triplicate DNA samples and 16 % between triplicate cDNA samples (Table 2.6), demonstrating sample heterogeneity and the importance of replicates when assessing community composition in this manner.

Table 2.6: Total number of raw and filtered reads for the three replicate DNA & three replicate cDNA samples respectively. Sum of all OTUs identified for all three replicates (Total OTUs) and OTUs shared by all three replicates are shown.

Nucleic acid	Total reads	QC'd [1]	Total OTUs [2]	Shared OTUs [3]
DNA	109683	66868	3479	957
cDNA	91235	63515	2495	934

[1] Read number after quality trimming and chimera removal. [2] OTU – Operational Taxonomic Units as clustered at 97 % similarity. [3] OTUs shared between three replicate samples.

Rarefaction curves, were constructed to determine species richness as a function of sampling effort, where curves that plateau signify adequate sampling depth (Gotelli & Colwell 2001). From this analysis (Figure 2.7) we can see that we did not access all of the species present in our sample, however this is a frequently observed occurrence in the context of highly diverse microbial communities such as those found in soils. A selection of similar results can be seen in Lauber *et al.* (2009), Nacke *et al.* (2011) and Meng *et al.* (2016).
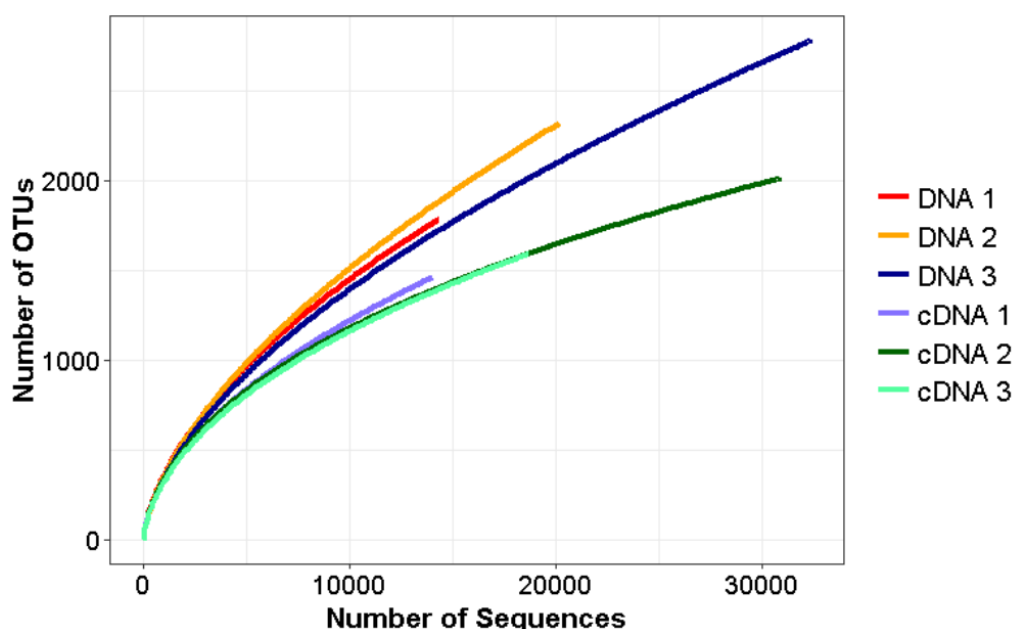
Figure 2.7. Rarefaction curves for 3 replicate DNA and cDNA samples. The number of OTUs detected as a function of sequences allows for the comparison of richness of each sample as a measure of the sampling intensity.

Sample coverage for DNA (92 %) and cDNA (95 %) datasets was above the limit of 90 % typically regarded as sufficient resolution to allow for OTU-based analyses (Lemos *et al.* 2011) (Table 2.7). cDNA samples had reduced richness (1407 OTUs) but were more even (0.05) than DNA samples, with a richness of 1771 OTUs and an evenness of 0.02. This increased evenness in the cDNA samples resulted in a higher measure of alpha diversity than in the DNA samples, as calculated with the inverse of the Simpson's index.

Table 2.7. Alpha diversity metrics for the DNA and cDNA fractions of Soil 2. Included are the 16S rRNA library coverage; species richness, assessed by OTU count per sample; species evenness (using Simpson's evenness index) and the inverse of the alpha diversity estimator detailed by Simpson (1949). Values show the mean and standard deviation of replicates (n=3).

Fraction	Coverage (%)	Richness (OTUs observed)	Simpson's evenness	Simpson's diversity
DNA	92 ± 1.0	1771.12 ± 79.69	0.02 ± 0.00	31.30 ± 7.06
cDNA	95 ± 1.0	1407.89 ± 45.34	0.05 ± 0.01	64.75 ± 9.46

Considering community composition, the dominant phyla of *Proteobacteria, Verrumicrobia, Acidobacteria* and *Bacteroidetes* were present in both the DNA and cDNA samples, but their relative abundances varied between the two nucleic acid fractions (Figure 2.8). Members of the *Proteobacteria* were detected at higher relative abundances in cDNA samples (50 %) compared to 20 % in the DNA samples.
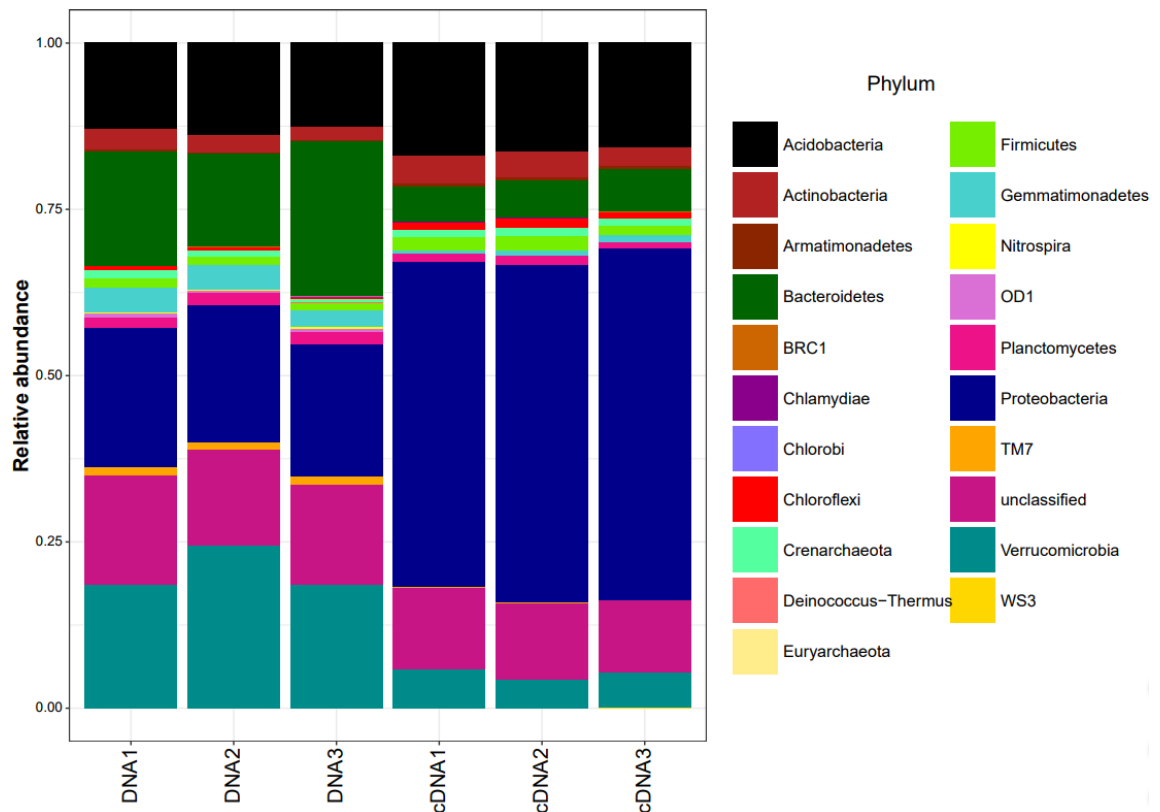
Figure 2.8. Prokaryotic community composition from the DNA and cDNA fractions. Relative abundances are shown at the phylum level, for the three replicates extracted from Soil 2 (DNA1, DNA2, DNA3 and cDNA1, cDNA2, cDNA3).

Meanwhile *Verrumicrobia* showed the opposite trend and were in higher relative abundances in the DNA fractions that the cDNA fractions. Thus, we can conclude that sequencing depth was sufficient to observe differences between relative abundance of species present (DNA) compared to those active at the time of sampling (cDNA), even when observed at the phylum level (Figure 2.8). To investigate difference between the fractions at a finer taxonomic scale, one replicate (replicate b) from Soil 2 was chosen for further analyses. This sample was chosen to mirror protein data presented later in the chapter, where it was selected as it had the median number of meta-proteins identified (Table 2.8).

Krona plots were used for this analysis and depict the relative abundances of OTUs from Soil 2 replicate b at the levels of domain, phylum and class (Figure 2.9). Marked

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differences were seen at the level of class, with *Spartobacteria* being the most dominant in the DNA fraction, making up 22 % of the samples relative abundance (Figure 2.9a). Meanwhile, in the cDNA sample, *Alphaproteobacteria* was the most abundant class, at 23 % (Figure 2.9b). Each Krona plot is coloured according to relative abundances of individual OTUs within the phylotypes presented; the most abundant OTU in the DNA sample, at 18.4 %, was a member of the *Spartobacteria,* while in the cDNA sample, it was a member of the *Gammaproteobacteria*, with a relative abundance of 7.3 %.

Figure 2.9. Relative abundances of prokaryotic communities from Soil 2 (replicate b) from a) the DNA fraction and b) the cDNA fraction. Abundances are depicted at the levels of phylum (inner ring), order (central ring) and class (outer ring). Plots are

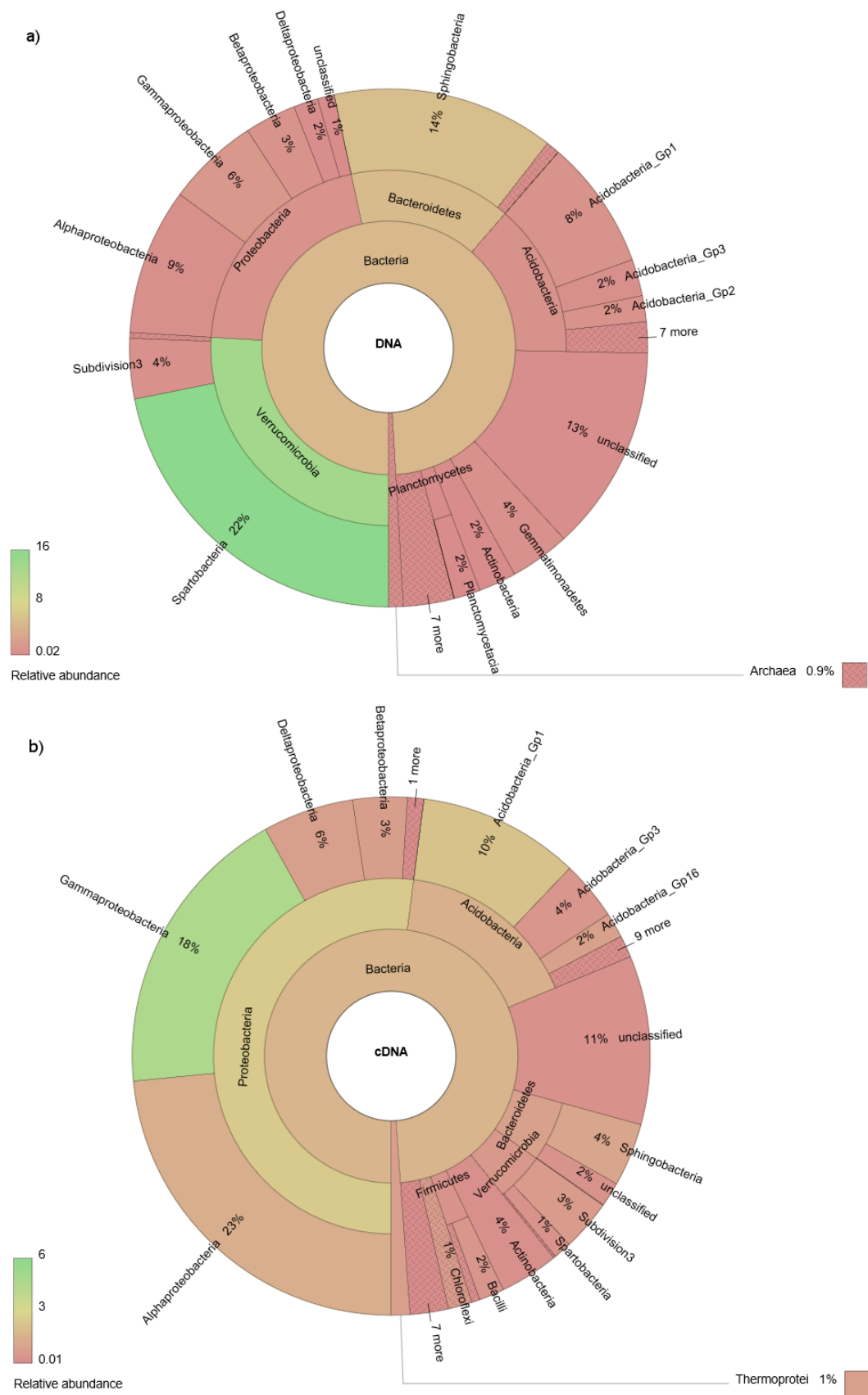61

coloured by relative abundance at the OTU level, with the scale depicted in each of the relevant panels. For the purposes of clarity, data from only one replicate sample are plotted (replicate b). This sample was chosen based on protein data, as it had the median number of metaproteins identified (after removing contaminants; Table 2.8).

2.3.3.2 Metaproteomics

Metaproteomic analysis of protein fractions from the triplicate Soil 2 samples revealed that while high total numbers of spectra were observed, roughly only 1 to 2 % were identified (Table 2.8), which is in line with other soil studies (Keiblinger *et al.* 2012).

Table 2.8: Total number of spectra, those assigned to peptides and the number of distinct and unique peptides identified using the MPA, for each of the 3 replicate protein extracts obtained from Soil 2.

ID	Total spectra	Identified spectra	Distinct peptides	Unique peptides	Meta-proteins
Replicate a	132028	840	516	397	327
Replicate b	127914	907	513	338	282
Replicate c	124790	900	445	304	252

This low identification rate is most likely due to insufficient availability of soil-relevant proteomic databases, but also to the well documented interference of humic substances with soil-extracted proteins (Bastida *et al.* 2009). The use of a matched metagenome, allowing for the 'meta-peptide' approach detailed by May *et al.* (2016) would surely have improved the identification rate (Tang *et al.* 2016), however was beyond the scope of this work. A concerted effort was made to run the peptide searches against an unmatched metagenome, originating from a grassland at Rothamstead research station (Delmont *et al.* 2012). However, as this metagenome feature of the MetaProteomeAnalyser was recently implemented, we were unable, at the time of analysis, to perform the searches adequately in the time available. To reduce data redundancy, protein hits were binned into meta-proteins using MPA (with the peptide

rule of at least on peptide in common), and between 252 and 327 meta-proteins were identified per sample (Appendix II).

To determine protein fractions accessed by this co-extraction method, an aspect known to vary according to extraction protocols (Maron *et al.* 2007), meta-proteins were grouped according to their cellular component ontology (Figure 2.10).
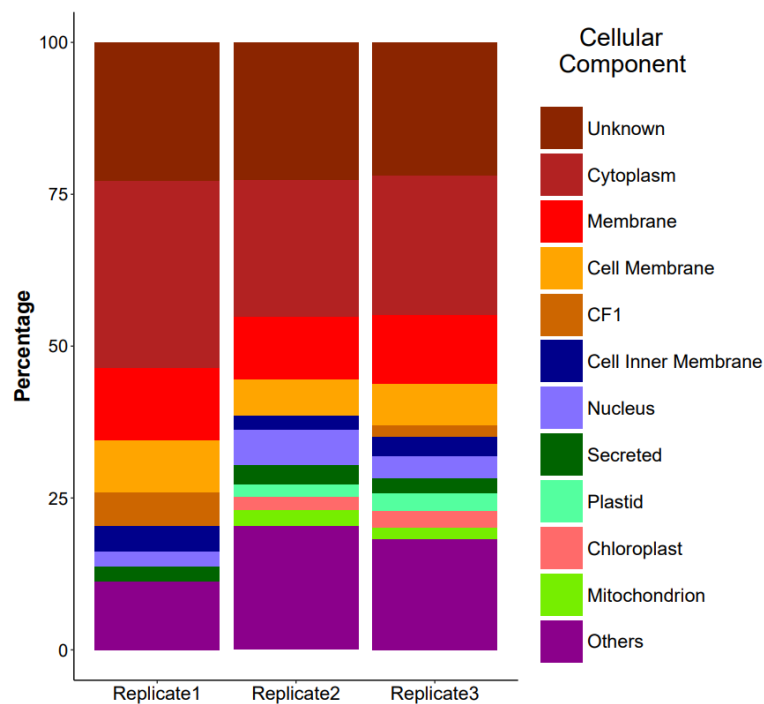
Figure 2.10. Cellular component ontology per replicate sample, showing the cellular location of the meta-proteins identified. Components not present at more than 2 % in any sample are grouped into 'Others' while meta-proteins where no cellular component ontology information was available are grouped into 'Unknown'

The most represented component, across all replicates, was the cytoplasm, followed by the membrane and cell membrane. Many meta-proteins could not be classified as a function of their cellular location, likely a consequence of insufficient database information exacerbated by the fact that many are expected to be extra-cellular proteins (Bastida *et al.* 2009).

For the purpose of investigating protein function, meta-proteins were grouped according to their molecular function ontology (Figure 2.11). While a diverse range of ontologies were seen, meta-proteins assigned to the ontologies of transferases and hydrolases were the most frequently observed in all samples. Highly represented functions were consistently observed in all three replicates however, differences can be seen in less represented functions. This highlights sample heterogeneity and the need for co-extraction.
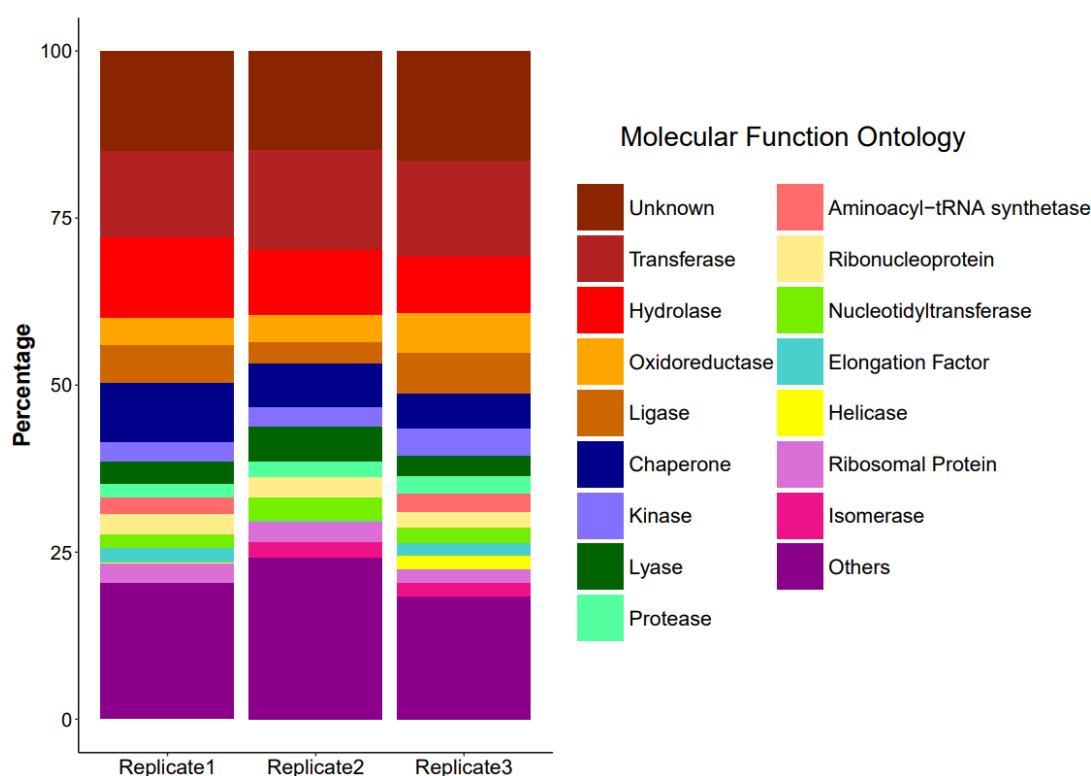
Figure 2.11. Grouping of meta-proteins by molecular function ontology, per replicate sample extracted from Soil 2. Functions represented at less than 2 % in any sample are grouped into 'Others' and meta-proteins where no molecular function ontology information was available are grouped into 'Unknown'.

Metaproteomics is a powerful tool for linking phylogeny with function, and adequate data visualisation needs to be applied to capitalise on this valuable information. For this purpose, Krona plots were used in order to efficiently represent these two facets of the metaproteomics data. For clarity only one replicate sample was used for the

Krona plot analysis, replicate 2 was chosen for this purpose as it had the median number of protein identified (Table 2.8). The KO functional categories of 'Metabolism' and 'Genetic Information Processing' were the most represented within the taxa detected (Figure 2.12).
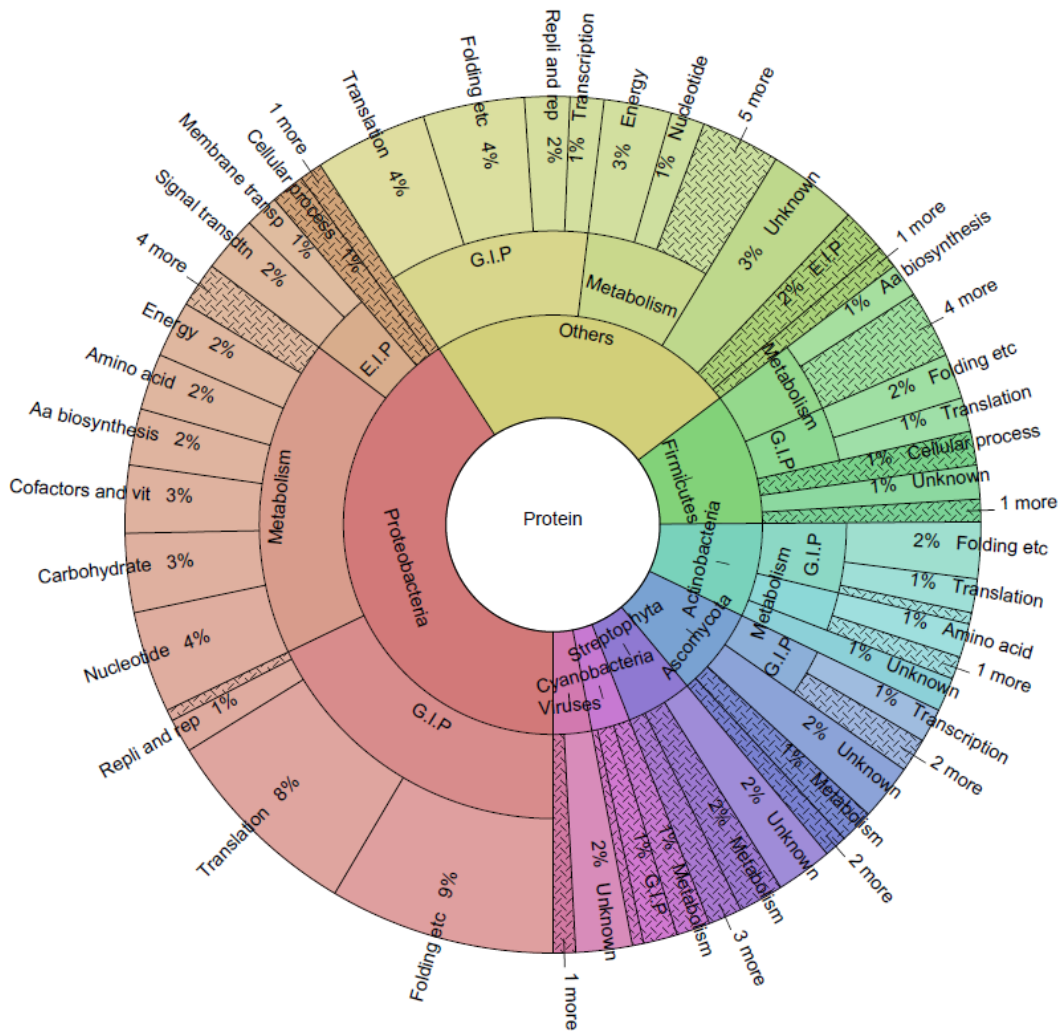
Figure 2.12. Krona plot showing taxonomic classification of metaproteins detected at the phylum level, (in the interior segments), with their associated KEGG ontologies (in the outer segments). Any phylum present at < 3 % relative abundance of this sample (Soil 2; replicate b) were grouped into 'Others'. Abbreviated KEGG ontologies are depicted as follows: G.I.P = Genetic information processing; E.I.P = Environmental information processing; Folding etc = Folding, sorting and degradation; Repli & rep = Replication and repair; Cofactors & vit = cofactor and vitamin metabolism; Aa biosynthesis = Biosynthesis of amino acids; Signal transdtn = signal transduction; Terps & polyks = Metabolism of terpenoids and polyketides. For an interactive version of the Krona plot see Data Accessibility section.

Within the 'Metabolism' category, 'Energy metabolism' is the most frequently observed across all taxa, although not the most abundant. Regarding 'Genetic Information Processing', across all proteins and phyla, the sub-categories 'Translation' and 'Folding, sorting and degradation' (in particular chaperones) were the most highly represented. Looking at function and taxonomy together, it appears that across the phyla, the categories of 'Genetic information processing' and 'metabolism' are the most represented (Figure 2.12). In this sample, the majority of the proteins identified were assigned to *Proteobacteria* (41 %). The dominance of this phylum is also observed within the 16S rRNA dataset, but of note is the increased relative abundance of *Proteobacteria* at the cDNA level (52 %) compared to the DNA level (21 %) (Figure 2.9). Taken together these observations highlight that caution must be applied when inferring functional contribution of specific taxa to ecosystem functioning based on DNA relative abundance data alone. It is worth noting that proteins assigned to viruses, protists, fungi and plants could also be detected as metaproteomics is by nature untargeted (Figure 2.12; Appendix II).

2.4   Discussion

Systems biology based investigations of complex, mixed microbial communities are now within reach thanks to the multi-omics revolution. These omics tools have allowed for increasingly in-depth explorations of previously uncharacterised complex microbial communities, however despite this, significant hurdles are still to be overcome. A frequently occurring theme in recent years has been the need for extensive culture-dependent work to corroborate molecular based assumptions, and considerable advances in this field have been achieved, as reviewed by Lagier *et al.*

(2015). Additionally, logistical issues exist, relating to computational efficiency and data storage when analysing the mega-data produced by such techniques (Hahn *et al.* 2016). We must also consider the appropriate use of sequencing-based tools if we are to answer ecologically relevant hypotheses (Prosser 2012). As detailed by Muller *et al.* (2013), sound experimental design and the use of suitable analytical tools and methods to interpret -omics data will help to progress the field. This includes taking a holistic approach where data are integrated for more meaningful inferences of systems processes. Due to the inherently complex and heterogenous nature of mixed microbial communities, the biomolecules required to produce these integrable data sets should be isolated from the same biological sample. This point of view, as well as a co-extraction method appropriate for their samples of interest (wastewater sludge, water and faeces), has been detailed by Roume *et al.* (2013). Due to the paucity of databases with regards to soil microbiome representatives, as well as the minute spatial heterogeneity, it seems especially pertinent that biomolecules used for culture independent techniques originate from the same biological sample. Additionally, the sample should be handled in such a way that each isolation step does not introduce further bias. The method presented herein was developed to provide a robust soil co-extraction method that fulfilled these requirements. The method developed here allowed for a cost-effective, robust means of obtaining sufficient yields of DNA, RNA and proteins from all sample types tested and its applicability was proven through the investigation of community composition (DNA and RNA 16S rRNA analysis) and function (metaproteomics) of a complex soil microbial community. The sample to sample variation between the biological replicates used for these analyses was clearly demonstrated and further emphasizes the importance of extracting from the same biological sample when integrating data sets for either improved annotation and/or

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biological interpretation. We hope that the accessibility of this method, both in terms

of cost-effectiveness and ease of implementation, together with its adaptability will

allow for its use with a diverse range of environmental samples in order to expand

knowledge of microbial community assemblages and microbial ecology.

2.5   Data Accessibility

16SrRNA sequence data were deposited on NCBI's Sequence Read Archive under the accession number SRP111658, which can be accessed through the following link to the Biosample: http://www.ncbi.nlm.nih.gov/biosample/7345359.

Data associated with metaproteomics was deposited to the ProteomeXchange Consortium via the PRIDE partner repository:
http://www.ebi.ac.uk/pride/; with the dataset identifier PXD007077
Reviewer account details for PRIDE:
Username: reviewer93755@ebi.ac.uk
Password: ViPdTa0l

Commands used in Mothur as well as Krona plots are available at the research group's GitHub page:
https://github.com/FEMLab/DNA-RNA-Protein-Coextraction-Method-Soil

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--------- Chapter III ---------

Investigation into the effects of lime application and barley crop development on the rhizosphere - associated microbiome

3.1 Introduction

Soils classed as acidic, with a mean annual pH of less than 5.5, cover approximately 30 % of the globe, with increased coverage in high rainfall areas such as North Western Europe (Merry 2009). To improve plant productivity many of these soils are amended with lime, typically crushed rock rich in carbonates, to increase the pH (Truog 1947). However, the process of weathering such rock chips in order to release the carbonate which alters pH, is not quick and typically the effects of liming are only seen one to two years post-application (Lukin & Epplin 2003). As many farmers in Ireland lease land on a year-to-year basis, known as the 'conacre' scheme, incentive to invest in a treatment with benefit only seen after the lease has ended is understandably low (Donnellan *et al.* 2015). Hence, 'quick lime' products (calcium oxide), which typically have a faster effect on soil pH, mostly due to their much finer particle size (Peters *et al.* 1996), represent a more cost-effective solution.

The work presented in this chapter was part of a collaboration involving the testing of such a product (Growmax), new to the Irish market, on spring barley (Hordeum vulgare) in terms of both plant productivity and changes in the soil microbial community. This is the largest spring crop grown in Ireland, with an average of 150,000 hectares sown annually (Boyle 2015) thus representing significant economic value for the country. Barley has an optimum pH of 6.5 (Fageria *et al.* 1990) and thus often cultivation of this crop in Ireland is associated with lime application.

We believe there existed a research gap in this field, whereby deeper characterisation of the microbial community changes associated with lime-induced pH shifts was needed. To tackle this, we employed culture-independent techniques, namely 16S rRNA profiling, for microbial community analysis. The use of DNA as a template for

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investigating complex microbial community membership via 16S rRNA analysis is widespread, however there are some caveats to consider. This includes the fact that the presence of DNA does not necessarily infer that the corresponding organism is active and contributing to any phenotype seen. Secondly, DNA has been shown to be relatively stable in the soil environment, resulting in 'relic' DNA that can inflate estimates of bacterial abundance and diversity (Carini *et al.* 2016). Looking at microbial community structure based on the 16S rRNA transcripts, in conjunction with 16S rRNA genes can help to overcome these caveats and thus DNA and RNA were co-extracted from the same soil samples prior to 16S rRNA sequencing. One of the benefits of RNA in the context of microbial community analysis is its more rapid turnover in contrast to that of DNA; for example Ostle *et al.* (2003) estimated, using isotope analysis, that microbial RNA turnover was around 20 % per day in their soil microcosms which equated to a total RNA pool replacement roughly every five days. They also demonstrated how rapidly photosynthetically derived carbon is transferred and respired at the root-soil interface (within 5 hours to 2 days) and then incorporated into rhizospheric bacterial RNA (4 to 8 days) thus illustrating the strong effect of the plant over soil dwelling microbiota in their vicinity. Indeed, soil surrounding plant roots represents a hotspot for microbial activity, with increases in microbial biomass between bulk soil and the rhizosphere shown to be around 200 %, where plant derived carbon accounted for 68 % of this increase (Helal & Sauerbeck 1986). The rhizosphere therefore represents the most suitable environment to sample from when investigating the effects of agricultural amendments such as lime upon the soil microbiota. Additionally, due to variations in root exudates as a function of the developmental stage of plants (Chaparro *et al.* 2013) and the strong influence this has on the root-

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associated microbiota (Chaparro *et al.* 2014), it is prudent to follow such communities on a temporal basis.

A field trial of Spring Barley grown on acidic soil, in the presence and absence of quick lime amendment, was set up and the overall aims of the study were to evidence the rapid action of Growmax, in terms of i) improvement in plant productivity and ii) the corresponding rhizosphere-associated soil microbial communities, assessed by 16S rRNA profiling, at three distinct growth stages of the barley, namely stem elongation, heading and ripening.

3.2   Materials and methods

3.2.1   Experimental design and sample collection

The field trial was undertaken in Kilavullen, Co. Cork on a site where Spring Barley had been grown for the two prior years (2011 and 2012). The soil at this site is a loamy brown-earth soil. The trial was set out as follows, plots measuring 1.8 by 21.5 metres were treated with a commercial agricultural lime (CaO) product (Growmax). Among the application rates tested were: negative control (0 kg/hectare) and high lime (950 kg/hectare). Each treatment was repeated over four plots making a total of 8 plots set out in a random block design (Figure 3.1)..

Figure 3.1. Field trial plots immediately following lime application. The photograph is taken during lime application and different loading rates can be seen.

The drill planting method was then used to sow all plots with Spring barley (*Hordeum vulgare* cv. Propino) at a density of 155 kg ha^{-1}. All plots were then subject to the same treatments of fertiliser and herbicide throughout the trial, the last of which was on week 10 of the trial (details in Appendix III). The plots were established in mid-April 2014, and throughout the field trial, pH was monitored fortnightly and crop productivity measured as function of the plant weight of 10 randomly selected plants/plot. Three time points were chosen for taking soil samples for microbial community analysis week 12, week 15 and week 19. These corresponded to following growth stages of barley; stem elongation, heading and ripening, respectively. On the day of microbial sampling, ten plants were randomly selected from each plot and the closely attached soil was gently removed from the root mass and immediately sieved through a 0.5 mm sieve to remove rocks and large roots. For each plot, duplicate aliquots of 0.5 *g* soil were placed in 2 ml screw top micro centrifuges tubes and flash frozen in liquid nitrogen. These duplicates will here-in be referred to as 'technical' replicates while 'biological' replicates will refer to samples from the 4 × replicated plots. All samples

remained immersed and stored in liquid nitrogen until returning to the laboratory where they were placed at -80 °C until nucleic acid extractions were performed.

3.2.2 Soil pH

Soil pH was measured following the guidelines of AOAC Official Method 994.16. Due to limited sample amount, roughly 3 g of soil was removed from storage at -80 °C and dried for 48 °C hours at 40 °C. From this, 2 g of dry soil was weighed into a 15 ml centrifuge tube and 2 ml of dH_2O was added. The tubes were then shaken at 25 °C and 90 rpm for one hour (New Brunswick™ Innova® 2300), after which they were left to settle for 10 minutes. After calibration, pH was measured using a Hanna HI 2210 pH meter and data recorded.

3.2.3 Sample preparation for downstream analyses

In order to reduce any bias associated with nucleic acid extractions, these and other handling steps including DNase treatment and cDNA generation were performed on samples chosen in a randomised manner.

3.2.3.1 Nucleic acid extraction

DNA and RNA were extracted following a protocol based on that of Griffiths *et al.* (2000) as detailed in section 2.2.2.1. A modified 5 % hexadecyltrimethylammonium bromide (CTAB) extraction buffer was used, consisting of 0.2 mM casein added to the buffer of Griffiths *et al.* (2000) prior to autoclaving (Wang *et al.* 2012). Soil samples were removed from -80 °C and placed on dry ice while 500 µl of extraction buffer was added and very briefly vortexed to ensure an even contact of the pre-treatment solution with soil while ensuring minimal cell disruption. Zirconia beads (0.5 g of a mix of 0.1mm and 0.5mm zirconia beads) were inserted into each tube with 500 µl of Phenol:

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Chloroform: Isoamyl Alcohol (P:C:I; 25:24:1). In order to improve RNA yields, P:C:I at a slightly acidic pH of 6.1 was used. Tubes were vortexed at high speed for 2 minutes 45 seconds, followed by centrifugation at 16 000 g for 10 minutes at 4 °C. The resulting top aqueous phase was removed and placed in a sterile, pre-cooled, 1.5 ml micro-centrifuge tube with 500 µl of Chloroform: Isoamyl Alcohol (24:1). Samples were briefly vortexed then centrifuged at 4 °C and 16 000 g for 5 minutes after which the top aqueous phase was again removed and placed in a sterile 2 ml centrifuge tube. Nucleic acids were precipitated *via* the addition of two volumes of a polyethylene glycol (PEG) 6000 solution (30 % PEG; 1.6 M NaCl) and samples were refrigerated for two hours. Nucleic acids were collected by centrifugation at 16 000 g and 4 °C for 20 minutes and the resulting pellet was washed in 1 ml of ethanol. Samples were mixed by inverting the tubes several times, and DNA and RNA were then pelleted via centrifugation at 16 000 g and 4 °C for 25 minutes. Ethanol was poured off and the tubes briefly centrifuged to allow removal of any residual ethanol by pipette, followed by a brief air dry under a flame. The pellet was re-suspended in 35 µl of nuclease free water and 4 µl was run on a 1 % agarose gel to check for DNA and RNA integrity. Nucleic acid concentrations were quantified using Qubit assays and quality was assessed using NanoDrop ratios (260:230 and 260:280). Samples either proceeded directly to the DNase treatment or were snap frozen in liquid nitrogen and stored in aliquots at -80 °C.

3.2.3.2   DNase treatment and cDNA synthesis

RNA was rendered DNase treated to remove genomic DNA, where complete removal of DNA was verified *via* PCR, followed by normalisation and cDNA generation following the methods in section 2.2.3.1.

3.2.3.3 Amplification of 16S rRNA gene with Golay Barcoded primers

A pool of purified, barcoded, 250 bp amplicons of the 16S rRNA V4, from both the DNA and cDNA fractions were prepared as detailed in section 2.2.3.3. Samples were pooled in equimolar amounts of 2.1 ng resulting in a final pool of 100 ng of purified amplicons. DNA and cDNA pools were shipped on ice to the Centre of Genomic Research (Liverpool) to undergo paired-end sequencing on the Illumina MiSeq platform. Due to the large number of samples, 48 for each fraction, the DNA and cDNA samples were run on two separate MiSeq runs. We believe that running all 96 samples on one run could have significantly compromised the sequencing depth.

3.2.4 Quantitative PCR analysis

Changes in abundance of 16S rRNA gene copy numbers, at the DNA and cDNA level, were determined using quantitative PCR (qPCR). Standard curves were generated using serial dilutions of purified, 16S rRNA gene amplicons constructed using the primers 27F and 1087R according to the method and cycle conditions in section 2.2.3.2. Aliquots of purified amplicons were stored neat, at -80 °C, with each aliquot undergoing a single freeze-thaw cycle to minimise the risk of degradation (Dhanasekaran *et al.* 2010). Serial dilutions were made using PCR-grade water on the day of PCR quantitation and then discarded. All soil DNA / cDNA samples were normalised to a concentration of 2 ng/µl prior to amplification. Each 20 µl reaction contained 10 µl of 2 Takyon™ SYBR® MasterMix (Eurogentec), 0.6 µl of each 10 µM primer (515F/806R), 6.8 µl of PCR-grade water and 2 µl of template. Standards were amplified in triplicate and samples in duplicate. Amplification was performed on a Roche LightCycler 480, where initial denaturation / activation was performed at 95 °C for 3 minutes followed by 40 cycles of 95 °C for 10 seconds and 60 °C for 45

seconds, with light acquisition performed at 77 °C for 1 second at the end of each cycle. Melt curve analysis was performed for all samples, and amplicons were run on a 1 % agarose gel to verify correct amplicon size. A regression equation was acquired from standard curves and used to calculate gene copy numbers from the Cp values. Samples were tested for inhibition by spiking a known concentration of standard with 2 µl of sample and checking for any reduction in the expected Cp value (based on a non-spiked sample).

3.2.5 Bioinformatic analysis of 16S rRNA gene sequence data.

De-multiplexed files were received from the sequencing facility, and sequences were quality trimmed, de-noised and clustered into OTUs at a 97 % threshold, using the same databases and protocol detailed in section 2.2.5.1.

3.2.6 OTU based analyses

Alpha and beta diversity metrics were calculated using Mothur (Schloss *et al.* 2009) and the statistical programme R (R Core Team 2017), as detailed in section 1.2.5.2.

3.2.6.1 *Alpha diversity of soil microbial communities*

Rarefaction curves were constructed and percentage coverage, observed richness, species diversity and evenness were calculated as implemented in Mothur. To check for statistically significant differences in means of alpha diversity indices between treatments, samples were first tested for normality using a Shapiro–Wilk test as implemented in R. If data was normal (Shapiro $p > 0.05$), a one-way ANOVA followed by a Tukey's post-hoc test was performed. For non-normal data (Shapiro $p < 0.05$) a

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Kruskal-Wallis followed by a post-hoc Dunn test was performed. Resulting p values were collated and appropriately adjust for multiple testing (FDR approach).

3.2.6.2 Beta diversity of soil microbial communities

Beta diversity was calculated *via* the generation of distance matrices determined using the occurrence based Jaccard index where J represents Jaccard similarity and is calculated as follows:

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$$J(A,B) = \frac{|A \cap B|}{|A| + |B| - |A \cap B|}$$

Where:

$A \cap B$ = the number of OTUs shared between communities A and B
$|A|$ and $|B|$ = the number of OTUs in communities A and B respectively.

Differences in community structure were calculated using the relative abundance based index of Yue and Clayton theta (θ) as follows:

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$$\theta = \frac{\sum_{i=1}^{AB} \left(\frac{Xi}{n_{total}}\right)\left(\frac{Yi}{m_{total}}\right)}{\sum_{i=1}^{A} \left(\frac{Xi}{n_{total}}\right)^2 + \sum_{i=1}^{B} \left(\frac{Yi}{m_{total}}\right)^2 - \sum_{i=1}^{AB} \left(\frac{Xi}{n_{total}}\right)\left(\frac{Yi}{m_{total}}\right)}$$

Where:

AB = the number of shared OTUs between samples A and B;
X_i, and Y_i = the abundances of the ith shared OTU in samples A and B, respectively
n_{total} = the total numbers of sequences sampled in A and
m_{total} = the total numbers of sequences in sample B.

Further details regarding these formulae can be found in Schloss & Handelsman (2006).

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Hierarchal clustering using the UPGMA (Unweighted Pair Group Method using arithmetic Averages) algorithm (Sneath & Sokal 1973) was performed and visualised using dendrograms plotted in FigTree (Rambaut 2012). Non-metric multidimensional scaling (nMDS) was also used to visualise the dissimilarity of the samples, based on the Yue and Clayton theta distance matrix. To test if observed clustering of samples was statistically significant, a non-parametric analysis of similarities (ANOSIM) was performed (CLARKE 1993), in addition to a non-parametric calculation of analysis of variance, namely the analysis of molecular variance test (AMOVA) (Excoffier 1993). Homogeneity of variance (non-parametric) was calculated (HOMOVA) (Stewart and Excoffier 1996). To determine how the OTUs influenced the distribution of samples within the nMDS plot, Spearman's correlation coefficient of the relative abundance of each OTU with the nMDS axes was calculated (Ramette 2007). This was done in order to determine which OTUs were contributing to positioning the corresponding samples along the nMDS axes. Linear discriminant analysis (LDA) as implemented in the LEfSe tool of Segata *et al.* (2011) was employed to identify differentially abundant OTUs between experimental groups. These were identified by a Wilcoxon rank-sum test (with alpha set to 0.05), where the effect size of each OTU detected was denoted by an LDA score (where a threshold score of 2 was used). For both alpha and beta diversity analysis, the R packages phyloseq (McMurdie & Holmes 2013) and ggplot2 (Wickham 2009) were used for data handling and visualisation.

3.3 Results

3.3.1 Effect of lime on soil pH and crop yields

Growmax application did not significantly affect the soil pH (Figure 3.2 and Table 3.1) or the crop yields throughout the trial, where no statistically significant differences were seen in grain yield between the untreated control and the Growmax treated plots (data not shown). While the pH did slightly increase in the plots treated with Growmax (Figure 3.2), this increase was not statistically significant at any points of the trial (Table 3.1).
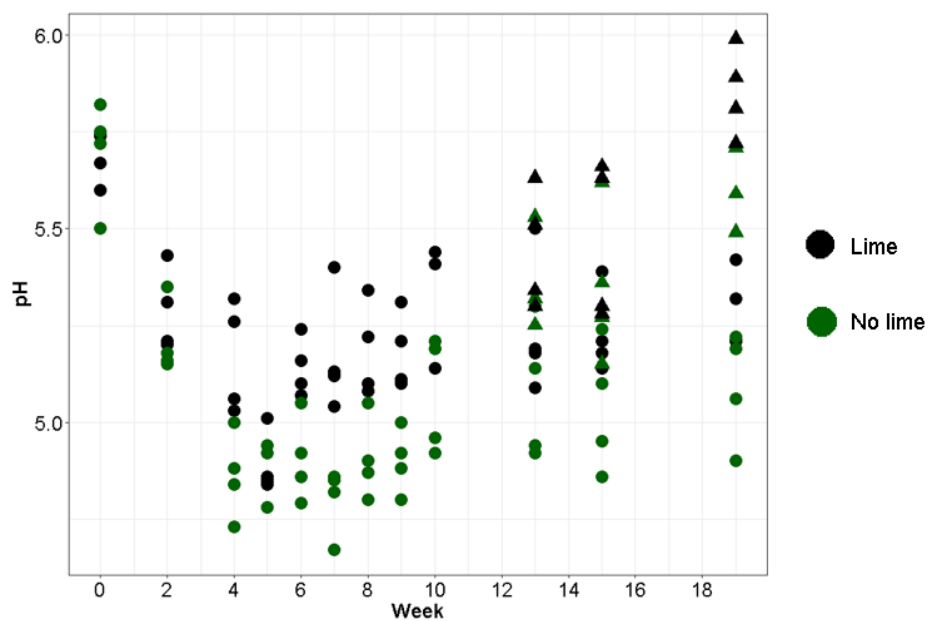

Figure 3.2. Soil pH throughout the field trial. Each circular data point represents the pH measured from composite soil samples from each of the four replicate plots (one data point per plot) in the presence (black) and absence (green) of Growmax application. Triangular shapes represent pH values obtained from the same root-associated soil from which nucleic acids were extracted.

Table 3.1: Mean soil pH from control and Growmax treated plots throughout field trial. Calculated using data from four replicate plots in the presence and absence of Growmax. Statistical significance is represented using FDR-adjusted p values determined by two tailed t-tests, subsequent to testing for equality of variance.

Week of trial	Mean pH Control	Mean pH Growmax	FDR adjusted p values
0	5.70	5.63	0.50
2	5.21	5.29	0.50
4	4.86	5.17	0.13
5	4.89	4.89	1.00
6	4.91	5.14	0.12
7	4.80	5.17	0.12
8	4.91	5.19	0.12
9	4.90	5.18	0.12
10	5.07	5.28	0.24
13	5.21	5.34	0.16
15	5.19	5.35	0.12
19	5.37	5.57	0.12

3.3.2 Microbial community analysis

3.3.2.1 *Bacterial abundance based on 16S rRNA copy numbers.*

Changes in mean 16S rRNA copy number as a result of lime treatment were determined for each nucleic acid fraction and at each barley growth stage (Figure 3.3), where the only statistically significant difference was observed in the DNA fraction in samples taken from during the elongation phase (Table 3.2). Comparison of 16S rRNA gene copy numbers, per gram of soil, demonstrated an approximate 10-fold increase between gene copy numbers (DNA) and transcript copy numbers (cDNA) (Figure 3.3), with numbers of transcript copies being higher at all three sample points. These differences were statistically significant for all time points (Table 3.2).
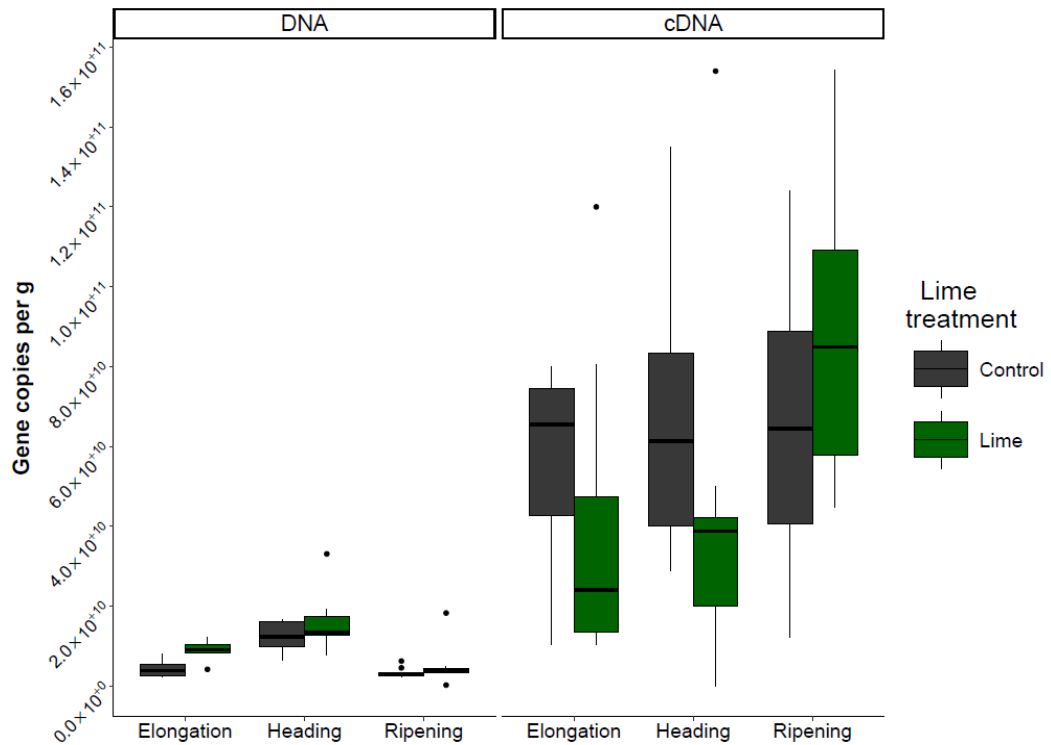
Figure 3.3: 16S rRNA gene and transcript copy numbers, in DNA and cDNA samples as a function of lime treatment at each barley growth stage. Copy numbers are calculated per gram (dry weight) of soil. Statistically significant difference in mean copy number are detailed in Table 2.2.

Considering variation in each nucleic acid fraction as a function of time, there was more statistically significant variation in copy numbers in the DNA samples, with higher copy numbers present during heading compared to both elongation and ripening phases. The only significant change in the number of rRNA transcripts (cDNA) observed as a function of time / crop development, was between stem elongation and the ripening phase where higher copy numbers were seen in the latter. This difference however, showed borderline significance at $p = 0.04$.

Table 3.2: Statistical significance of changes in mean 16S rRNA gene or transcript copy numbers. Significance was tested both within and between treatments (i.e. crop development), where T1 = stem elongation; T2 = heading and T3 = ripening. FDR-adjusted p values were determined by t-tests (two-tailed), subsequent to testing for equality of variance.

Interaction		FDR adjusted p value
DNA Lime vs control	Elongation	0.009
	Heading	0.38
	Ripening	0.39
cDNA Lime vs control	Elongation	0.39
	Heading	0.39
	Ripening	0.39
DNA vs cDNA	Elongation	0.001
	Heading	0.004
	Ripening	< 0.0001
DNA	T1 vs T2	0.001
	T1 vs T3	0.16
	T2 vs T3	0.0001
cDNA	T1 vs T2	0.55
	T1 vs T3	0.04
	T2 vs T3	0.12

3.3.2.2 *Alpha diversity of prokaryotic communities*

The rarefaction curves for DNA and cDNA samples (Figure 3.4 and Figure 3.5 respectively) depict the number of new OTUs observed upon randomly selecting 100 sequences from the dataset. When curves plateau, this implies that sampling effort was sufficient to acquire an adequate representation of the community of interest. In the present study, the sequencing depth appeared to be sufficient for DNA samples, as sample rarefaction curves begin to plateau (Figure 3.4), which was not the case for the

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corresponding cDNA samples (Figure 3.5). This latter observation is in line with a

number of other soil sequencing projects reporting rarefaction curves that do not

plateau (see section 2.3.3.1).
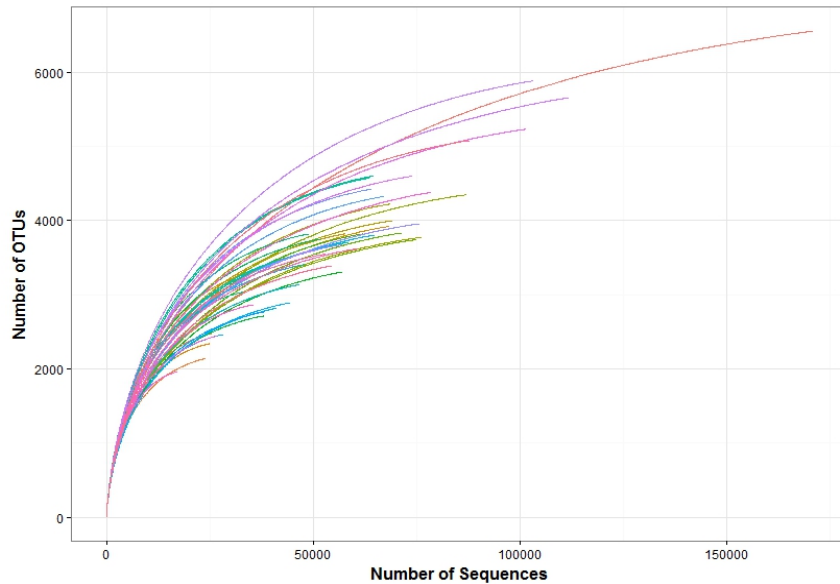
Figure 3.4: Rarefaction curves for all 48 DNA samples. The number of OTUs detected as a function of sequences allows for the comparison of richness of the un-subsampled DNA dataset, from all samples, as a measure of the sampling intensity. Each sample is represented by a single line, and no legend is included.
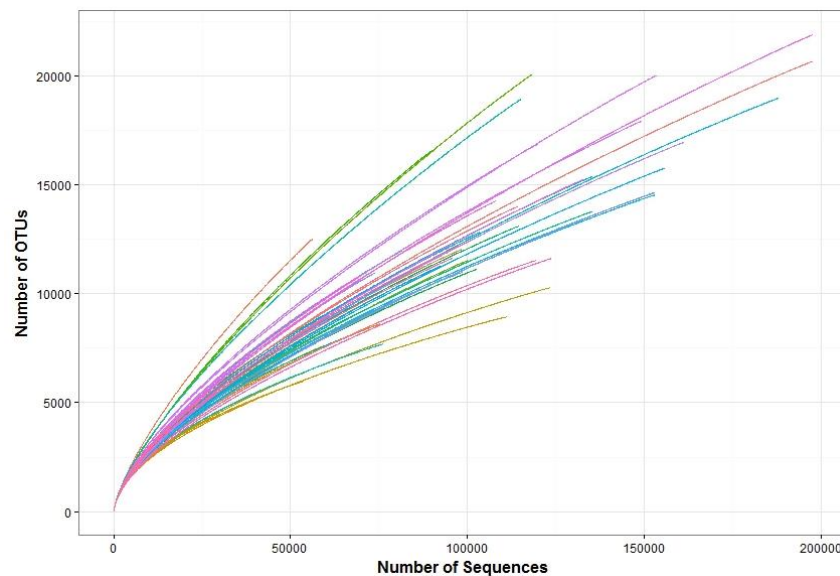
Figure 3.5: Rarefaction curves for all 48 cDNA samples. Comparing richness of the un-subsampled cDNA dataset, from all samples, as a measure of the sampling intensity. Each sample is represented by a single line, and no legend is included.

This difference between DNA and cDNA sampling depth is reflected in sample coverage. (Figure 3.6A). For DNA samples, average coverage was ~ 94 %, meaning that 6 % of sequences are from OTUs that appear only once in the DNA samples. In turn, when looking at the cDNA data, the average sequence coverage was lower at 89 %, suggesting that around 11 % of sequences were singletons. It should be noted that despite these differences, for both sequencing runs, the coverage provided was close to 90 % and therefore regarded as sufficient resolution to allow for OTU-based analyses (Lemos *et al.* 2011). The observed richness (Figure 3.6B) was significantly higher in the cDNA samples, with average of 8570 OTUs, compared to the corresponding DNA samples (average of 2555 OTUs). In each of the fractions (DNA and cDNA), there was no statistically significant difference in coverage or observed richness as a function of time. The fact that coverage within each nucleic acid fraction was consistent over the three time points (Figure 3.6A) suggests that any changes observed within the microbial communities are not due to artefacts related to sequencing depth discrepancies.
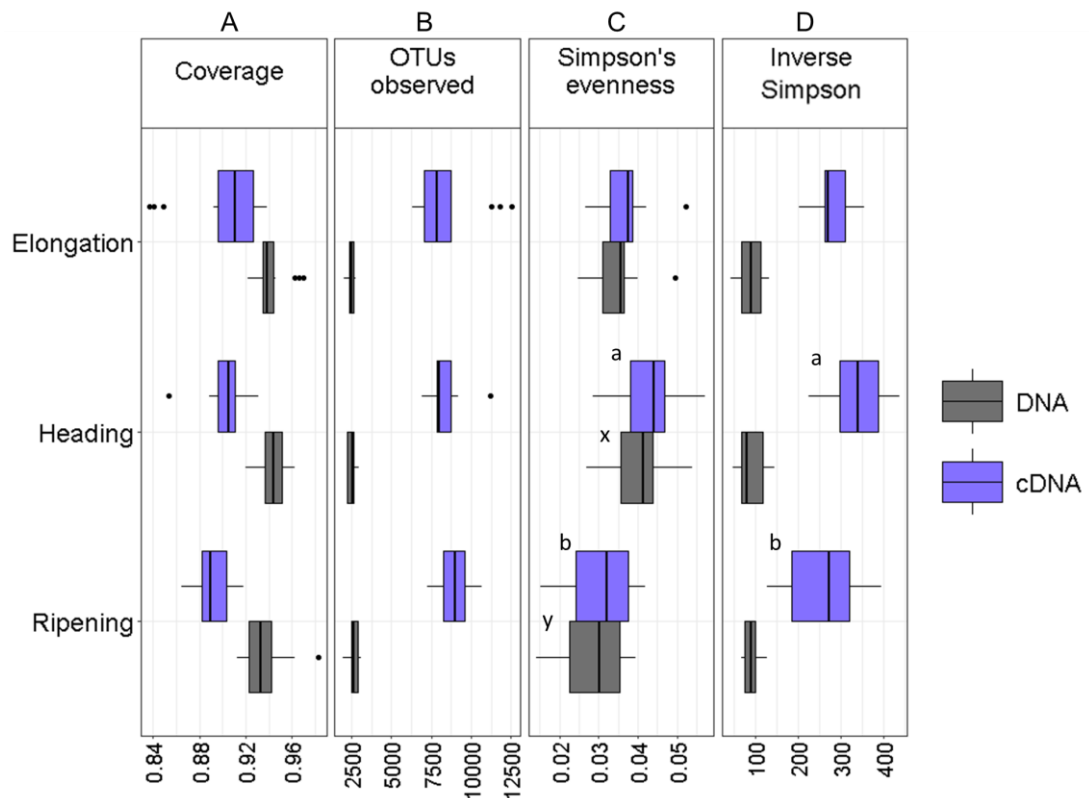
Figure 3.6. Alpha diversity metrics at the DNA and cDNA level for the three crop growth stages. Included is A)16S rRNA library coverage based on Good's estimator (with 0.9 or 90 % being acceptable) B) the OTU count per sample, when sequences are clustered to 97% similarity C) Simpson's evenness for defining numerical equality of the community and D) the inverse of the alpha diversity estimator detailed by Simpson (1949). Any statistically significant differences occurring as function of time, per fraction, are denoted by different letters when p < 0.01 (a – c for cDNA samples and x – z for DNA samples). FDR-adjusted p values were determined using one-way ANOVA with Tukey's post-hoc tests, subsequent to testing for data normality.

Evenness of the communities was very similar between fractions, where the same temporal pattern was followed for DNA and cDNA samples (Figure 3.6C). While there was no difference in community evenness between elongation and heading, a statistically significant decrease in evenness was observed between the heading and ripening phases for both nucleic acid fractions. Alpha diversity was calculated using the inverse Simpson's index, which takes into account species number and abundance. Thus, the higher OTU number observed at the cDNA level, implying increased richness, equated to a significantly larger measure of diversity, at the cDNA compared

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to the DNA level (Figure 3.6D). As it is not biologically possible to have more species present at the cDNA level than the DNA level, we propose that this could be partly due to artefacts relating to problems during the DNA sequencing run (namely sub-optimal formation of sequencing clusters, as reported by the sequencing facility). Additionally, it is worth noting that the majority of microbial species in soil are present in very low numbers within any given locale. As such, when DNA is extracted many of these rare taxa will remain undetected due to insufficient gene copy numbers present in the DNA pool compared to that from more abundant members of the community. Many bacterial species have multiple 16S rRNA gene copies within their genome, however, and as soon as favourable conditions are encountered, these ribosomal genes will be translated. If, for example, a bacterium is harbouring 3 x 16S rRNA copies, it would acquire $3 \times n$ copies of rRNA transcripts (where n is the number of transcription events), thus increasing correspondingly its chance of being detected at the RNA level compared to the DNA level.

3.3.2.3   Microbial community composition

The major bacterial phyla present remained relatively similar between all three crop growth stages sampled, and there was no effect of lime treatment at either the DNA level (Figure 3.7) or the cDNA level (Figure 3.8). When considering changes within each nucleic acid fraction as a function of time, the only evident change was an increased relative abundance of *Bacteroidetes* at the ripening stage in cDNA samples (Figure 3.8), while no differences were observable for any phylum in DNA samples (Figure 3.7).

Figure 3.7: Microbial community composition of all DNA samples. Where the relative abundances for all biological and technical replicates for heading, stem elongation and ripening are displayed, at phylum level. Samples from the stem elongation, heading and ripening phase are represented in the first, second and third panel respectively, where each bar represents an individual sample. Samples taken from plots receiving no lime treatment are denoted by a green dot on the x axis, while those receiving Growmax are identified by a black dot.

When comparing samples from the DNA and cDNA fractions however, a number of differences existed between the relative abundances of several phyla. This included a more significant representation of *Proteobacteria* and *Planctomycetes* in the cDNA (Figure 3.8) compared to the DNA samples (Figure 3.7), and conversely an increased relative abundance of *Bacteroidetes* and *Verrucomicrobia* in the DNA fraction compared to cDNA. OTUs belonging to the phylum of *Fusobacteria* were observed at low abundances in DNA samples (Figure 3.7), but were not detected in cDNA (Figure 3.8) samples. *Deinococcus thermus* and *Tenericutes* were detected in very low abundances in the cDNA fraction and were absent from the DNA fraction.
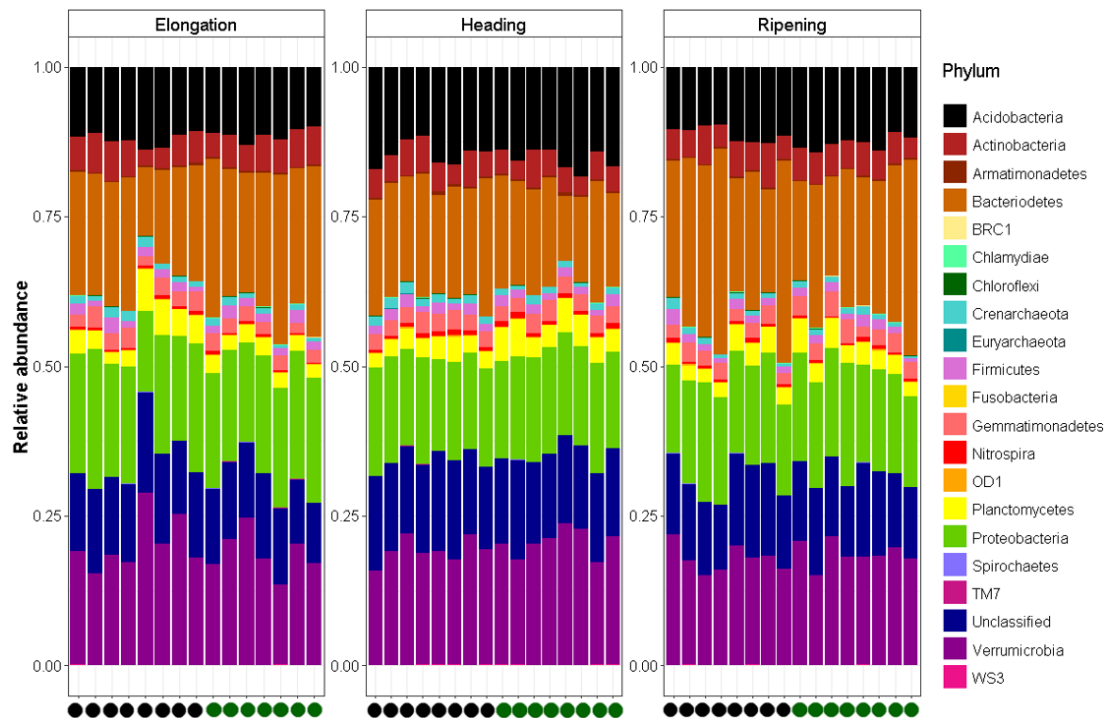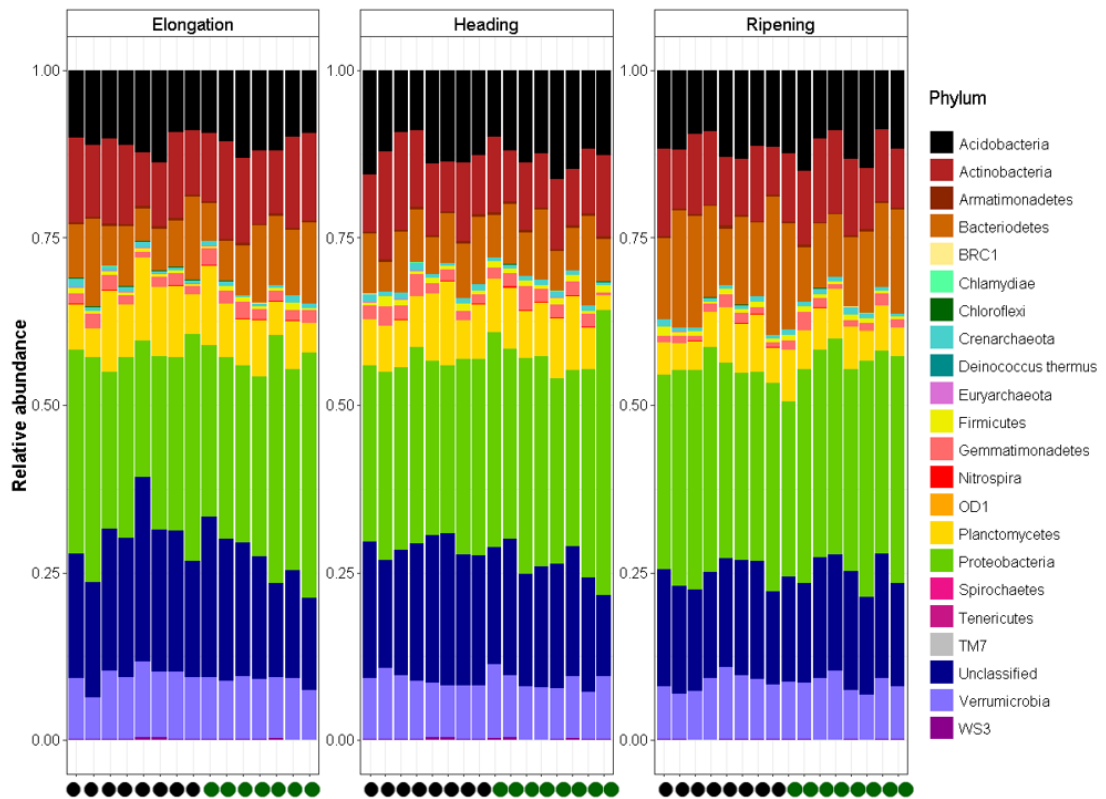
Figure 3.8: Microbial community composition of all cDNA samples. Where the relative abundances for all biological and technical replicates for heading, stem elongation and ripening are displayed, at phylum level. Samples from the stem elongation, heading and ripening phase are represented in the first, second and third panel respectively, where each bar represents an individual sample. Samples taken from plots receiving no lime treatment are denoted by a green dot on the x axis, while those receiving Growmax are identified by a black dot.

As no easily observable treatment effects were seen at this resolution, the next analyses involved detecting differences between the samples at an OTU level, thus providing a much finer resolution. It is worth noting that the sequencing strategy we employed was pair-end and targeting 250 bp of the 16S rRNA V4 region, which allows for fully overlapping paired-end reads, thus significantly reducing error rates and inflated numbers of erroneous OTUs (Kozich *et al.* 2013). Therefore, such a sequencing strategy permits some confidence when employing an OTU-centric analysis.

3.3.2.4 Differences in community composition: beta diversity

As the number of sequences was different between samples, OTU tables of the DNA and cDNA samples were both rarefied by subsampling datasets to the lowest number of sequences observed in each. This serves to reduce the bias that differences in sequence depth may have when looking at sample dissimilarity (Weiss *et al.* 2017).

Differences in microbial community structure between samples were represented with distance matrices constructed using a selection of dissimilarity indices. Hierarchal cluster analysis of DNA sample dissimilarity calculated with the Jaccard index of presence/absence was visualised in a dendrogram (Figure 3.9). This analysis revealed that DNA samples from the three time points varied little in the context of community membership as all the samples appear closely clustered under one branch of the tree (Figure 3.9).
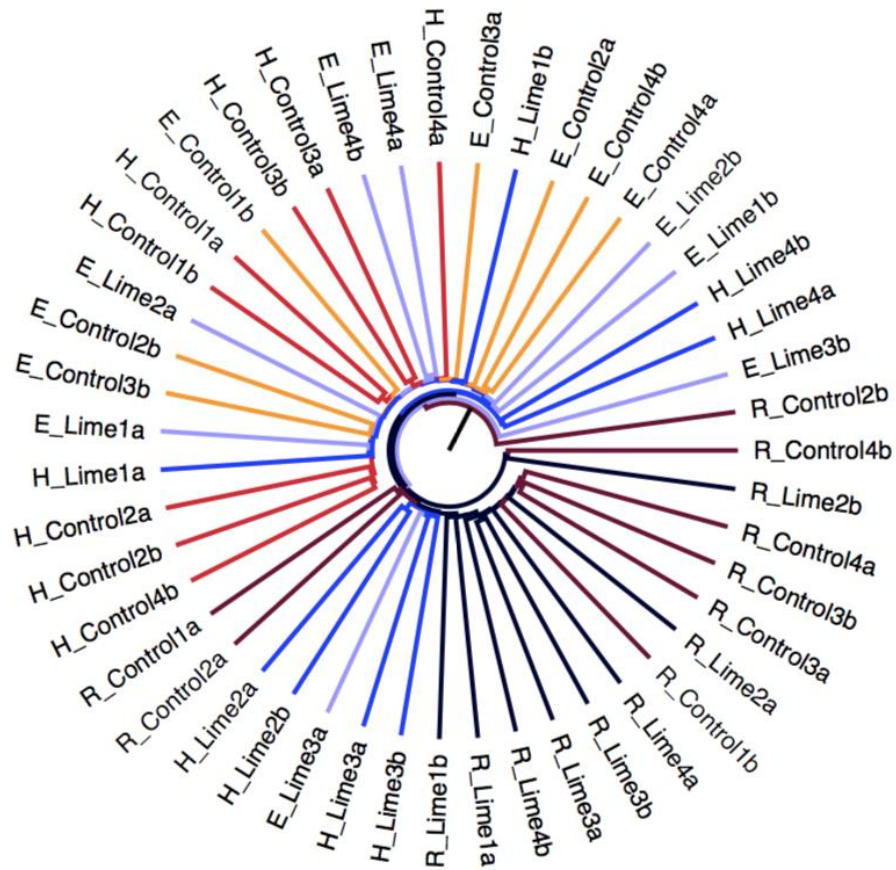
Figure 3.9: Dendrogram of hierarchal clustering of DNA samples, as calculated with the Jaccard dissimilarity index (presence/absence). Clustering is performed with the UPGMA algorithm. Colours represent technical replicates from the same sample plot. The prefix 'E' identifies samples from the stem elongation phase, 'H' from the heading phase and 'R' from the ripening phase. 'High and 'Zero refer to plus and minus lime application; numbers indicate replicate plots and letters a and b denote technical replicates.

To determine if there were differences between sample populations due to changes in relative abundance, sample distances calculated using the Theta Yue and Clayton index, which considers relative abundance (Yue & Clayton 2005), were then used to generate a dendrogram (Figure 3.10). There was more distinct clustering using this index, suggesting that differences between microbial communites of different samples were a result of changes in relative abundance, and not presence or absence of different OTUs. The same trend was seen when comparing the dendrograms of hierarchal clustering from the cDNA fraction.
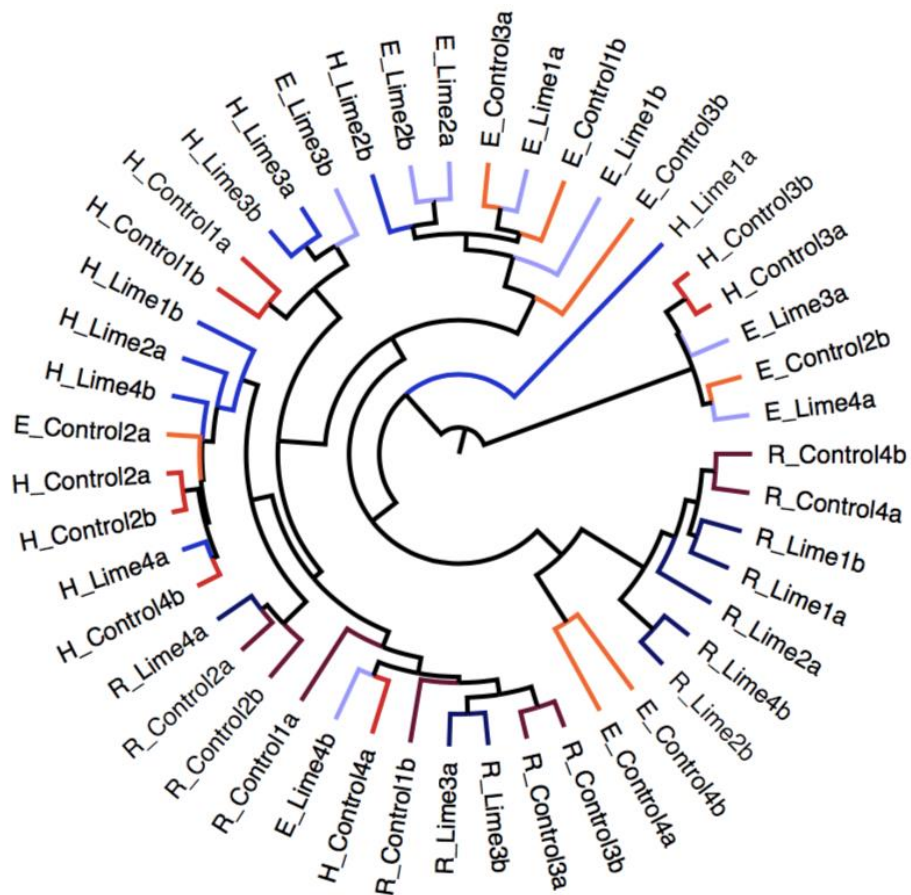
Figure 3.10: Dendrogram of hierarchal clustering of DNA samples, as calculated with the theta Yue & Clayton index (relative abundance). Clustering is performed with the UPGMA algorithm. Colours represent technical replicates from the same sample plot. The prefix 'E' identifies samples from the stem elongation phase, 'H' from the heading phase and 'R' from the ripening phase. 'Lime' and 'control' refer to plus and minus lime application; numbers indicate replicate plots and letters a and b denote technical replicates.

Within these dendrograms, there is no consistent clustering of samples as a function of lime treatment in either the occurrence based (Figure 3.9) or the abundance based (Figure 3.10) dissimilarity analyses. As the lime product did not have the desired effect upon soil pH, this result is not entirely unexpected. However, samples from the same crop growth phase do appear to cluster together, irrespective of lime treatment. Parsimony analyses, as implemented in Mothur, was performed on tree files constructed with the Yue and Clayton index, to determine if the observed grouping of samples was statistically significant (Table 3.3). To verify there was no significant

clustering as a function of lime treatment, this variable was also included in the Parsimony analyses, and for trees constructed from DNA and cDNA data, no p values were smaller than 0.05. For DNA fractions, the elongation and heading phase samples did not group separately from each other ($p = 0.07$), however samples from the heading and ripening phases ($p < 0.001$) as well as from the elongation and ripening phases ($p < 0.001$) did indeed form statistically significant clusters. For cDNA, samples from all three phases formed clusters that were statistically significant at $p < 0.05$ (Table 3.3).

Table 3.3. Results of parsimony analysis to test significance of clusters observed for dendrograms constructed for DNA and cDNA datasets. Statistically significant results are denoted by an asterisk.

Interaction	DNA p value	cDNA p value
Elongation: Lime – No lime	0.966	0.978
Heading: Lime – No lime	0.615	0.923
Ripening: Lime – No lime	0.312	0.315
Elongation - Heading	0.065	0.017 *
Heading - Ripening	< 0.001 *	< 0.001 *
Elongation - Ripening	< 0.001 *	0.004 *

Due to the highly dimensional nature of the data, non-metric multidimensional scaling (nMDS) was chosen to most effectively represent sample dissimilarity (Tzeng *et al.* 2008). Distances calculated with the Yue and Clayton theta metric, from both the DNA samples (Figure 3.11) and the cDNA samples (Figure 3.12) were plotted. Coloured polygons were used to aid visualisation of samples as a function of barley developmental stage. In order to test if these clusters were statistically significant, samples underwent non-parametric analysis of similarity (ANOSIM), as implemented in Mothur (Table 3.4).

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Figure 3.11: nMDS of DNA sample dissimilarity as calculated with the Yue & Clayton metric. Coloured polygons indicate the grouping of samples according to growth stage. Clustering of samples were tested for statistical significance by analysis of molecular variance (ANOSIM) and results are recorded in Table 3.4. Arrows represent Spearman's rank correlations of ten discriminatory OTUs with the largest effect size (LDA score), as identified by LEfSe analysis.

In agreement with the hierarchal clustering and parsimony analyses, the grouping of cDNA samples at all three growth phases was distinct at $p < 0.01$ (Table 3.4). DNA samples from the ripening and elongation phases clustered separately from each other ($p < 0.01$), as did those from the ripening and heading phases ($p < 0.01$). However, DNA samples from the elongation and heading phase did not form statistically significant clusters ($p = 0.096$). Thus, the outcome of this method of ordination of DNA samples, in conjunction with statistical testing, is in accordance with that provided by the hierarchal clustering and parsimony analyses.
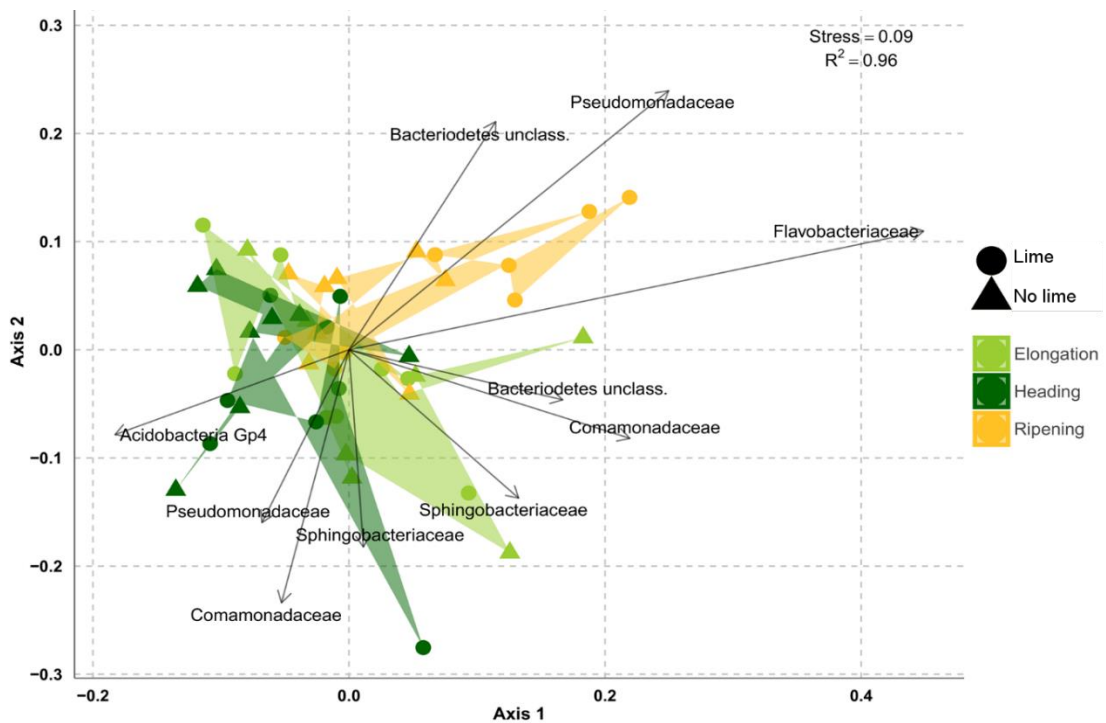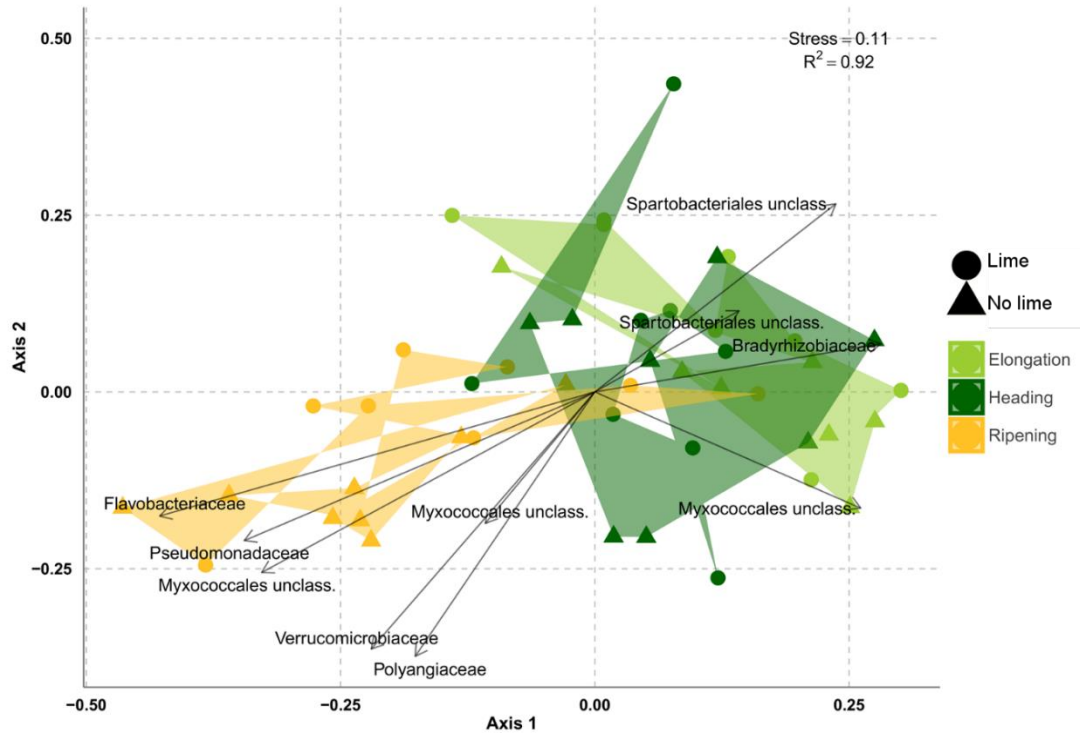
Figure 3.12: nMDS of cDNA sample dissimilarity as calculated with the Yue & Clayton metric. Coloured polygons indicate the grouping of samples according to growth stage. Clustering of samples as a function of lime treatment and sample day were tested for statistical significance (ANOSIM) and results are recorded in Table 3.4. Arrows represent Spearman's rank correlations of ten discriminatory OTUs with the largest effect size (LDA score), as identified by LEfSe analysis.

Table 3.4: ANOSIM results for all interactions tested. Statistically significant clustering is indicated by asterisks where $p < 0.017$ (adjusted Bonferroni)

Interaction	DNA		cDNA	
	R value	*p* value	R value	*p* value
Elongation - Heading - Ripening	0.28	<0.001*	0.49	<0.001*
Elongation – Heading	0.10	0.039	0.32	<0.001*
Heading - Ripening	0.38	<0.001*	0.63	<0.001*
Elongation - Ripening	0.36	<0.001*	0.52	<0.001*

In short, these two different methods of representing sample dissimilarity, namely

hierarchal clustering (Figure 3.9 and Figure 3.10) and nMDS (Figure 3.11 and Figure

3.12) each result in the same conclusions; they suggest that the drivers of community

shifts were not related to lime application, but instead to the barley developmental stage. In light of this, it was proposed to undertake a more in-depth analysis of community succession with time and crop development, looking at only the control samples which received no lime treatment.

3.3.2.5   Differential abundance analysis between barley growth stages

The linear discriminative analysis (LDA) effect size (LEfSe) method (Segata *et al.* 2011) was used to identify OTUs that were differentially abundant between stem elongation, heading and ripening phases. This analysis returns an LDA score for the identified OTUs, thereby demonstrating the effect size of each OTU in discriminating between treatments. LDA scores are logarithmic, and the more abundant an OTU is in all samples of a particular treatment *vs* the other treatments, the higher its LDA score will be, where a score of > 2 is considered significantly discriminatory (Segata *et al.* 2011). As there are more than 200 OTUs for each DNA and cDNA dataset that fit this criterion (LDA score > 2), a stricter LDA score threshold of 3 was set for the purposes of these analyses. A selection of differentially abundant OTUs are represented by arrows on each of the nMDS plots (Figure 3.11 and Figure 3.12), with only the ten highest scoring OTUs displayed for the purposes of clarity. The direction of the arrows represents the Spearman's rank correlation of the relative abundance of each LEfSe discriminatory OTU with the nMDS axes. OTUs are labelled at class and order, or if no classification is available at these taxonomic ranks, then phylum is used. As only ten LEfSe discriminatory OTUs can be clearly represented on each nMDS plot, Figure 3.13 and Figure 3.14 represent all OTUs which had an LDA > 3, for DNA samples and cDNA samples respectively. OTUs are labelled at the level of class and order, in

order to identify trends between certain phylotypes and barley growth stage, for DNA (Figure 3.13) and cDNA (Figure 3.14).

Fewer OTUs with discriminatory power are identified during the heading phase, meaning fewer OTUs consistently experienced significant increases in their relative abundances, for all samples, during this growth phase. This trend is seen for both DNA samples (Figure 3.13), with 6 OTUs having an LDA > 3, and cDNA samples (Figure 3.14) with 4 OTUs with LDA > 3. In the DNA fractions, OTUs assigned to *Sphingobacteriales* and *Burkholderiales* are the most frequently observed discriminative OTUs for the elongation phase (Figure 3.13), along with two OTUs from *Pseudomonadales*. The heading and ripening phases of DNA samples are each dominated by LEfSe discriminative OTUs from a particular phylotype, with the heading phase dominated by *Acidobacteria* and the ripening phase by *Bacteroidetes,* and more specifically *Flavobacteria, Sphingobacteria* and other *Bacteroidetes* that were unclassified at the level of class or order (Figure 3.13).

Figure 3.13. Linear discriminative analysis (LDA) effect size (LEfSe) analysis between the three growth stages, for DNA samples. OTUs with an LDA score of > 3 are plotted and are coloured according to the growth stage for which they provide discriminatory power, due to markedly increased abundances. OTUs are labelled at the level of class and order, where only statistically significant discriminatory OTUs are represented ($p < 0.01$).

When looking at cDNA samples (Figure 3.14), during the elongation and heading phases, there is no phylotype to which the majority of discriminatory OTUs are assigned. However, during the ripening phase discriminatory OTUs were mainly assigned to *Myxococcales*, representing more than 50 % of the discriminatory OTUs (Figure 3.14). While diverse members of the *Bacteroidetes* phyla were identified as discriminatory for the ripening phase in both DNA and cDNA samples, no *Myxococcales* were identified as discriminatory for ripening, or indeed any phase, in the DNA samples, representing a significant contrast to the cDNA samples where this group dominates.
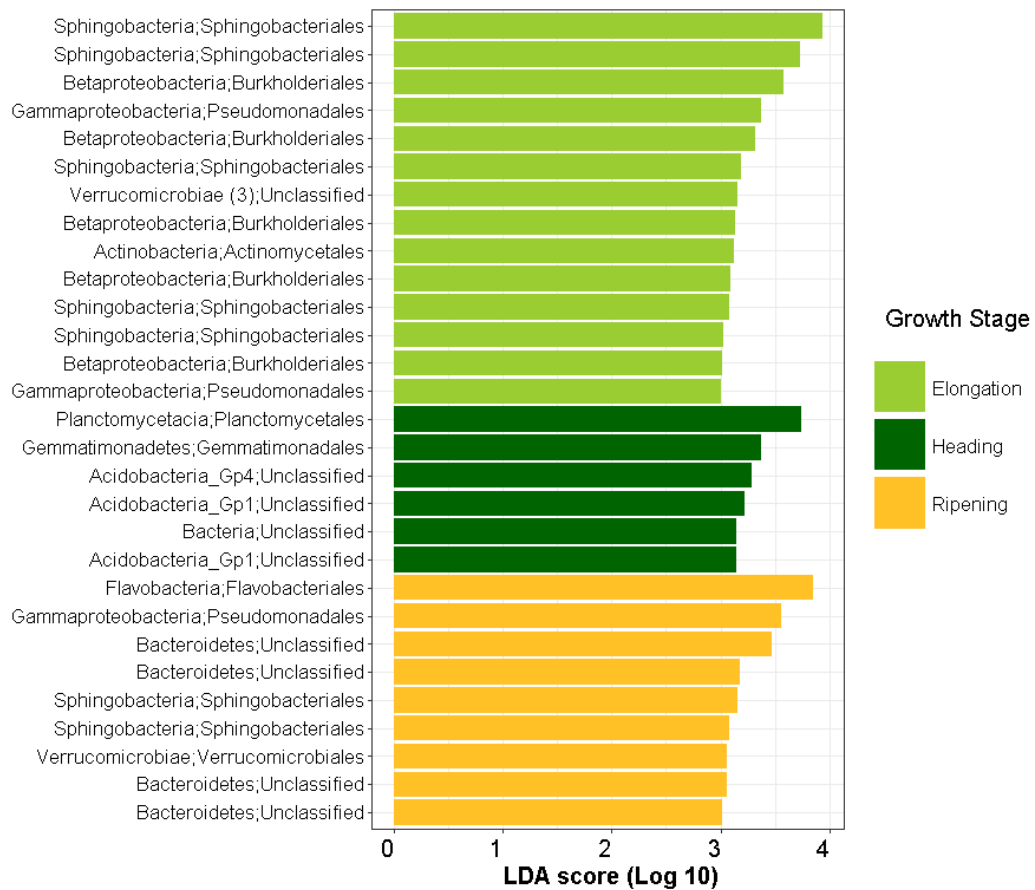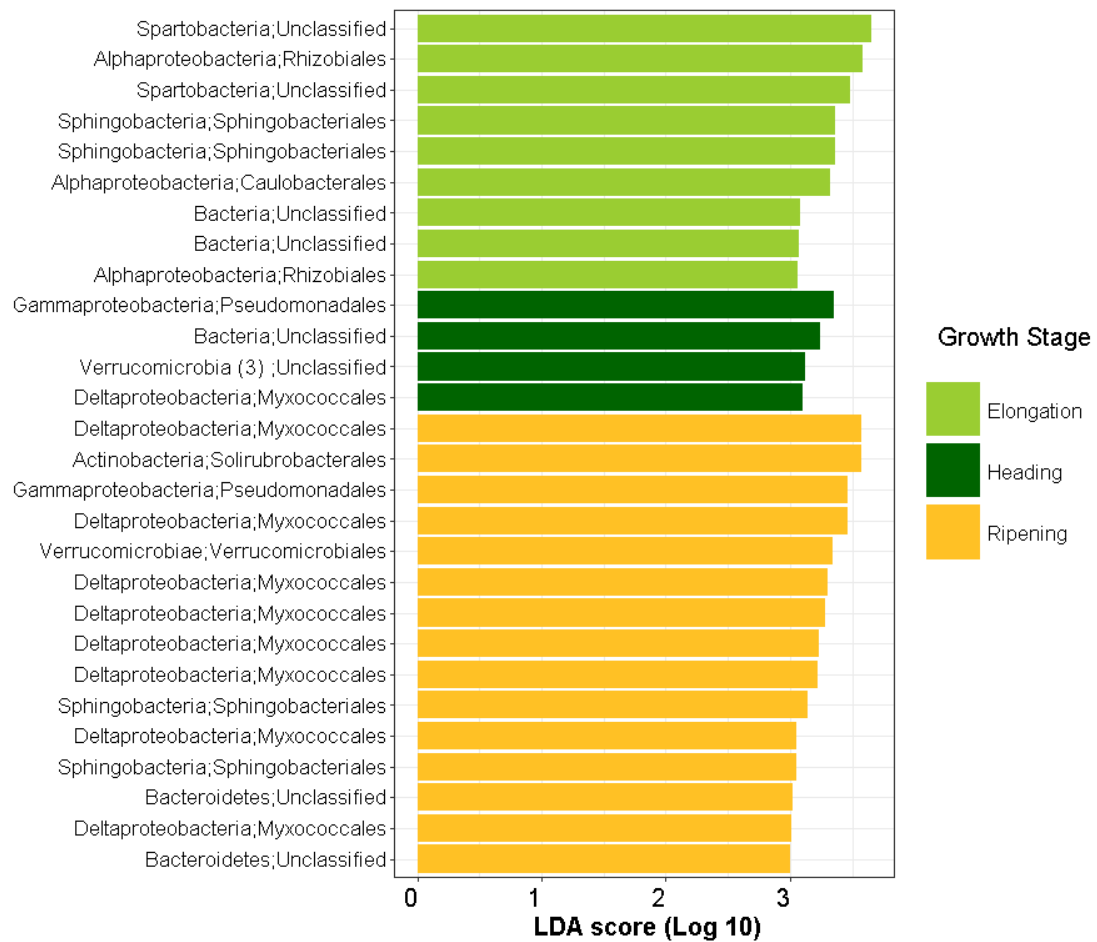
Figure 3.14. Linear discriminative analysis (LDA) effect size (LEfSe) analysis between the three growth stages, for cDNA samples. OTUs with an LDA score of > 3 are plotted and are coloured according to the growth stage for which they provide discriminatory power, due to markedly increased abundances. OTUs are labelled at the level of class and order, and only statistically significant discriminator OTUs are represented ($p < 0.01$).

As the OTUs in Figure 3.13 and Figure 3.14 were identified as significantly differentially abundant between growth phases, we next investigated how exactly their abundance varied with time. This was in an effort to reveal any temporal trends, for example increases or decreases with time, or indeed if a particular OTU was absent at one sample point yet highly abundant in another. Changes in relative abundances of

the LEfSe discriminative OTUs were plotted as a function of crop development, for

both DNA (Figure 3.15) and cDNA (Figure 3.16) samples.

Figure 3.15. Changes in relative abundance of LEfSe discriminative OTUs from the DNA fractions, as detected in all phases, as opposed to only the phase where they provide discriminatory power. Box and whisker plots represent relative abundances of OTUs defined as being discriminatory for A) stem elongation B) heading and C) ripening phases as detected in all samples from each time point (as represented by the colour scale). The upper, middle and lower line of each box indicate the 75th percentile, median and 25th percentile respectively, while whiskers indicate the highest and lowest relative abundances for each OTU, and dots denote outliers. OTUs are labelled at the level of order, where 'unclass' stands for unclassified.

In the DNA samples, *Sphingobacteriales* assigned OTUs were discriminative for both the elongation Figure 3.15A and the ripening phase Figure 3.15C. It is evident, that despite belonging to the same phylotype, particular OTUs from the *Sphingobacteriales* respond very differently to plant growth phase. The *Sphingobacteriales* OTUs that discriminate for the elongation phase experience a stepwise decrease in relative abundance with time. Meanwhile, those identified for the ripening phase show exactly the opposite trend. The 6 OTUs identified as highly discriminatory (LDA > 3) for the heading phase in DNA samples, are indeed more abundant during this phase, however they remain at similar relative abundances for all growth phases (Figure 3.15B), a trend that is not generally seen for the other phase-discriminatory OTUs (Figure 3.15 A and C). In particular, all OTUs (except the *Flavobacteriales*) that discriminated for the ripening phase in DNA samples, were present in very low abundances until this final timepoint (Figure 3.15C). Indeed, this same pattern was shared by ripening-phase discriminatory OTUs from cDNA samples (Figure 3.16C): one OTU (in this case from *Solirubrobacterales*) was far more abundant than all others, at all time points. Meanwhile, most other OTUs were at very low abundances during the elongation and heading phases, before experiencing a notable increase in relative abundance in the final phase. All OTUs identified as discriminative in the heading phase of cDNA samples were present at very low abundances, even during the phase for which they were identified as differentially abundant (Figure 3.16B).
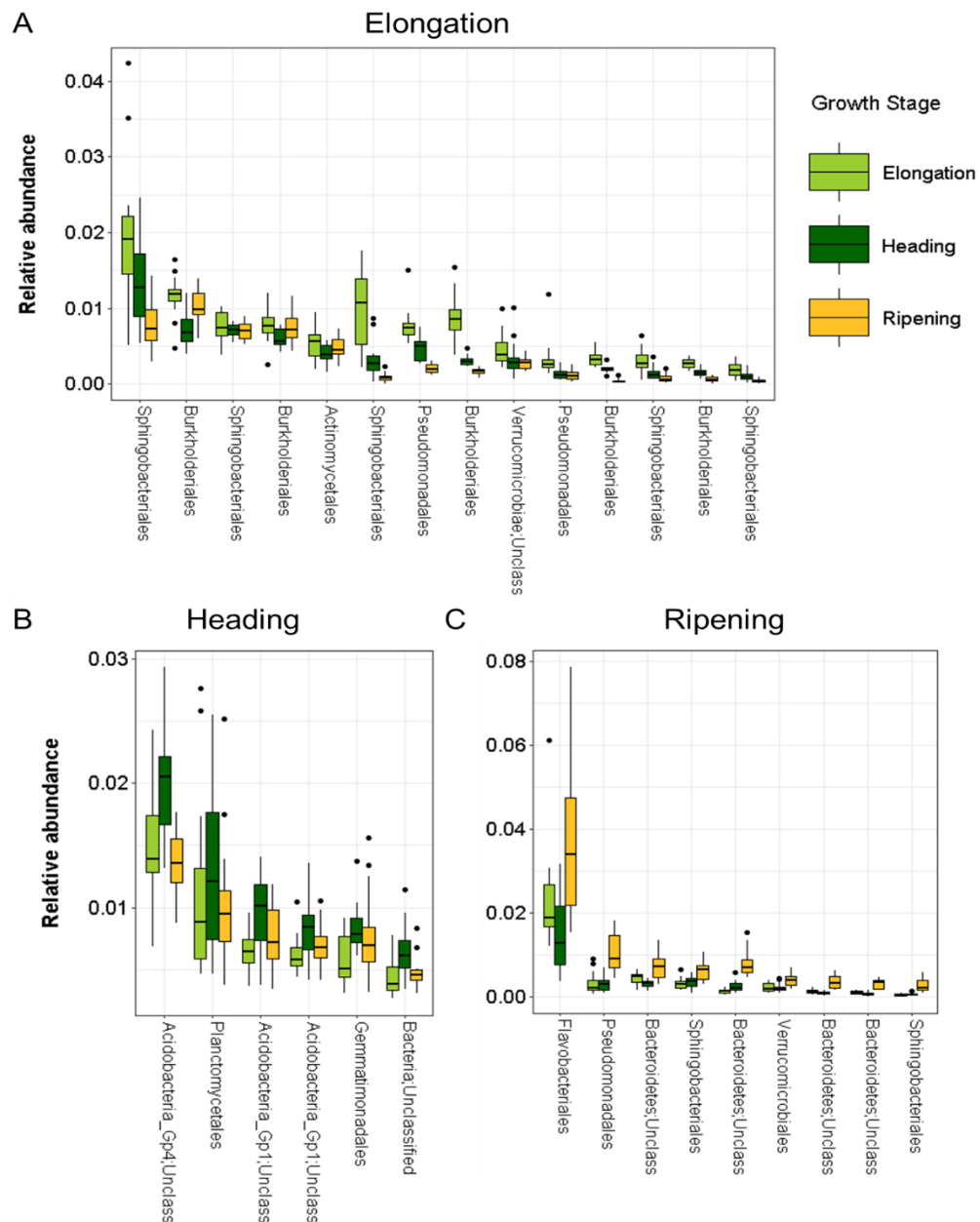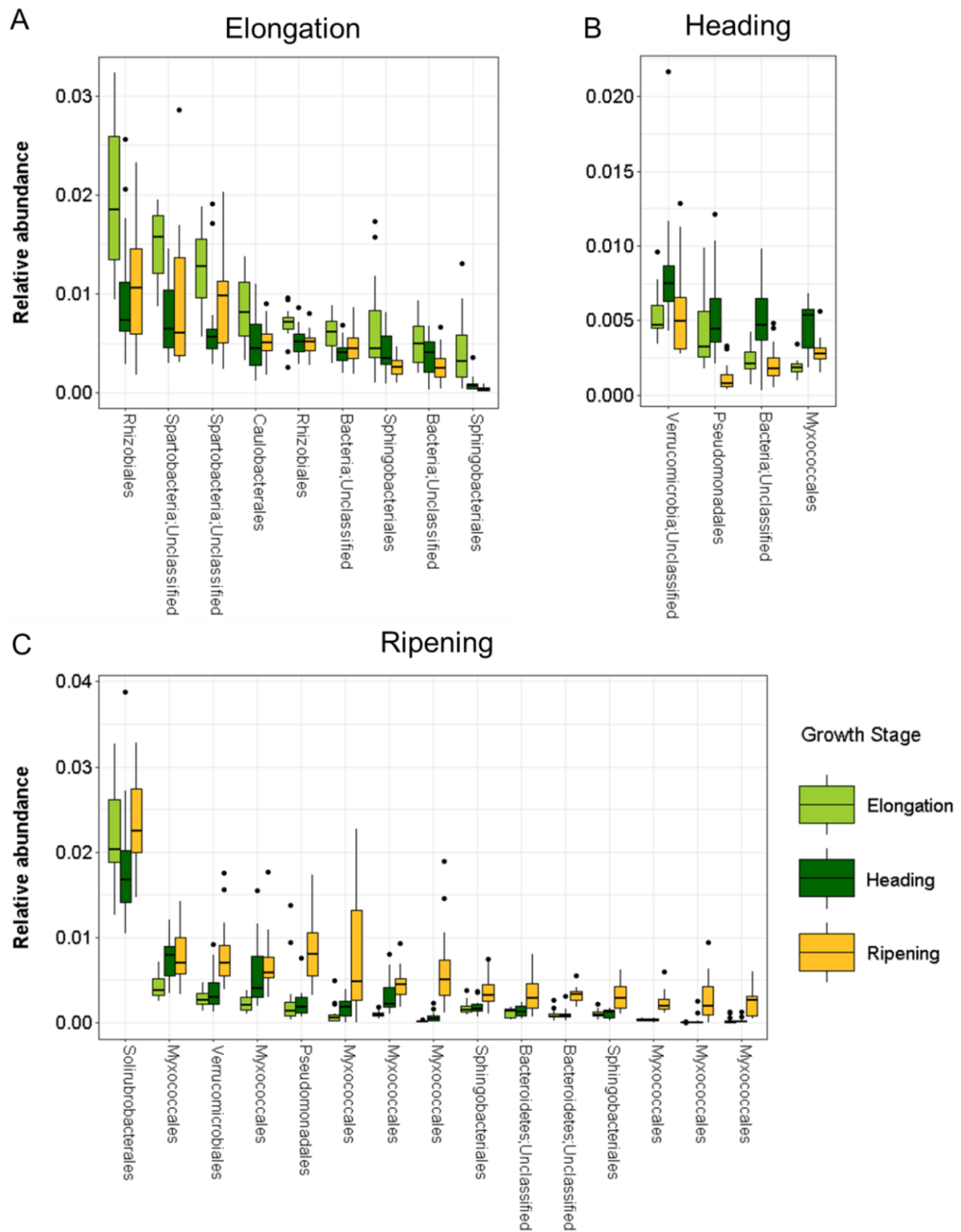
Figure 3.16. Changes in relative abundance of LEfSe discriminative OTUs from the DNA fractions, as detected in all phases, as opposed to only the phase where they provide discriminatory power. Box plots represent the relative abundances of OTUs defined as being discriminatory for A) stem elongation B) heading and C) ripening phases as detected in all samples from each time point (represented by the colour scale). The upper, middle and lower line of each box indicate the 75th percentile, median and 25th percentile respectively, while whiskers indicate the highest and lowest relative abundances for each OTU and dots show outliers. OTUs are labelled at the level of order.

All *Myxococcales* assigned OTUs identified as discriminatory for the ripening phase in cDNA samples followed the same trend with time, whereby a stepwise increase in relative abundance was observed at each growth phase sampled (Figure 3.16C). We then examined the temporal dynamics of all the OTUs assigned to *Myxococcales* (non-LefSe and LefSe discriminative) in order to investigate this phylotypic pattern further. Additionally, we wished to investigate why no OTUs from the *Myxococcales* were identified as discriminative for any phase in the DNA samples. The relative abundance of the most abundant members of this order where identified and, in the interests of clarity, the 15 most abundant were analysed (Figure 3.17).

It is evident that *Myxococcales* are present in the DNA fractions (Figure 3.17A) at much lower abundances than in the cDNA fractions (Figure 3.17B). This suggests that these microbial groups are potentially more active than the relative abundance of their 16S rRNA gene implies. While the relative abundances of *Myxococcales* OTUs fluctuate significantly in the cDNA samples (Figure 3.17B), those identified in the DNA fractions remained relatively constant as a function of time (Figure 3.17A). This might suggest that these bacteria are present in similar numbers throughout the trial, but their activity changes significantly as a function of time/crop development. The notable exceptions to this trend of stable abundance in the DNA samples are OTU 646 and more particularly OTU 607 which were present at extremely low abundance in the stem elongation phase, yet by the ripening phase were among the most abundant members of this order in the DNA fractions (Figure 3.17A).
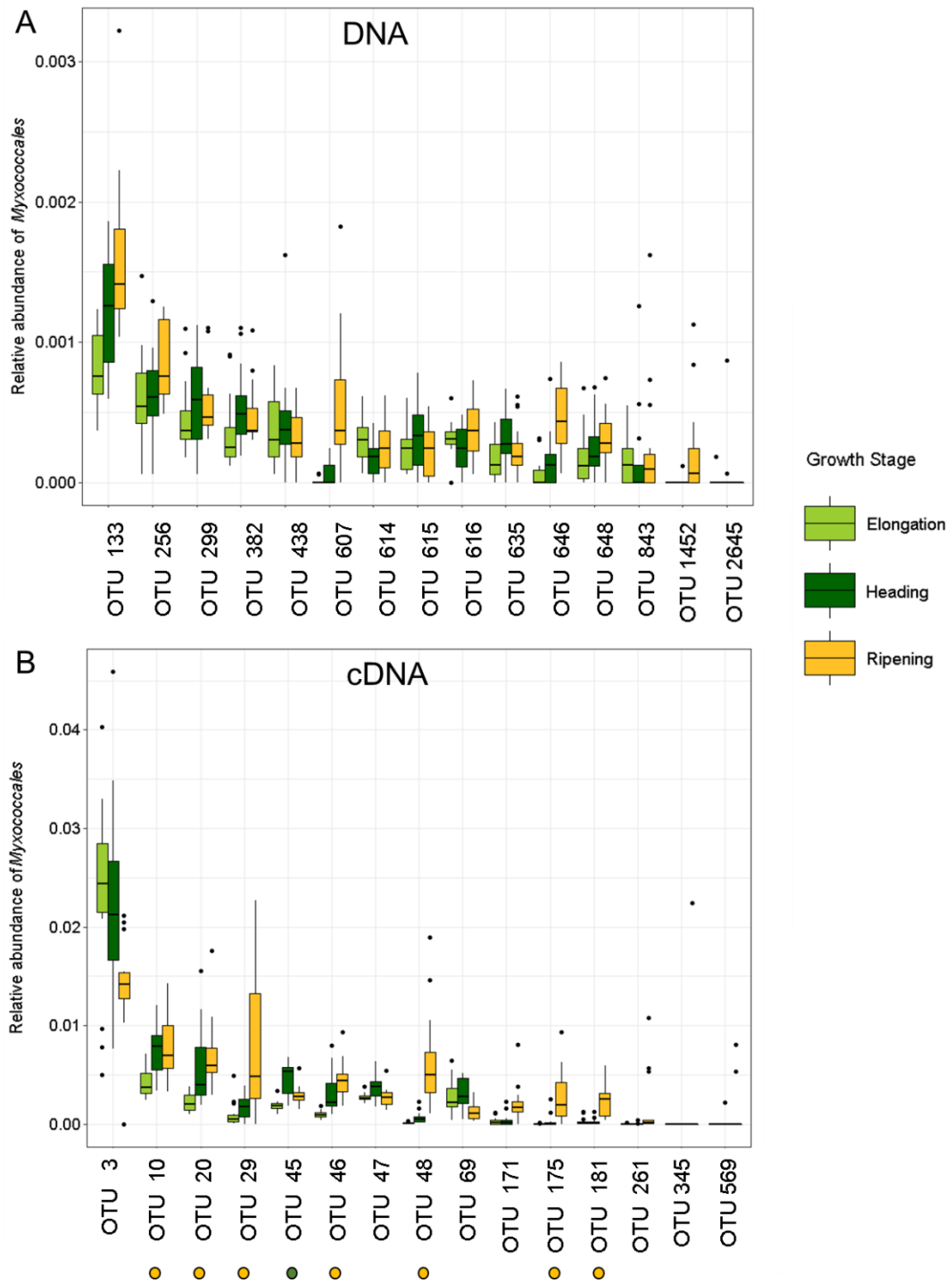
Figure 3.17. OTUs with the highest relative abundance from the order of *Myxococcales,* from A) the DNA and B) the cDNA samples. The box plots represent the relative abundances of each OTU as observed in all samples per time point. The upper, middle and lower line of each box indicate the 75th percentile, median and 25th percentile respectively; whiskers indicate the highest and lowest relative abundances for each OTU and black dots show outliers. OTUs already identified as having high discriminatory power by LEfSe analysis (LDA > 3) are identified by dots below the x axes, coloured according to the phase for which they are discriminatory.

In the cDNA fractions, the majority of *Myxococcales* follow the same temporal pattern, where their relative abundances are highest during the ripening phase (Figure 3.17B). The most abundant member of this order, OTU 3, however shows the reverse trend, with a decreasing relative abundance as a function of time/plant growth. Unfortunately, all the *Myxococcales* presented in Figure 3.17 are unclassified at the genus level, as well as at family level. Thus, it is not possible to determine if these variable responses are attributable to differences in phylotype.

3.4   Discussion

Using root-associated soil taken from a field trial designed for testing a quicklime product, we aimed to investigate the effect of lime on the stability of microbial communities associated with a barley rhizosphere, in a time dependent manner. Unfortunately, the lime product did not have the desired effect upon soil pH, potentially a result of any number of reasons including soil type, and rainfall, meaning that perhaps at this field site, the Growmax application rate was not sufficient to alter pH. In weeks 6 to 10 of the trial there was an observable, albeit small, increase in pH in plot treated with Growmax, however, this increase was not statistically significant, when using the appropriately adjusted *p* values. This lack of effect was mirrored by the microbial community, where no significant grouping of samples according to lime treatment was observed. Clear temporal shifts in community structure were observed at the three distinct barley developmental stages of stem elongation, heading and ripening. In this study it is not possible to disentangle the stochastic, temporal changes in the soil microbial community from those resulting from barley growth stage, as no bulk soil samples were concomitantly analysed. It is widely accepted that the

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rhizosphere supports a larger, and more active, community, both at the micro- and macro- scale, than the surrounding bulk soil (Brimecombe *et al.* 2007) and as such plant interaction is typically recognised as one of the principle drivers of community succession in root associated soil (Breidenbach *et al.* 2016; Shi *et al.* 2015). For the purposes of this conclusion therefore, changes potentially associated with plant association will be discussed. The change in microbial community with crop developmental stage has been observed in a number of plants including, but not limited to, *Arabidopsis* (Chaparro *et al.* 2014), soybean (Sugiyama *et al.* 2014), pea and sugar beet (Houlden *et al.* 2008). Similarly to the study of Chaparro *et al.* (2014), little change was observed in terms of richness, evenness and diversity, however beta diversity analysis revealed that communities at the three growth stages were distinct. In this study, statistically significant grouping of samples was seen between all three plant developmental stages at the cDNA level, and between two of the stages at the DNA level. Between the phases of elongation and heading (DNA) no significant grouping was seen, however at the cDNA level there was a clear separation of these samples. This could be a result of relic DNA obscuring temporal or treatment effects (Carini *et al.* 2016), again demonstrating the significance of looking at cDNA in conjunction with DNA.

When investigating the prokaryotic soil communities in terms of presence/absence, there was little difference, implying that changes in beta diversity were related to changes in relative abundance, and not changes in community composition. Indeed, the same core assemblage seems to be present at all growth stages studied. Therefore, it could be proposed that temporal changes in the relative abundances of particular microbial groups occur as a result of changing conditions that favour the metabolic strategy of particular microbes. The increase in microbial activity seen in the

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rhizosphere compared to bulk soil is typically attributed to the continual supply of carbon provided by plant roots (Brimecombe *et al.* 2007). Root exudates vary with the growth stage of plants, whereby the amount of exudate has been previously shown to correlate with the growth rate of the plant (Aulakh *et al.* 2001; Lucas García *et al.* 2001). It has further been demonstrated that this correlation depends on active root growth. For example, Přikryl and Vančura (1980) showed that only actively growing wheat roots produced detectable root exudates. In annual plants, root growth and production typically begin to decline after flowering (Rees *et al.* 2005). Swinnen *et al.* (1995) estimated that in a conventionally farmed crop of spring barley, such as the one used in the present study, by the end of the growing season, approximately 55 % of the root system has decayed. This represents a significant source of carbon deposition for the surrounding microbes. Indeed, some research suggests that the roots of graminaceous plant such as wheat, oats and barley, undergo a progressive form of senescence, whereby the cortical cells of more mature roots gradually die off, and this can occur in roots only 20 days old (Henry & Deacon 1981). It could then be assumed that a shift in available carbon sources would occur as the crop ages: from soluble, organic compounds such as amino acids, sugars, organic acids and vitamins during active root growth, to more recalcitrant carbon sources, namely decaying plant material such as cell wall components, lignin and cellulose as root growth slows and senescence begins. This would select for microbial community members more adept at decomposing insoluble organic materials. Members of the myxobacteria are one such group, known to decompose macromolecular substances through the production of a suite of extra-cellular enzymes (Mohr *et al.* 2016). Under nutrient limiting conditions, *Myxococcales* spp. undergo fruiting-body formation and sporulation. Groups of these species 'clump' together and achieve cooperative feeding (Shimkets

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1990). As shown by the LEfSe analysis, the majority of the highly discriminatory cDNA OTUs for ripening, were *Myxococcales* spp. We could thus perhaps hypothesise that their ability to decompose complex organic matter, as well as their 'social' lifestyle, gave these species an advantage over their competitors and allowed for the observed increase in relative abundance at the cDNA level. It is worth noting that this phenomenon was not seen when looking at discriminatory OTUs identified from the DNA fraction, demonstrating the importance of looking at both nucleic acid fractions. OTUs belonging to the order *Sphingobacteriales* were shown to be discriminatory for the heading stage, at both the DNA and cDNA level. As flowering/heading has been shown to be when plants are most actively growing and thus producing root exudates, the rhizosphere represents an ideal niche for these chemoorganotrophic bacteria to survive during this growth phase. Using stable isotope probing in the presence of four different $^{13}CO_2$-exposed plant types, Haichar *et al.* (2008) demonstrated that OTUs which incorporated plant root exudates consistently, independent of plant species were predominantly members of both the *Myxococcales* and the *Sphingobacteriales*. In our study, growth phase also seemed to control the abundance of these groups. In contrast to *Myxococcales*, the *Sphingobacteriales* do not exhibit the same 'social' response to nutrient limiting conditions and perhaps were therefore outcompeted when plants began entering the senescent phase and this could account for the decrease in their relative abundance at this time point. Within these two microbial groups, there were of course a number of OTUs that responded differently with respect to changes in relative abundances as a function of plant developmental stage. Additionally, the OTUs of interest were often unclassifiable at the finer resolutions of family and genus, preventing any phylotype-based conclusions being drawn. This variation in relative abundance of OTUs within the same taxonomic guild demonstrates the improved

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resolution provided by OTU-based as opposed to phylotype-based investigations of microbial communities. Phylotype-based analysis are convenient when investigating the multitude of interactions occurring as microbial communities respond to an event (Fierer *et al.* 2007). However, it must be kept in mind the variety of genetic potential, acquired through horizontal gene transfer or otherwise, that exists within each phylotype can complicate these efforts (Schloss & Westcott 2011).

Soil microbial community dynamics result from the interaction of a number of abiotic and biotic factors including soil type, climate, precipitation regime, soil organic matter content, plant type and agricultural practices to name but a few (Bossio *et al.* 1998; Lauber *et al.* 2013; Wieland *et al.* 2001). To determine if any of the observations seen in this study can be attributed specifically to barley growth stage, it would be of real interest to find 16S rRNA datasets from other studies investigating the soil microbial communities of barley and other crops. This could allow us to determine if any microbial groups consistently respond in the same manner with barley development. Indeed, a recently published tool was created with exactly this type of analyses in mind (Rodrigues *et al.* 2017). Ideally such datasets would include samples taken from the bulk soil, which unfortunately was not possible in this study, in order to unpick temporal from crop-associated responses.

There has been much research into the use of inocula of plant growth promoting bacteria as a means of sustainably improving plant productivity. This plant growth promotion can be achieved in a number of ways, including disease suppression, root growth stimulation and mobilisation of recalcitrant forms of nitrogen and phosphorous (Lugtenberg & Kamilova 2009). However, a major limitation to this includes the fact that once inoculated, many bacteria do not remain in high enough numbers within the rhizosphere to have the desired effect (Bashan *et al.* 2014). As the authors therein

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detail, this can be as a result of inappropriate 'carrier' of the inoculant but also due to the inability of the introduced population to find a niche in which they can persist. This can be a result of the highly competitive surroundings in which allochthonous bacteria find themselves. Resource availability in the bulk soil is often extremely low, indeed even some of the ecosystems services we frequently attribute to soil (such as nitrogen fixation) are often unable to occur at any meaningful rate in these root-free environments, due to lack of substrate (Postgate 1974). Thus, the rhizosphere is a highly competitive niche already occupied by a plethora of well adapted microorganisms. This poses complications for the survival and success of bacterial inoculants. However, perhaps observing the naturally occurring changes in relative abundance of a close relative (to the inoculant), might allow for more informed decisions when trialling plant growth promoting bacteria as biofertilizers or biocontrol agents. Understanding which plant developmental stages might be more opportune for the application of these inoculants could indeed enhance the likelihood of the inoculant persisting and resulting in the desired phenotype. Thus, through the investigation of the natural succession of microbial communities in response to crop growth, we can begin to gain some insights into rhizosphere-associated microbial dynamics and use this knowledge to guide the implementation of sustainable means of agricultural management

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--------- Chapter IV ---------

Soil microbial community responses to a compounded soil management and climatic disturbance

4.1   Introduction

Upon an extensive literature review, we identified a number of research gaps within the field of microbial community stability, including the need for an in-depth characterisation of the effects of flooding upon the soil microbiota. Additionally, we hoped to investigate flooding in combination with a prior ecosystem disturbance to determine if this impacted the resistance and/or resilience of the microbial community. For the imposition of a compounded perturbation, the application of an organic amendment was chosen as Allison & Martiny (2008) identified it as a stress commonly experienced by agricultural soil ecosystems. Additionally, we believe the combination of slurry amendment followed by waterlogging represents a condition which Irish grassland soils may indeed be subjected to.

Thus, the aim of this work was to test the following three hypotheses: 1) the application of cattle slurry will impose a stress on the soil microbial community. This will be as a result of an influx of i) microbiota which will alter soil microbial community composition and function and ii) nutrients, including labile forms of carbon, which will lead to alterations in trophic strategy of native soil microbiota (K- vs r- strategists). The effect of slurry amendment on the soil microbiota has been reported to be transient, with microbial community resilience observed within 10 (Stark *et al.* 2007) to 50 days (Suleiman *et al.* 2016). Thus, in order to impose a compounded disturbance, flooding must occur within this time frame. 2) The addition of a compounded disturbance of a heavy rainfall event three days post slurry application will result in further effects on microbial community composition and functional capacity and/or activity. Additionally, the extent to which the microbial community responds to the second disturbance, i.e. flooding, will be affected by the presence or absence of slurry,

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due to changes in carbon availability and ratio of K to r strategists (Hammesfahr *et al.* 2008; Wallenstein & Hall 2012). Flooding may affect the typically rapid die-off seen of slurry-associated microbes (Cools *et al.* 2001) and hence reduce the usually-observed resilience of the community to slurry disturbances. Finally, 3) if the flooded soils return to the same moisture content as the controls, this will allow for resilience of the community, which may be improved by the increased resource availability in slurry amended soils (Griffiths & Philippot 2013). In order to test these three hypotheses, the community composition of bacteria and archaea was investigated using 16S rRNA profiling, while physiological profiles were assessed via substrate induced respiration using MicroResp (Campbell *et al.* 2003). Finally, rates of litter decomposition, potential nitrification and potential denitrification were assessed throughout the 140 day experiment, to allow interpretation of the results in terms of functions associated to the ecosystems services of nutrient cycling.

4.2   Materials and methods

4.2.1   Soil sampling and processing

Soil used for this experiment was collected from the top 20 cm of a permanent pasture land situated at the Johnstown Castle Research Facility (Teagasc) in County Wexford, Ireland, with GPS coordinates of 52°17′32″N, 6°30′. Soil from this site has been used by Harty *et al.* (2016), during which full characterisation of the soil was performed, as described therein and summarised in Table 4.1. The soil was characterised as a moderately drained, sandy loam consisting of 52 % sand, 34 % silt and 14 % clay.

After collection, soil was transported and sieved to 2 mm for the removal of large roots and stones, and after sieving the soil was kept for two weeks at 10 °C.

Table 4.1. Summary of soil physicochemical characteristics as determined by Harty *et al.* (2016).

Soil pH	Total carbon %	Total nitrogen %	Loss on ignition %	Sand : Silt : Clay %
5.69	2.83	0.284	7.02	52 : 34 : 14

4.2.2 Moisture and organic content of soil and slurry

Slurry was obtained from a cattle dairy farm. Moisture content of soil and slurry was performed following the European Standard ISO 11465 protocol for calculating gravimetric moisture content and dry matter on a wet weight basis. An aliquot of fresh soil was placed at 110 °C for 12 hours. Samples were cooled in a desiccator and weighed to the nearest mg on a fine balance scale. The moisture content was calculated using the following formula:

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$$Soil\ moisture\ (\%) = \left[\frac{fw - dw}{fw}\right] \times 100$$

Where:
fw = soil fresh weight
dw = soil dry weight

Organic matter content was calculated by loss on ignition, (European standard EN 15935:2012) by incubating oven dried samples in a furnace at 550 °C for two hours. After cooling in a desiccator, samples were weighed to the neared mg and percentage organic matter content calculated as follows:

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$$Soil\ organic\ matter\ (\%) = \left[\frac{dw - iw}{dw}\right] \times 100$$

Where:

dw = soil dry weight minus dish weight

iw = ignited dry weight minus dish weight

4.2.3 Microcosm set up

Microcosms were set up in 250 ml plastic containers, modified to each include 10 regular sized drainage holes. In each container, 150 g fresh weight (fw) of soil was placed and gently packed to achieve a bulk density of $1.02\ \text{g}/\text{cm}^3$ (Figure 4.1). Packing was performed in 'thirds' to achieve an even density throughout the pots. Microcosms were weighed for monitoring moisture content, covered with loosely fitting lids and placed at 15 °C in the dark for two weeks prior to the application of any experimental treatments.

Figure 4.1. Microcosms constructed from plastic containers, filled with sieved soil at a bulk density of $0.77\ \text{g}/\text{cm}^3$

4.2.3.1 Slurry application

Microcosms were numbered (1 – 200) and randomly assigned to each of the four treatments, with 6 surplus microcosms for each treatment. One litre of cattle slurry (7.53 % dry matter content) that had been stored at 10 °C for two weeks since collection, was homogenised in a blender for 45 seconds. A 25 ml pipette tip, with the end cut to 1 cm to remove the narrow tip, was used to apply slurry to half of the microcosms. The application rate equated to 33 m^3 per hectare, within the suggested limits of slurry application to grasslands by Teagasc (Humphreys & Lawless 2006). In each microcosm, 10.1 ml of slurry was therefore applied to the soil surface area of 30.6 cm^2. To account for the increased moisture content of microcosms receiving slurry, those microcosms not amended with slurry received 7.6 ml of distilled water. In order to achieve saturated soil conditions later in the experiment, all microcosms were placed inside sterile, 500 ml Ravenhead Kilner® jars, with the lids removed. Parafilm was used to cover the jars, and pierced with a needle to allow gaseous exchange but reduce water loss. Jars containing microcosms were placed, in numerical order (thereby preventing storage in treatment 'blocks'), inside loosely closed cardboard boxes, at a constant temperature of 15 °C in the dark. Weekly, throughout the experiment, any unflooded microcosms were weighed, and lost moisture was replaced by spraying with distilled water. Three sample points were performed during this phase, T0, T1 and T2 (Table 4.2; section 4.2.4).

4.2.3.2 Flooding of microcosms

Three days after the slurry had been applied, 45 microcosms from each treatment, with and without slurry, were flooded. This resulted in four treatments, each with 45 microcosms; i) untreated control ii) slurry iii) flood iv) slurry + flood.

Flooding was achieved by adding distilled water gently into the microcosms until water remained at a height of 5 mm above the soil horizon. Microcosms were again covered in parafilm, pierced with a needle ($\times$ 10) before being placed in boxes in the dark at 15 °C, in numerical order to prevent treatment blocks. The sampling regime continued, with sample points T3 to T7 (inclusive) occurring during the flooded phase (Table 4.2, section 4.2.4).

4.2.3.3   Draining of microcosms

After 26 days of water-logged conditions, excess water was removed from the jars and gently poured off the top of the microcosms. To remove pooled water from within the base of the plastic microcosm pots, these were placed on tissue paper and left to sit for 10 minutes. Microcosm weights were recorded, and pots returned to the Kilner jars, sitting atop inverted weigh boats to allow drainage. To keep conditions the same as the non-flood treated controls, jars were once again covered in parafilm. Once a week, the inside of the jars was wiped to remove any drained water and pots left to sit on tissue paper for 10 minutes, again to allow any water pooled at the bottom of the pots to be drained. After 66 days of natural draining, the microcosms had reached a moisture content within 2 % that of the non-flood treated controls, and sampling continued, thus resulting in the final 'recovery phase' of sampling. This sampling phase consisted of timepoints T8 to T13 (inclusive) over 48 days (Table 4.2; section 4.2.4).

4.2.4 Microcosm sampling

Sampling was performed in a destructive manner, whereby at each sample point, three microcosms were removed per treatment. Soil from each microcosm was used to determine soil moisture content and soil organic matter content (section 4.2.2) as well as for microbial community analysis; community level physiological profiling;

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potential nitrification and denitrification rate assays; litter decomposition assays; extractable inorganic nitrogen quantification and total carbon and nitrogen determination. The methods for each of these analyses are detailed below (section 4.2.6 to 4.2.12).

4.2.5 Summary of sampling regime

In total, thirteen sample points were investigated, with the experiment divided into three phases: i) Disturbance 1: slurry ii) Disturbance 2: flooding and iii) Recovery phase, as summarised in Table 4.2.

Table 4.2. Details of each of the 13 sample points, showing the experimental days on which they occurred and the phase to which they belonged.

Time point ID	Days post slurry	Days post flood	Days post drainage	Phase
T0	0	-	-	Disturbance 1
T1	1	-	-	Disturbance 1
T2	3	-	-	Disturbance 1
T3	6	3	-	Disturbance 2
T4	9	6	-	Disturbance 2
T5	15	12	-	Disturbance 2
T6	22	19	-	Disturbance 2
T7	29	26	-	Disturbance 2
T8	92	-	66	Recovery
T9	99	-	73	Recovery
T10	107	-	81	Recovery
T11	114	-	88	Recovery
T12	120	-	94	Recovery
T13	140	-	114	Recovery

4.2.6 Microbial community analysis

Using a 15 ml tube, three soil cores were taken from each microcosm and mixed gently in a 50 ml tube, before placing three aliquots of 1 g (fw) subsamples into 2 ml screw cap tubes, which were immediately flash frozen in liquid nitrogen and placed at − 80 °C until DNA extractions.

4.2.6.1 DNA extraction and 16S rRNA amplicon library preparation

DNA extractions were performed following the protocol of Griffiths *et al.* (2000). DNA was normalised to 50 ng / µl and 1 µl was amplified in a 25 µl reaction using Golay barcoded 16S rRNA universal primers 515F/806R (Caporaso *et al.* 2011) as detailed in 2.2.3.3. Due to the large sample number, it was not possible to perform 16S rRNA profiling on all 14 time points, thus using phenotypic data to guide selection, 6 time points were chosen. These included two sample points (T0 and T2) from the pre-flood phase, three sample points (T3, T5 and T7) from the flooded phase and finally three sample points (T8, T11 and T13) from the recovery phase. This corresponded to the first and last sample point of each phase and a middle sample point for the flood and recovery phases. In addition, a negative control (no template DNA) and a mock community were also included in the MiSeq run in an effort to determine sequencing error rates.

4.2.6.2 Mock community construction

Using the phenol:chloroform method (Griffiths *et al.* 2000) described in 3.2.3.1, DNA was extracted from axenic, overnight cultures of *Escherichia coli, Listeria monocytogenes, Halomonas titanicae, Vibrio parahaemolyticus, Streptococcus pyogenes, Pseudomonas aeruginosa* and *Micrococcus luteus.* These were chosen as

representing a variety of cell wall structures, phylogenies, genome sizes and rRNA copy numbers (Table 4.3). 16S rRNA copy number and genome size details were obtained from the Ribosomal RNA Operon Copy Number Database (rrnDB) (Stoddard *et al.* 2015). DNA was quantified using the Qubit assay, and samples normalised to the lowest DNA concentration before pooling 1 µl of each. The resulting pool, consisting of equimolar amounts of DNA from each strain, was used as template for 16S rRNA gene amplification, at 1 µl per 25 µl reaction, using the same primer set and conditions as the soil samples (section 4.2.6.1).

Table 4.3: Details of 7 bacterial strains used to construct a mock community for 16S rRNA amplicon sequencing

Bacterial strain	Phylogeny	16S rRNA copy no[1]	Genome size bp[2]
Escherichia coli	*Gammaproteobacteria*	7	4641652
Listeria monocytogenes	*Firmicutes*	6	2944528
Halomonas titanicae	*Gammaproteobacteria*	3	5339792
Vibrio parahaemolyticus	*Gammaproteobacteria*	11	5165770
Streptococcus pyogenes	*Firmicutes*	6	1852433
Pseudomonas aeruginosa	*Gammaproteobacteria*	4	6264404
Micrococcus luteus	*Actinobacteria*	2	2501097

[1] Estimated 16S rRNA copy number and [2] estimated genome size, as taken from rrnDB.

Soil extracted and mock-sample extracted DNA for all samples was amplified in triplicate and pooled after amplification. Resulting PCR amplicons for each sample were size selectively purified using Promega's Wizard® SV Gel PCR Clean-Up System (section 2.2.3.3). Purified amplicons were quantified using pooled in equimolar amounts before shipping on ice to the Centre for Genomic Research (University of Liverpool) where they underwent paired-end sequencing on a single run of the Illumina MiSeq platform.

4.2.6.3   16S rRNA data analysis

Paired-end sequencing reads were analysed using Mothur (Schloss *et al.* 2009), as detailed in section 2.2.5.1 and following the protocol of Kozich *et al.* (2013). Briefly, low quality sequences were removed, and remaining sequences aligned against the SILVA database release 128, trimmed to the V4 region of the 16S rRNA for improved alignment quality (Pruesse *et al.* 2007). VSEARCH, as integrated in Mothur, was used to remove chimeras (Rognes *et al.* 2016). Classification of sequences was performed using the method of Wang *et al.* (2007) (kmer size of 8) against the same V4 trimmed SILVA database used for sequence alignment. Sequences were clustered into OTUs in a two-step process; first datasets were split by classification, after which they were clustered at 97 % similarity using the OptiClust method (Westcott & Schloss 2017). A mock community was included in the sequencing run in an effort to determine sequencing error rates. OTUs represented by only one sequence (singletons) were removed prior to alpha diversity analysis using Mothur's remove.rare() function and subsequent alpha and beta diversity metrics were calculated as detailed in section 3.2.6 unless otherwise stated. In order to improve diversity estimates, the recommendation of Bokulich *et al.* (2013) was implemented, whereby any OTUs representing less than 0.005 % of the total read abundance in the dataset were not included for beta diversity analyses. Within R (R Core Team 2017), the package vegan (Dixon 2003) was used to calculate sample dissimilarity using the Bray Curtis index. Nonmetric Multidimensional Scaling (nMDS) was then performed using the metaMDS function in vegan and output was plotted using ggplot2 package (Wickham 2009). To assess persistence of slurry-derived microbial groups within the soil microcosms, the tool SourceTracker was employed and run in R (Knights *et al.* 2011).

4.2.7   Community level physiological profiling using MicroResp.

Changes in carbon utilization profiles were measured using the MicroResp™ Soil Respiration System developed by Campbell *et al.* (2003). In this system, CO_2 production rate from the soil microbiota is determined as a function of the substrate added, where a range of different substrates are used. Detection of evolved CO_2 is colorimetric, whereby a pH indicator dye embedded in agarose changes colour as CO_2 reacts with bicarbonate. This consists of a detection solution of 12.5 μg ml^{-1} cresol red, 150 mM potassium chloride and 2.5 mM sodium bicarbonate suspended in a 1% purified agar solution within a standard 96 well plate.

4.2.7.1   MicroResp standard curve generation for CO_2 quantification

A standard curve was prepared in order to convert colour change of the detection solution to percentage CO_2. A 96 well MicroStrip plate was prepared containing the cresol red indicator solution. The absorbance of the solution at time zero was measured at 570 nm. A strip of four wells were placed in a 40 ml universal tube which was then sealed with a turnover septum stopper. This was repeated for 17 tubes, from which air was removed (using a syringe and needle) and 20 % CO_2 added in increments to create a series of CO_2 concentrations from 0.039 % (ambient) up to 5 % CO_2 (gel saturation), thereby creating a standard curve. Sealed tubes were incubated for 5 hours at 25 °C, after which the strips were placed in the microstrip rack and absorbance of the detection solution at 570 nm was recorded. Absorbance readings were normalised by averaging the absorbance at time zero per 96 well detection plate, and then applying the following formula to the post-incubation 5 hour data:

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$$Normalised\ absorbance = \left(\frac{At5}{At0}\right) \times mean(At0)$$

Where:

At0 = absorbance at time zero (570 nm)

At5 = absorbance after 5 hours incubation (570 nm)

Regression analysis was performed using the open-access 'MyCurveFit' software in order to curve fit percentage CO_2 data against absorbance readings (software available at mycurvefit.com). The best fitting standard curve was a four parameter logistic model (4PL), where goodness of fit was represented by $R^2 = 0.99$. The formula obtained is detailed below:

10

$$y = 0.1767 + \frac{14221229 - 0.1767}{1 + \left(\frac{x}{0.0089}\right)^{4.7352}}$$

Where:

x = absorbance at 570nm

y = percentage CO_2.

4.2.7.2   Preparation of carbon sources

Nine carbon sources were chosen for analysing substrate induced respiration patterns; L-arginine, L-cysteine-HCl, D-(+)-glucose; γ-amino butyric acid; α-ketoglutaric acid; L-lysine-HCl; L-malic acid, N-acetyl glucosamine, oxalic acid and protocatechuic acid. These were chosen to represent carbon sources with a range of complexity including amino acids, carbohydrates, carboxylic acids and phenolic acids (Colombo *et al.* 2016). The final concentration of each carbon source, prepared per gram of soil water is presented in Table 4.4. While the recommended substrate concentration is 30 mg / ml soil water, some substrates had to be used at lower concentrations. This was a

result of the high water content of flooded microcosms, and the fact that substrate concentrations are based on soil water content.

Table 4.4: Carbon sources used in the MicroResp system. Substrates were prepared per gram of soil water.

Carbon Source	Concentration (mg/ml)
L-arginine	15
L-cysteine-HCl	30
D-(+)-glucose	15
γ-amino butyric acid	30
α-ketoglutaric acid	30
L-lysine-HCl	30
L-malic acid	30
N-acetyl glucosamine	30
oxalic acid	15
protocatechuic acid	2

4.2.7.3 Substrate induced respiration of soil microbial communities

Deep, 96-well plates were filled with fresh soil directly from microcosms, using the MicroResp filling device, and weighed. Substrates were added in triplicate (technical replicates), at 25 µl per well, along with three wells per microcosm that received 25 µl of water alone, as a control indicating basal respiration. A detection plate was inverted above the deep well plate. A specialised rubber seal was placed between the two 96-well plates, allowing air exchange between one deep well and its corresponding detection well above, thus creating 96 separate, air-tight 'chambers' (Figure 4.2).
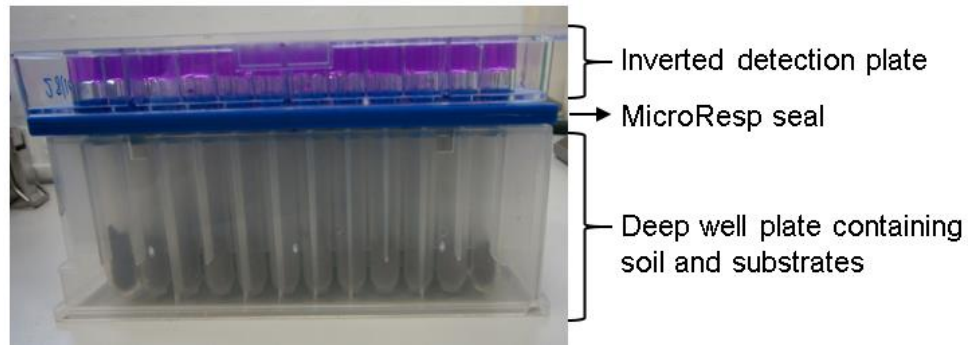
Figure 4.2: The assembled MicroResp system, consisting of a deep, 96-well plate containing fresh soil amended with a range of carbon substrates. A 96-well plate containing cresol red CO_2 detection gel was inverted above, with a specialised seal between the plates. A MicroResp clamp applied around the entire system maintained the seal between each set of corresponding soil and detection wells during incubation.

The plates, as shown in Figure 4.2, were placed in a MicroResp clamp to maintain the seal, and incubated at 25 °C for five hours. The unit was disassembled, and absorbance of the detection plate was recorded at 570 nm. Post incubation absorbance readings were normalised (Equation) and converted to percentage CO_2 using the 4PL model determined from the standard curve (Equation). Production rate of CO_2 (in µg CO_2-C/g/h) was then calculated by applying equation 11, and technical replicates were averaged to give one rate per substrate per microcosm.

11

$$CO_2 rate = \left[\frac{\left(\frac{\% \, CO_2}{100}\right) \times V \times \left(\frac{44}{22.4}\right) \times \left(\frac{12}{44}\right) \times \left(\frac{273}{273 + T}\right)}{Soil \, fw \times \left(\frac{\% \, dw}{100}\right)} \right] \Big/ hr$$

Where:
V = well headspace volume (µl);
44 is the molecular weight of CO_2 and 22.4 is the volume, in litres, occupied by one mole of a gas at standard pressure and temperature (ideal gas law);
12/44 accounts for the molecular weight of carbon (12) in CO_2 (44);
T = the incubation temperature in ° C which is then converted to Kelvin;
soil fw = average soil fresh weight per well (g);
% dw = percentage dry weight (gravimetric) of the soil and
hr = incubation duration in hours.

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4.2.7.4 Analysis of MicroResp datasets

Mean and standard deviation of CO_2 production rate were calculated from the three replicate microcosms per treatment at each time point. Samples to which only water was added were used to represent basal respiration rates. Within R (R Core Team 2017), the package vegan (Dixon 2003) was used to calculate sample dissimilarity using the Bray Curtis index. Nonmetric Multidimensional Scaling (nMDS) was then performed using the metaMDS function in vegan and output was plotted using ggplot2 package (Wickham 2009).

4.2.8 Litter decomposition rate assays

To assess the capacity of the soil systems to decompose complex plant material, changes in respiration rate were monitored upon mixing soil with litter, based on the litter decomposition assay detailed by Griffiths *et al.* (2000). Grass clippings from a pesticide-free garden were collected and dried overnight at 40 °C. Aliquots of 30 g (fresh weight) of soil were mixed with 30 mg of grass clippings and placed in 250 ml vessels. Tight fitting lids allowed for CO_2 accumulation which was detected *via* the attachment of four micro-strip wells containing CO_2 indicator solution (as used in the MicroResp 96 well plates; section 4.2.7.1) to the lid of each experimental unit as in García-Palacios *et al.* (2013). Vessels were placed at 25 °C for three hours and changes in absorbance of the microstrip wells were measured at 570 nm. Absorbance data were normalised (Equation 9), converted to percentage CO_2 (Equation 10) and CO_2 production rate was calculated (Equation 11), adjusting the soil weight, headspace and incubation time parameters as appropriate.

4.2.9 Potential nitrification rate assays

Potential nitrification rates (PNR) were assessed by incubating soil with an ammonium source (ammonium sulphate) and blocking the oxidation of nitrite to nitrate with chlorate, thereby preventing conversion to nitrogen gas and allowing for quantification of total nitrite produced from ammonium. A PNR buffer was prepared by the addition of ammonium sulphate (100 µM) and chlorate (2.5 mM) to a 1 mM phosphate buffer prepared to the same pH as the soil (pH 6) (Hart *et al.* 1994). Fresh soil from each microcosm (10 g) was mixed with 100 ml of PNR buffer in a 250 ml flask, and stoppered with a sponge. 'Time zero' flasks immediately underwent extraction of NO_2^- *via* the addition of 30 ml of 2 M KCl to determine background nitrite concentrations. Remaining flasks were incubated for 24 hours at 90 rpm and 15 °C. After incubation, NO_2^- was extracted with 30 ml of a 2 M KCl solution, shaking at 90 rpm for 1 hour at 15 °C. Soil slurry was filtered through Whatman no. 1 filter paper and NO_2^- quantified as described below in section 4.2.11.2. Aliquots of soil were kept for determination of soil moisture content in order to convert data to per gram soil dry weight.

4.2.10 Potential denitrification rate assays

Short term anaerobic incubations, based on Smith and Tiedje (1979) were used to determine potential denitrification rates, where soils are incubated with nitrate whilst blocking the conversion of nitrous oxide to dinitrogen gas, thus allowing for quantification of total nitrous oxide produced from nitrate. A 20 g aliquot of soil from each microcosm was incubated in a 150 ml vial with 20 ml of a 1 mM phosphate buffer (pH 6) containing a mix of two carbon sources, 10 mM glucose and 10 mM succinate (Morley *et al.* 2014), and nitrate in the form of 10 mM KNO_3. Vials were stoppered

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with rubber bungs, flushed with N_2 and allowed to vent to atmospheric pressure via insertion of an empty, airtight syringe. Acetylene was added to a final volume of 10 % the headspace and vials were incubated at 15° C on a shaker at 90 rpm. Gas samples were removed at three time points (30, 60 and 90 minutes) during the short term incubation and injected into pre-evacuated, 7ml storage vials each fitted with a double-wadded septum. Samples were stored until analysis for N_2O concentrations. Gas chromatography for quantification of N_2O was performed using an electron capture detector (ECD) at 300 °C, as detailed by Roche *et al.* 2016. N_2O data were converted from parts per million (ppm) to µl N_2O L^{-1} headspace using the ideal gas law as in the following formula:

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$$C_m = \frac{C_v \times M \times P}{R \times T}$$

Where:
C_m = mass per volume concentration (µl N_2O / L) and
C_v = volume per volume concentration ppm.
M = molecular weight of trace gas (i.e., 28 to µg N_2O -N/ µmol N_2O);
P = barometric pressure in atmospheres;
T = air temperature in ° K and
R = the universal gas constant (i.e., 0.0820575 L atm•°K•mole).

The rate of denitrification (DR) was then calculated using the following formula as described in Groffman *et al.* (1999), as detailed below:

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$$DR = \frac{(C_{90} \times H) - (C_{30} \times H)}{(D \times T)}$$

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Where:

$C_{30} = \mu g \ N_2O$ -N/ L headspace after 30 minutes of incubation

$C_{90} = \mu g \ N_2O$ -N/ L headspace after 90 minutes of incubation;

H =the flask headspace (L);

D = the dry weight of soil used in the assay;

T = the duration between the two timepoints analysed, expressed in hours.

Rates were then expressed as the mean of three biological replicates.

4.2.11 Colorimetric quantification of extractable, inorganic nitrogen

Inorganic nitrogen was extracted by mixing aliquots of soil with 2 M KCl at a ratio of 1:2, followed by shaking at 90 rpm for 1 hour at 15 °C. Soil slurry was filtered through Whatman no. 1 filter paper and stored for 24 hours at 4 °C. Extracted ammonium, nitrate and nitrite were quantified as in Bollmann *et al.* (2011) and detailed below. All assays were performed in 48 well plates, with standards and blanks quantified in triplicate and samples in duplicate. After each reagent was added, plates were gently shaken by hand to mix.

4.2.11.1 Ammonium determination

Ammonium quantification was determined based on a protocol introduced by Kandeler & Gerber (1988). A series of standards was prepared by serial dilution of $(NH_4)_2SO_4$, to give a range of the following concentrations: 10, 20, 50, 100, and 200 $\mu M \ NH_4^+$. Blanks were included in every plate and consisted of the distilled water used to prepare all assay reagents. Standards, blanks and samples were dispensed in the 48 wells in 500 µl aliquots. Samples potentially having higher NH_4^+ concentrations (slurry and flood treated) were quantified neat as well as diluted with dH2O at 1 in 5 or 1 in 10, as appropriate. Sodium hydroxide (0.3 M) and sodium nitroprusside solution

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(0.5 M sodium salicylate and 2 mM sodium nitroprusside) were mixed in a 2:1 ratio of which 250 µl was added to the samples, followed by 100 µl of sodium dichloroisocyanurate (0.9 mM). Plates were placed in the dark and incubated for 30 minutes before absorbance was read at 660 nm using a microplate spectrophotometer (BioTek PowerWave).

4.2.11.2 Nitrite quantification

Nitrite (NO_2^-) was quantified following the method introduced by Keeney & Nelson (1965). Standards were generated by the dilution of a stock solution of $NaNO_2$ to give a series of concentrations of 5, 10, 25, 50, and 100 µM NO_2^-. The NO_2^- determination reagent consisted of 60 mM sulfanilamide and 2 mM naphthylethylene diamine dichloride (NEDD) in 8.5 % H_3PO_4. Standards, blanks and samples were placed in the 48 wells in 500 µl aliquots, to which 125 µl of determination reagent was added. Plates were incubated in the dark for 10 minutes and absorbance at 540 nm was recorded.

4.2.11.3 Nitrate and nitrite quantification

Quantification of nitrates and nitrites was performed using the method of Shand *et al.* (2008). Standards were generated by the dilution of a stock solution of KNO_3 to give a series of concentrations of 12.5, 25, 50, 75, and 100 µM NO_3^-. Standards, blanks and samples were placed in the 48 wells in 500 µl aliquots, to which 75 µl of a catalyst solution was added, (0.14 mM $CuSO_4$ and 5 mM $ZnSO_4 \cdot H_2O$) along with 75 µl of NaOH (1 M) and 75 µl hydrazine sulfate (13 mM). Plates were incubated in the dark before the addition of 250 µl sulphanilamide (60 mM) in HCl (3.5 M) and 75 µl of NEDD (4 mM). Plates were incubated for a further 10 minutes and absorbance was read at 540 nm.

4.2.11.4 Analysis of NH_4^+, NO_2^- and NO_3^- absorbance readings

For all plates analysed, background absorbance readings from blank samples (H_2O) were subtracted from standards and samples, and standards were used to generate a calibration curve. The equation of the line was used to determine the NH_4^+, NO_2^- and NO_3^- concentrations of samples, and a correction factor was applied for any that had been diluted. To determine NO_3^- concentrations, the concentration of NO_2^- was subtracted from the $NO_2^- + NO_3^-$ data acquired in 4.2.11.3. Accounting for percentage dry weight of the soil assayed, and the molecular weight of ammonium, data were converted to mg of N per g dry weight of soil. Data were collated and changes with time were plotting using R.

4.2.12 Total carbon and nitrogen

Soil samples were dried overnight at 40 °C and stored until being ball milled (Retsch 200) at 25 Hz and 90 seconds. Slurry samples that had been flash frozen and stored at -80 °C were freeze dried for 24 hours until all moisture was removed. Samples were transported to Teagasc's Environment Research Centre (Wexford, Ireland) where total carbon and nitrogen was determined using LECO Truspec CN analyser (LECO Corporation, St. Joseph, MI, USA), using combustion at 950 °C.

4.2.13 Physicochemical data and functional assays: statistical analyses and data visualisation

Statistical analysis was performed in R (R Core Team 2017) using the r-base statistics functions. Statistically significant differences in mean for soil functional assays and physicochemical properties were detected using two tailed t-tests, subsequent to performing an F-test for comparing sample variances. The variance-appropriate t-test (Student's or Welch's) was then performed and any p values that were presented in the same figure or table were corrected for multiple t-tests using the false discovery rate (FDR) approach (Benjamini & Hochberg 1995). For comparing differences in alpha diversity, data were first checked for normality using the Shapiro -Wilk test (Shapiro & Wilk 1965). If data were normal, an Anova test was performed, followed by a Tukey's post hoc test if $p < 0.05$ (Tukey 1949). For non-normal data, a Kruskal-Wallis analysis of variance test was performed (Kruskal & Wallis 1952), followed by a Dunn's post-hoc test if $p < 0.05$ (Dunn 1961).

For all box plots presented, the upper, middle and lower line of each box indicate the 75th percentile, the median and the 25th percentile of the data respectively, while the whiskers indicate the highest and lowest values, and dots denote outliers (data points more than two standard deviations from the mean).

Data representation was performed in R using the ggplot2 package (Wickham 2009) and in SciDavis (Standish *et al.* 2007).

4.3   Results

4.3.1   Soil moisture content

Untreated controls had a mean gravimetric soil moisture content of 24.8 %, similar to that of slurry amended samples with a mean of 25.6 % (Figure 4.3). During the flooding phase (T3 – T7 inclusive), mean gravimetric soil moisture content was 37.1 % and 36.6 % in un-amended and slurry amended microcosms respectively.
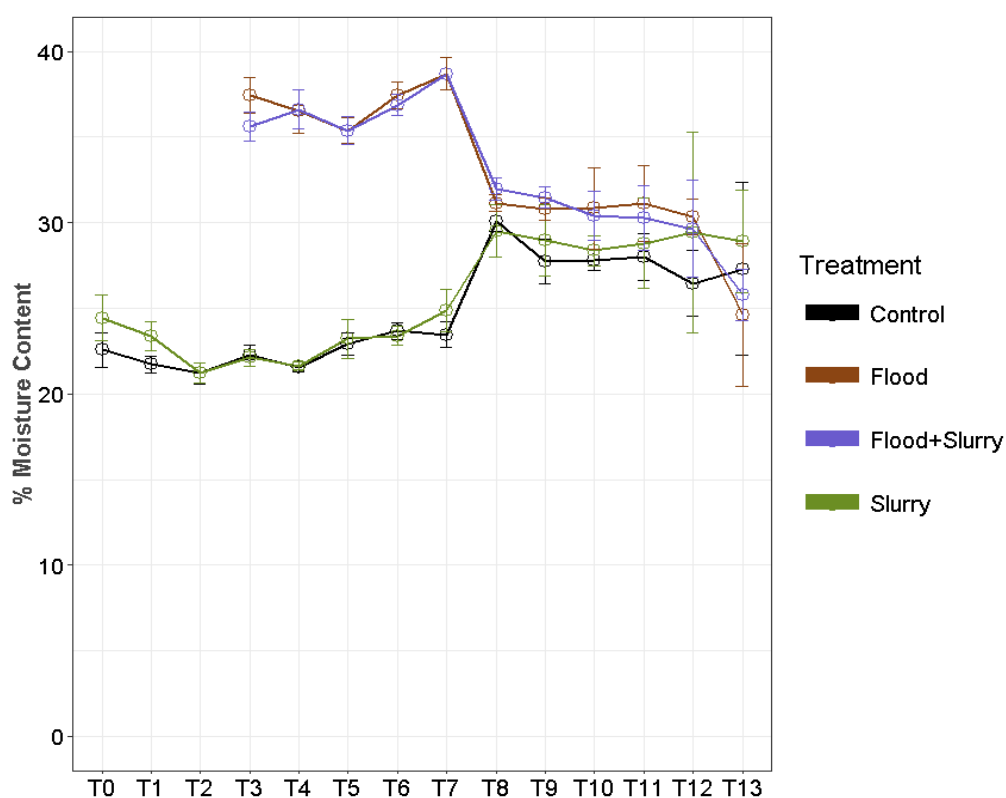

Figure 4.3. Gravimetric water content, on a wet weight basis, as a function of time. Each point represents the mean of biological replicates and error bars depict standard deviation (n=3).

4.3.2   Soil basal respiration rates

Immediately following slurry application (T0) there was roughly a three-fold increase in basal respiration rates, with the mean basal respiration rates in the control at 0.47 μg C-CO$_2$ g^{-1} hr^{-1} versus 1.45 μg C-CO$_2$ g^{-1} hr^{-1} in the slurry amended microcosms

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(Figure 4.4). This effect was relatively short lived, and after 6 days, no differences in mean basal respiration rates were observed between these two treatments.
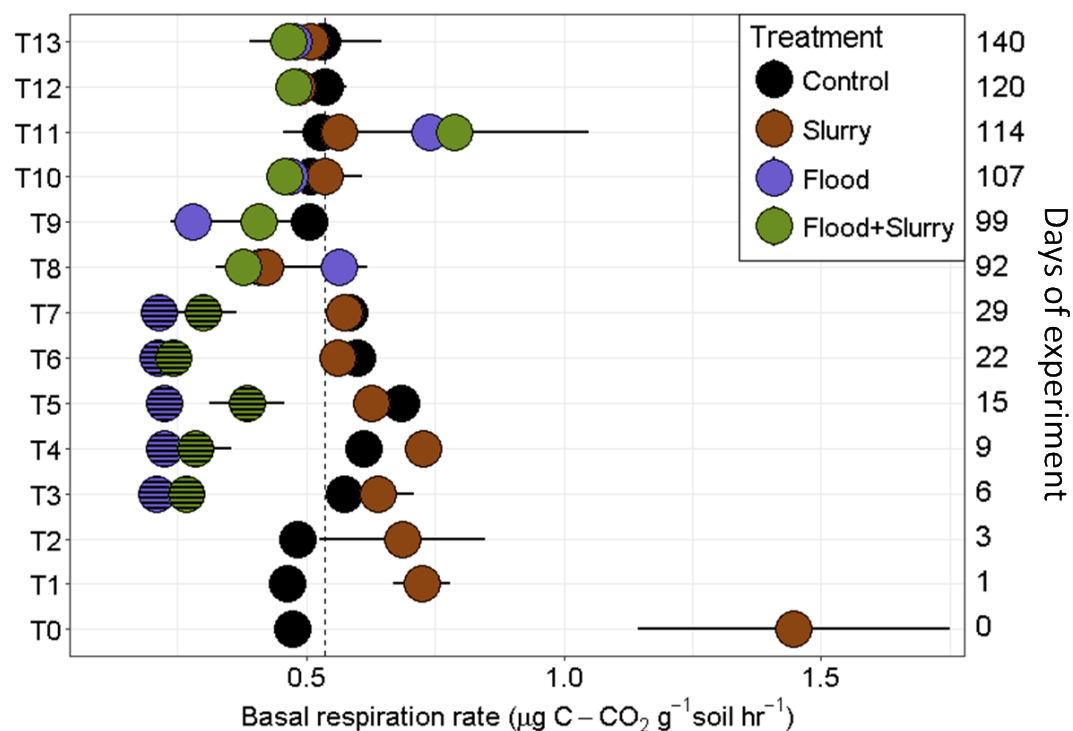

Figure 4.4. Changes in soil basal respiration rates for all four treatments, as a function of time. Each point represents the mean respiration of biological replicates, with standard deviation of the mean represented by horizontal whiskers (n=3). The left y axis shows the timepoint ID and for clarity, the right y axis shows the days since slurry amendement. The dotted line at 0.54 µg $C\text{-}CO_2$ g^{-1} hr^{-1} represents the mean respiration rate of the untreated controls, for T0 – T13. Points shaded with horizontal bars represent those in waterlogged conditions at the time of sampling, (i.e. 'flood' T3 – T7 and 'slurry+flood' T3 – T7).

Upon flooding, there was more than a two-fold decrease in basal respiration rates in the water-logged samples compared to the unflooded samples (Figure 4.4). This trend remained the same throughout the flooded period (T3 – T7). During this flooded phase, there was a trend whereby samples receiving slurry prior to the flooding event had slightly elevated basal respiration rates (0.3 ± 0.07 µg $C\text{-}CO_2$ g^{-1} hr^{-1}) when compared to those not receiving slurry amendment (0.22 ± 0.01 µg $C\text{-}CO_2$ g^{-1} hr^{-1}). This difference in mean basal respiration rates was statistically significant at $p < 0.01$. Once

in the 'recovery phase' (T8 – T13), basal rates for all three treatments were similar to the untreated control, and no consistent legacy effects from any of the disturbances were seen.

4.3.3 Litter decomposition rates

As with the basal respiration rates, the influx of nutrients and microbiota from the slurry resulted in a marked increase in litter decomposition rates at T0 in the slurry amended (5.2 µg C-CO$_2$ g^{-1} hr^{-1}) compared to control microcosms (1.5 µg C-CO$_2$ g^{-1} hr^{-1}), equating to more than a 3 fold increase (Figure 4.5).
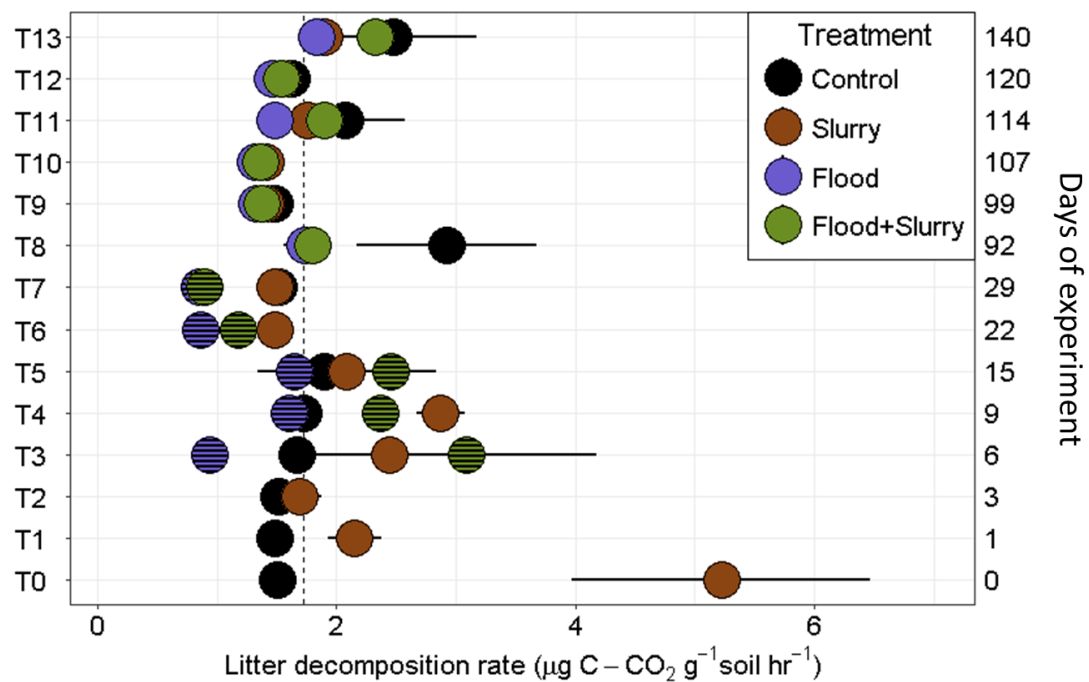

Figure 4.5. Changes in litter decomposition rates for all four treatments, as a function of time. Each point represents the mean respiration from biological replicates, with standard deviation of the mean represented by the horizontal whiskers (n=3). The left y axis shows the timepoint ID and for clarity, the right y axis shows the days since the first treatment was applied (slurry). The dotted line at 1.72 µg C-CO$_2$ g^{-1} hr^{-1} represents the mean litter decomposition rate of the untreated controls, for T0 – T13. Points shaded with horizontal bars represent those in waterlogged conditions at the time of sampling, (i.e. 'flood' T3 – T7 and 'slurry+flood' T3 – T7).

This higher rate of litter decomposition was seen in slurry treated samples until 9 days after amendment, except for T2 where no difference was seen between the control and the slurry treated samples (Figure 4.5). The impact of the flooding on litter decomposition rates appeared less immediate than on basal respiration rates, especially in microcosms receiving the compounded treatment of slurry and flooding. Litter decomposition rates were higher in the 'slurry+flood' microcosms, than the 'flood' microcosms. Indeed, the rates 'slurry+flood' soil were even higher than that seen in the untreated controls, for the first 12 days of the flood. After this, the litter decomposition rates appeared to converge (T6) and by the final flooded timepoint (T7) there was no evident legacy effect of the slurry amendment in either the flooded or unflooded samples. During the recovery phase (T8 – T13) no differences in mean decomposition rates occurred between the treatments, except for T8 where the control microcosms showed an uncharacteristic increase in decomposition rate.

4.3.4 Potential nitrification rates

Slurry amendment induced an evident increase in soil potential nitrification rates (PNR) and this trend was seen throughout the 140 days of the experiment, in both unflooded and flooded variants of the slurry treatment (Figure 4.6). During the flooding event (T3 – T8) there was no statistically significant reduction in the PNR of water logged soils with 'flood' treated samples having a similar PNR as the untreated controls throughout this period. Similarly, there was no effect of prolonged waterlogging on the PNR of flooded samples receiving the prior slurry amendment; these soils maintained similar PNR to those measured in soils with slurry and no flood (Figure 4.6).
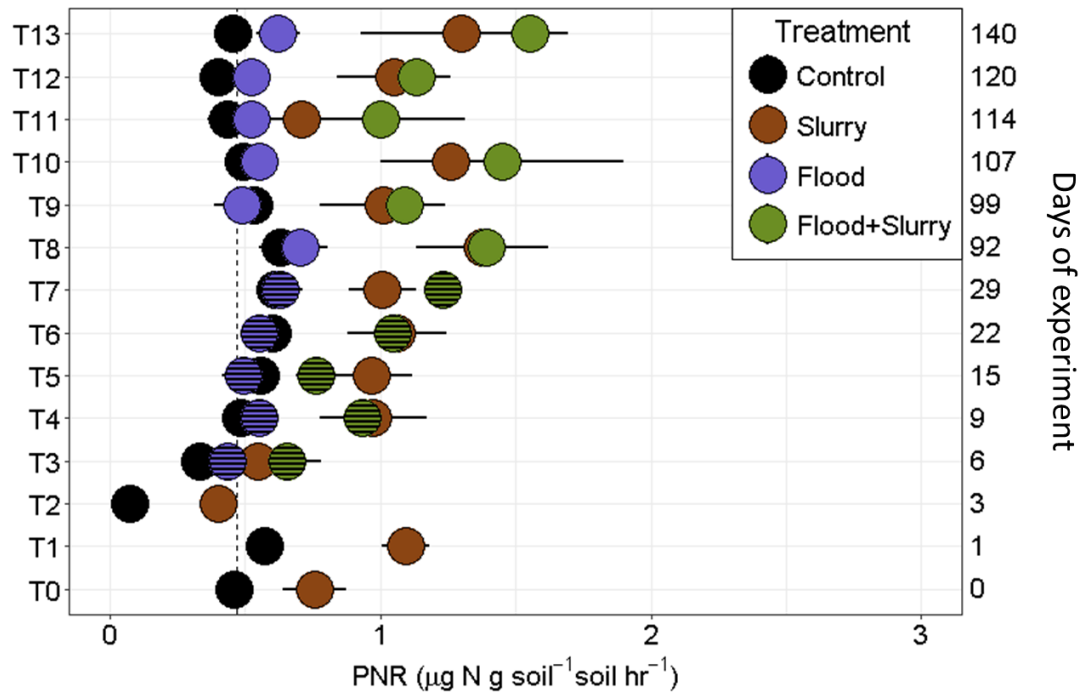
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Figure 4.6. Potential nitrification rates as a function of time, for all four treatments. Points shaded with horizontal bars represent those in waterlogged conditions at the time of sampling, (i.e. 'flood' T3 – T7 and 'slurry+flood' T3 – T7). The left y-axis shows the timepoint ID and for clarity, the right y-axis shows the days since the first treatment was applied (slurry). The dotted line at 0.47 μg N g^{-1} hr^{-1} represents the mean PNR of the untreated controls, for T0 – T13.

Due to variation seen in PNR in the untreated control, changes in potential nitrification rates (PNR) were then expressed in terms of percentage change from the untreated control (Figure 4.7). This provides a clearer demonstration of any treatments effects. After 3 days of flooding (T3) a 30 % increase in PNR was seen in 'flood' treated samples. This was perhaps a result of improved mobilisation of carbon and nitrogen sources as a consequence of the increased water content. The PNR of these 'flood' treated soils then remained approximately the same as the untreated control, until the final two timepoints, T12 and T13, where there was a statistically significant increase ($p < 0.01$) in PNR, relative to the untreated control (Figure 4.7).
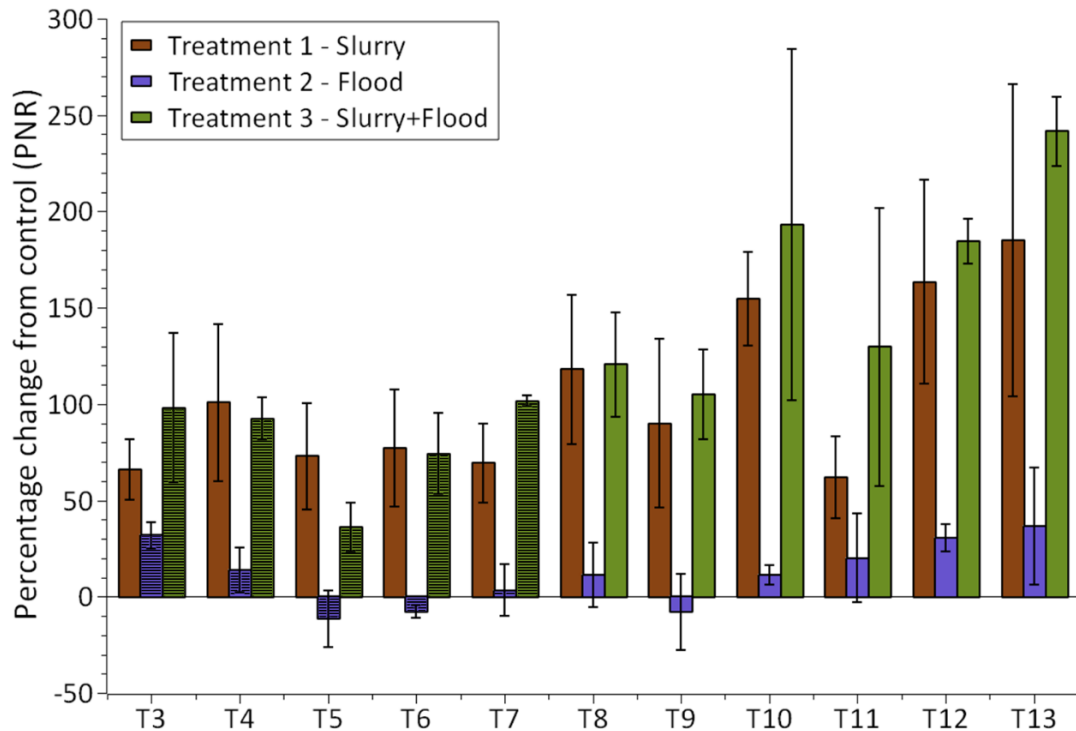
Figure 4.7. Percentage change in potential nitrification rates, compared to the untreated control. Each bar represents the mean PNR from biological replicates and the error bars depict standard deviation (n=3). The phases during which treatment 2 (flood) and treatment 3 ('slurry+flood') microcosms were waterlogged (T3 − T7) are shaded with black horizontal stripes.

When considering percentage change from the untreated control, the increase in PNR as a result of slurry amendment was again clear (Figure 4.7). While the percentage increase appeared larger in soils subjected to the compounded slurry and flood disturbance, compared to slurry disturbance alone, this difference was not statistically significant. Throughout the 140 days of the experiment, rates remained more than 50 % that seen in non-slurry amended soils (Figure 4.7).

4.3.5 Potential denitrification rates

Potential denitrification rates (PDR) of the untreated controls showed slight variation throughout the trial, where the mean PDR was 204 ± 28.9 µg N kg^{-1} soil h^{-1} (Figure

4.8). There was no change in PDR immediately following slurry amendment (T0), however after a further 24 hours (T1) the PDR rate was double that of the untreated control, and remained so until the end of the second phase of the experiment (29 days), by which stage the rate was the same as in the control. During the flooding phase (T3 − T8), soils from the 'flood' and 'slurry+flood' treated microcosms were capable of increased rates of denitrification (Figure 4.8; waterlogged samples represented by horizontal shading).
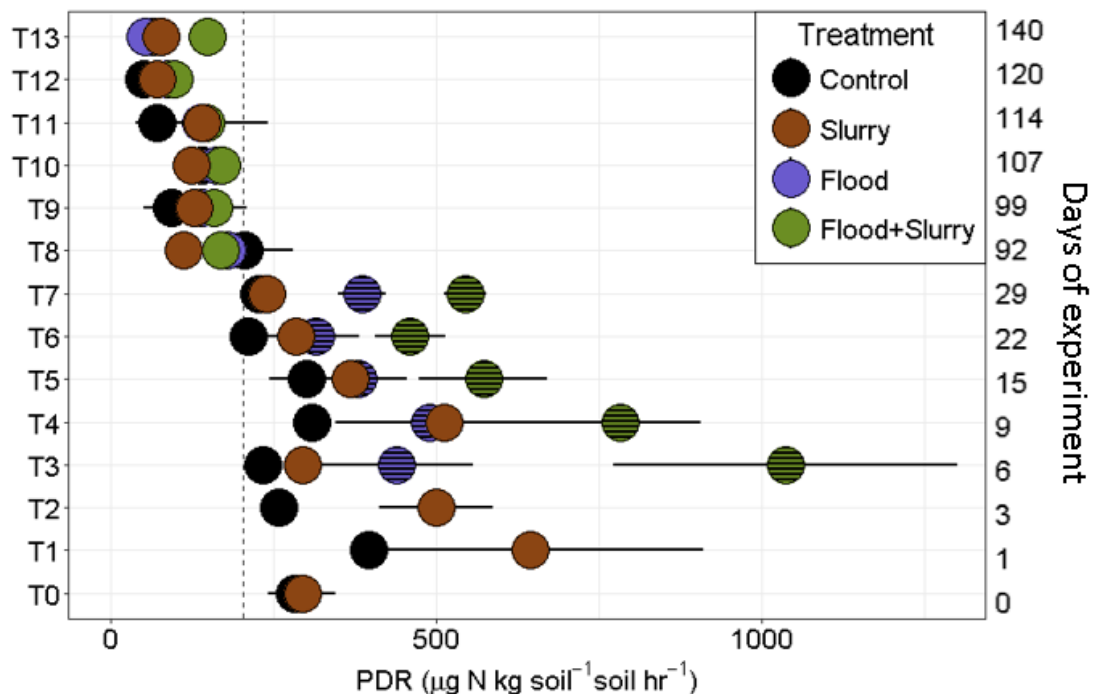

Figure 4.8. Changes in potential denitrification rates (PDR) as a function of time, for all four treatments. Points shaded with horizontal bars represent those in waterlogged conditions at the time of sampling, (ie 'flood' T3 − T7 and 'slurry+flood' T3 − T7). The left y-axis shows the timepoint ID and for clarity, the right y-axis shows the days since the first treatment was applied (slurry). The dotted line at 204 µg N kg^{-1} hr^{-1} represents the mean PDR of the untreated controls, for T0 − T13.

Amendment with slurry prior to the flooding event further increased PDR in the soils (Figure 4.8). Throughout the flooded phase (T3 − T8), this increase was statistically significant ($p < 0.001$) with the mean PDR of 'flood' treated soils at 387 ± 71 µg N kg^{-1} hr^{-1} versus a mean rate of 634 ± 170 µg N kg^{-1} hr^{-1} in 'slurry+flood' treated soils.

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During the recovery phase (T8 – T13), the PDR returned to that of the untreated controls, except for the final timepoint (T13) where there was a statistically significant increase ($p < 0.01$) in the PDR from soils subjected to the compounded disturbance. Due to the fluctuation in PDR seen in the control samples, in particular during the flooded phase, rates were analysed as percentage change from the untreated control for timepoints T3 – T7 (Figure 4.9).
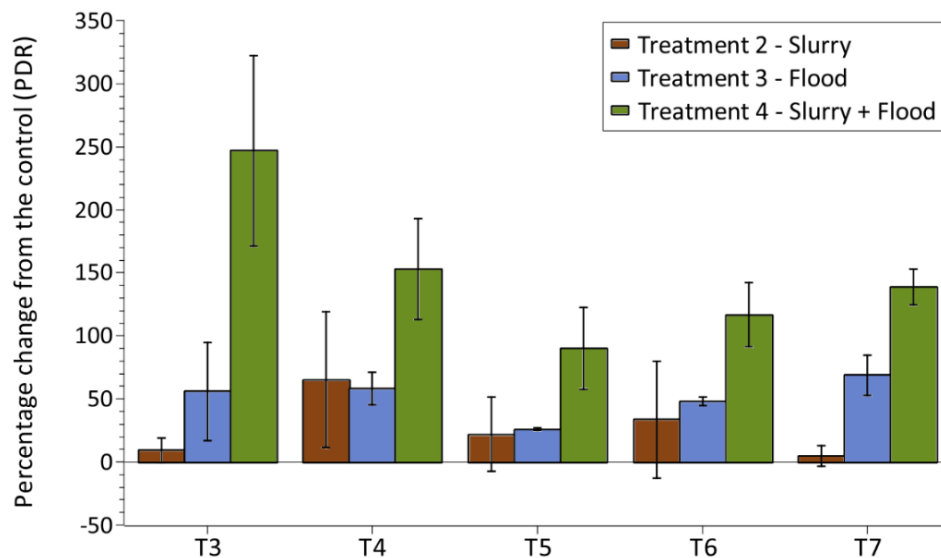
Figure 4.9. Percentage change in potential denitrification rates, compared to the untreated controls. Each bar represents the mean PDR from biological replicates and the error bars depict standard deviation (n=3).

Waterlogging induced significant increases in PDR relative to the controls during the flooded phase (Figure 4.9). The maximum increase in PDR was seen for the 'slurry+flood' treatment at T3, where PDR in these soils was approximately 250 % in excess of that measured in the untreated controls. After T3, the PDR stabilised to around 120 % that of the control for the remainder of the flooded phase. In soils exposed to the flood (without slurry amendment), PDR rates were 50 % higher than those measured in the control at T3 and remained so throughout this flooded period (Figure 4.9).

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To gain insights into the effects of the slurry and flooding stresses on *in situ* nitrogen cycling, extractable inorganic nitrogen concentrations were assessed.

4.3.6 Soil ammonium concentrations as a function of time.

The mean concentration of ammonium in the untreated soils was 2.8 ± 0.73 mg NH_4^+ - N kg^{-1} soil (dw) throughout the experiment. As expected, immediately following slurry application the soil ammonium concentration increased, to 7.8 ± 0.75 mg NH_4^+ - N kg^{-1} soil (Figure 4.10). While there was significant fluctuation in the ammonium concentration in slurry treated soils, they remained higher than that of the un-amended control ($p < 0.001$) until 9 days (T4) after the amendment was applied, when concentrations returned to that seen in the control. After 3 days of waterlogged conditions (T3) the ammonium in flooded, slurry amended soils was similar to that seen in their unflooded counterparts, suggesting some oxygen was remaining in the flooded soils after 3 days of water saturation, allowing for nitrification (Figure 4.10). However, after 6 days of waterlogging (T4) ammonium had started to accumulate (31.6 ± 10.37 mg NH_4^+ - N kg^{-1}) and three days later (T5) reached its maximum level of 93.3 ± 5.85 mg NH_4^+ - N kg^{-1} soil. Ammonium levels in these 'slurry+flood' treated microcosms remained high for the remainder of the flooded phase, however there was a slight decrease seen at each subsequent sample point, with 79.4 mg NH_4^+ - N kg^{-1} measured at T6 and 69 mg NH_4^+ - N kg^{-1} at T7 (Figure 4.10).
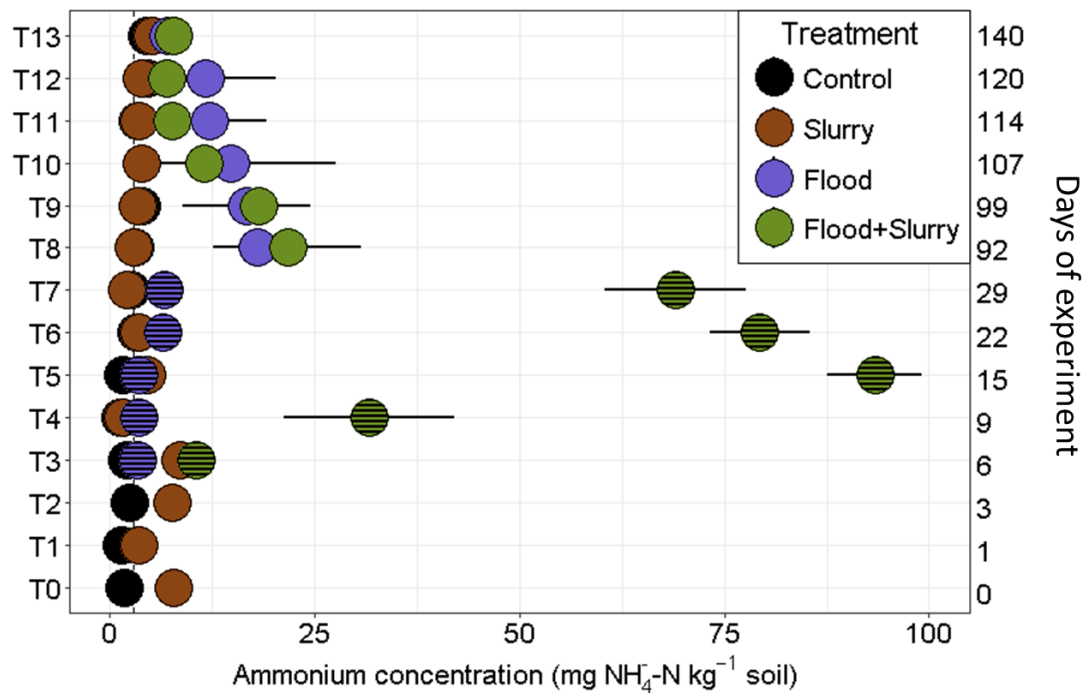
Figure 4.10. Extractable inorganic ammonium concentrations measured in control and stressed soils, in mg NH_4^+ - N kg^{-1} soil (dw). Points shaded with horizontal bars represent those in waterlogged conditions at the time of sampling, (ie 'flood' T3 – T8 and 'slurry+flood' T3 – T8). The dashed line represents the mean NH_4^+ concentration measured in the control soils.

In un-amended soils that underwent flooding, a step wise increase in ammonium concentration was seen in line with the duration of waterlogging (Figure 4.10). Again, 3 days post-flooding (T3) ammonium concentration remained the same as the unflooded control, however after 6 days (T4) it had doubled to 3.5 ± 0.34 mg NH_4^+ kg^{-1} soil and after 26 days of waterlogging, ammonium had accumulated to 11.1 ± 2.62 mg NH_4^+ kg^{-1} soil. The anoxic conditions during T3 – T7 appeared therefore to significantly reduce *in situ* nitrification rates, thereby leading to an accumulation of ammonium in these systems. This was especially apparent in samples receiving the prior stress of slurry application, where pre-flood nutrient content would have been in excess of that in un-amended soils.

After flooded microcosms were allowed to drain, ammonium levels in 'slurry+flood' treated soils fell to 21.7 ± 8.91 mg NH_4^+ kg^{-1} soil at T8, and continued to decrease with

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each sample point (Figure 4.10). By T11, ammonium concentrations had fallen to 7.5 $\pm$ 2.54 mg NH_4^+ kg^{-1} soil, and remained at a similar concentration for the remainder of the experiment (T12 and T13).

In microcosms subjected to flooding and no slurry amendment, there was a large increase in ammonium concentration between T7, the final flooded timepoint (11.1 $\pm$ 2.62 mg NH_4^+ kg^{-1} soil) and T8, the first recovery timepoint (18.0 $\pm$ 5.41 mg NH_4^+ kg^{-1} soil). This was likely a consequence of rates of nitrogen mineralisation being in excess of *in situ* nitrification rates at T7 and T8. Ammonium then steadily decreased in concentration at each subsequent sample point, and by the end of the experiment (T13) reached 7.1 $\pm$ 1.99 mg NH_4^+ kg^{-1} (Figure 4.10).

During the recovery phase, the lowest recorded ammonium concentration in 'flood' treated soils was seen at T13, while those receiving the 'slurry+flood' treatment, reached a similar ammonium concentration at T11; approximately three weeks earlier (Figure 4.10). Indeed, the rate at which ammonium falls during the recovery phase (T8 – T13) appears more rapid in 'slurry+flood' soils than in 'flood' soils, despite the lower ammonium concentration seen in the latter. Between T8 and T11 there was a 65 % reduction in ammonium concentrations in 'slurry+flood' treated samples, whereas during this same period, ammonium reduction in 'flood' treated soils was just 32 %. This suggests improved nitrification rates in 'slurry+flood' soils and corroborates the potential nitrification rate data (Figure 4.7), whereby slurry amendment improved PNR throughout the experiment.

To investigate the *in situ* reduction of nitrate *via* denitrification, changes in levels of inorganic extractable NO_3^- were investigated for the four treatments.

It should be noted that throughout the experiment, for most soils the level of nitrite remained below the detection limit of the assay employed for NO_2^- quantification,

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where the lowest standard had a concentration of 5 µM (section 4.2.11.2). The only exception was in slurry amended soils at T1 (24 hours post slurry application) where 0.08 ± 0.027 mg NO_2^- -N kg^{-1} soil was detected. These data are therefore not graphically presented.

4.3.7 Soil nitrate concentrations as a function of time

The mean concentration of soil nitrate in the untreated controls was 1.15 ± 0.07 mg NO_3^- -N kg^{-1} soil from T0 to T13. Immediately following slurry application, the concentration of nitrate was 2.2 ± 0.08 mg NO_3^- -N kg^{-1}, approximately double that measured in the control (Figure 4.11). Due to errors made in the quantification process, T5 and T11 are missing from this dataset.

Initially, during the flooded phase (T3 and T4) nitrate concentrations in the slurry amended and non-amended waterlogged samples remained similar to their respective unflooded counterparts; 'slurry' treated and control (Figure 4.11). However, despite the favourable conditions for denitrification, namely anoxic conditions and available nitrogen, nitrate accumulation was seen at T6 and T7 (19 and 26 days of flooding respectively). While this occurred faster in 'slurry+flood' treated soils, by the end of the flooding phase (T7) both flood treatments had accumulated nitrate to a concentration of ~ 6 mg NO_3^- -N kg^{-1}. This suggests perhaps that by T6 organic carbon sources, or access to these sources, was restricted in these microcosms and thus limiting the rates of denitrification seen *in situ* (Knowles 1982).
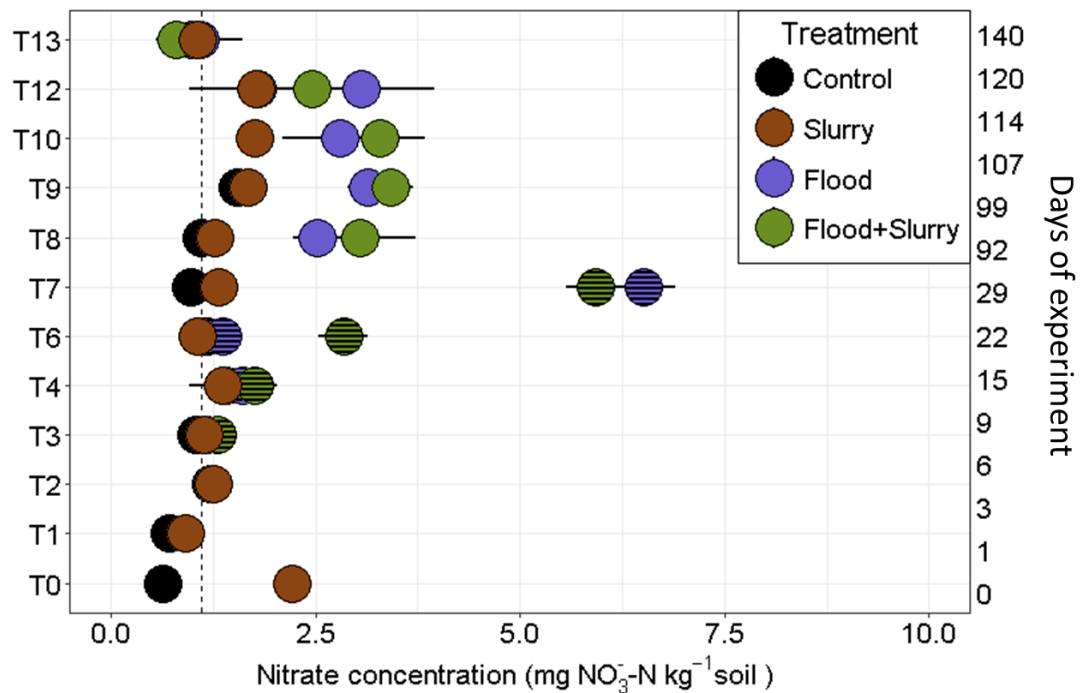
Figure 4.11. Extractable inorganic nitrate concentrations measured in control and stressed soils, in mg NO_3^- - N / kg soil (dw). Points shaded with horizontal bars represent those in waterlogged conditions at the time of sampling, (ie 'flood' T3 – T8 and 'slurry+flood' T3 – T8). The dashed line represents the mean NO_3^- concentration measured in the control soils (1.15 mg / kg soil). It should be noted that T5 and T11 are not presented due to errors during quantification.

In soils from both 'flood' and 'slurry+flood' treated microcosms, a reduction in nitrate concentrations, to approximately 3 mg NO_3^- -N kg^{-1}, was seen by T8, the first sample point of the recovery phase (Figure 4.11). Nitrate then remained at a similar concentration until T12. By the final sample point, T13, nitrate levels had fallen within the range of that measured in the untreated controls (1.0 ± 0.15 mg NO_3^- -N kg^{-1}), with concentrations in 'flood' treated samples at 1.1 ± 0.53 mg NO_3^- -N kg^{-1} and 'slurry+flood' treated samples at 0.8 ± 0.04 mg mg NO_3^- -N kg^{-1}.

While many of the functional assays indicated that the soil microbial community had somewhat recovered from the selection of stresses applied, we next wished to investigate the effects of the treatments on the physiological and taxonomic profiles of the soil microbiomes.

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4.3.8 Physiological profiling using carbon utilisation profiles

Microbial functional diversity was inferred from carbon utilisation profiles obtained using the MicroResp system. First, the cumulative substrate induced respiration (SIR) response was analysed. Upon slurry application, an increase in the total respiration response was seen, being double that of the un-amended control (Figure 4.12). While this elevated respiration response is still evident 9 days after slurry amendment (T4), it fell to the same as the untreated control by 15 days post slurry application (T5). This plot also demonstrates that slurry application increased the variance in the substrate induced respiration responses, where dots representing the three biological replicates per timepoint and treatments are generally more spread in 'slurry' and 'slurry+flood' treatments versus the control and 'flood' treatment (Figure 4.12).
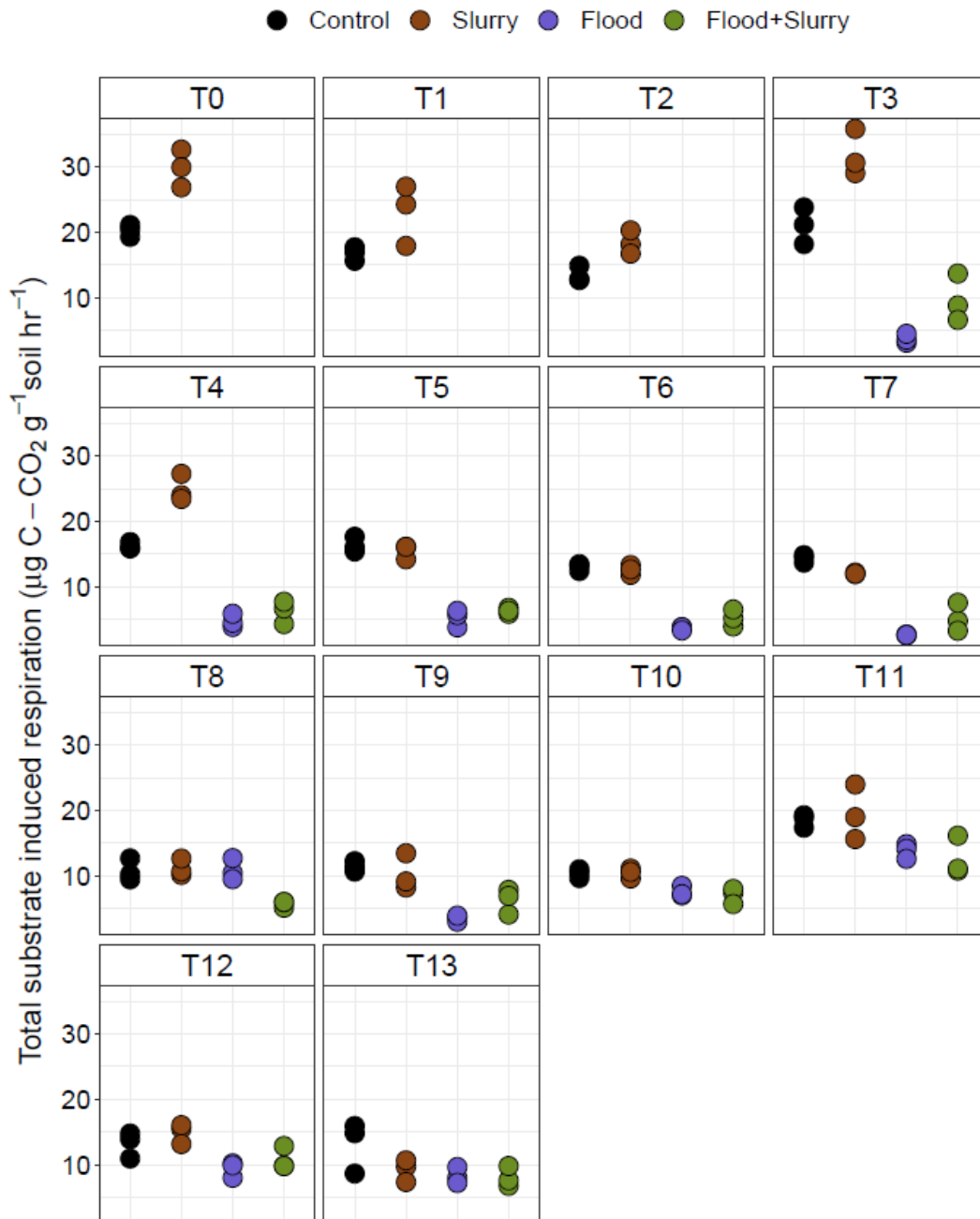
Figure 4.12. Total substrate induced respiration response for the four treatments at each of the fourteen timepoints. The first three timepoints represent the pre-flood phase and thus only two treatments appear for T0, T1 and T2.

During flooding, the total respiration response was significantly reduced in both 'flood' and 'slurry+flood' treatments (Figure 4.12). After 3 days of waterlogged conditions (T3) the total SIR response was slightly elevated in samples amended with slurry prior to flooding, compared with the flooded, un-amended soils, but this trend

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is no longer evident after 6 days of waterlogging (T4). During the recovery phase (T8 – T13) there is variability between the total SIR responses in the different treatments, but as a general trend rates tend toward that measured in the unperturbed control, particularly by the final two sample points (T12 and 13; Figure 4.12).

The patterns of SIR were analysed and in order to determine similarity between samples as a function of treatment and time, ordination was performed using non-metric multidimensional scaling (nMDS). To allow for meaningful patterns to be revealed within this large data set, SIR profiles were analysed per phase: pre-flood phase, flooded phase and recovery phase. Additionally, in order to allow comparison of physiological data with community composition data, only those time points where 16S rRNA profiling was performed are presented.

During the pre-flood phase (Figure 4.13A), there was an evident change in the SIR profiles as a result of slurry application. The first sample point, T0 was undertaken approximately two hours after slurry application and a clear divergence of slurry treated samples from the un-amended controls can be seen. This difference is still evident 3 days after the slurry amendment (T2) and indeed until 6 days post-amendment (T3 - Figure 4.13B). However, by 15 days post amendment (T5 - Figure 4.13B) slurry treated samples have converged toward the untreated controls.
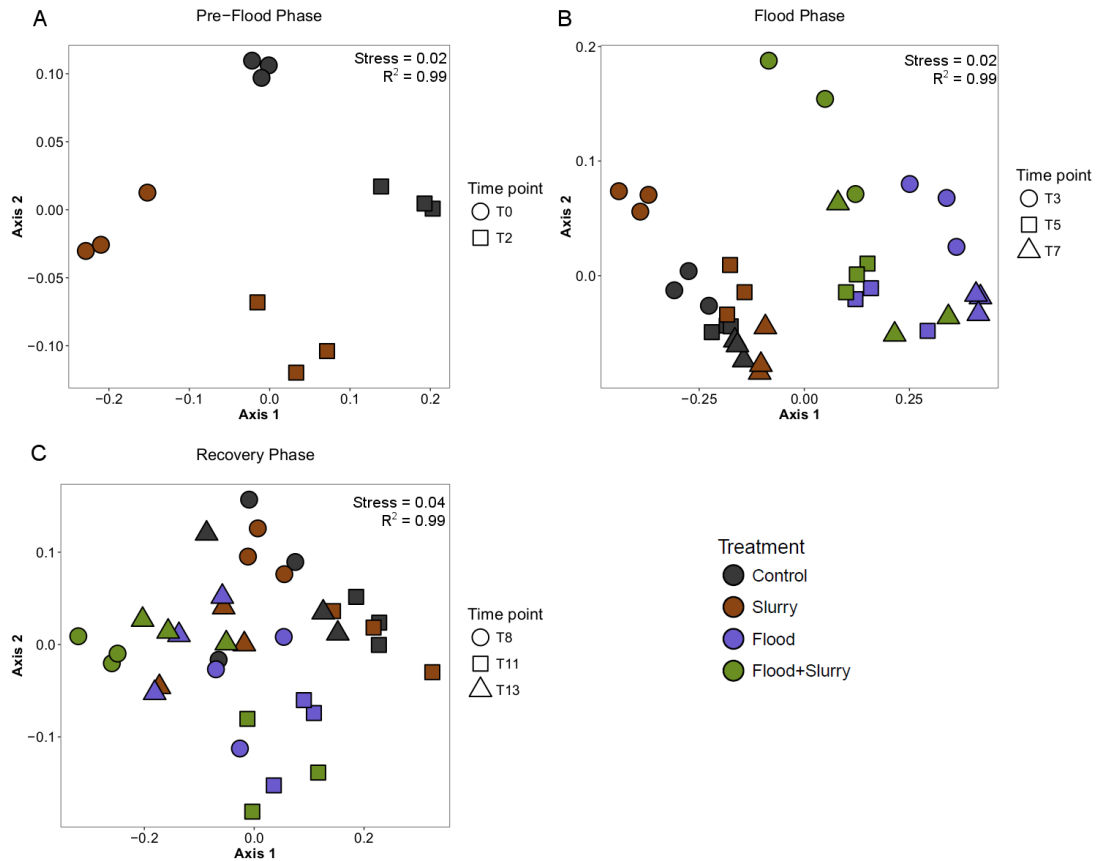
Figure 4.13. Non-metric multidimensional ordination of sample dissimilarity based on substrate induced respiration profiles, as calculated with the Bray Curtis index. Dissimilarities are represented for A) the pre-flood phase, B) the flooded phase and C) the recovery phase where soil moisture had returned to that of the unflooded control.

During the flooded phase (Figure 4.13B) there is an evident effect of the waterlogged status of the soils on SIR patterns, with a clear split between samples that underwent flooding ('flood' and 'slurry+flood' treated soils) versus those that did not ('control' and 'slurry' treated soils). Similarly to the non-flooded samples from this phase, there was an evident initial effect of the slurry upon SIR responses in 'slurry+flood' samples when compared to 'flood' treated samples (T3 - Figure 4.13B). However, by 15 days post application (T5) this effect appears reduced as 'slurry+flood' and 'flood' treated samples begin to converge at this point. Despite this convergence, the two flooded treatments do remain distinct from each other at all flooded time points (T3, T5 and

T7). In the initial stages of the recovery phase (T8 - Figure 4.13C), soils that had been flooded remain divergently ordinated from their non-flood treated counterparts. Also, at the first recovery sample point (T8), 'slurry+flood' treated samples group distinctly from samples recovering from the 'flood' treatment. But, as time progresses, these groupings become less distinct and by the final time point (T13 - Figure 4.13C) there is a clear convergence of 'slurry', 'slurry+flood' and 'flood' treated samples.

To determine how these changes in physiological profiles, as well as the functional data, compare with the microbial community structure, 16S rRNA profiling data was analysed.

4.3.9 Microbial community analysis using 16S rRNA profiling

To investigate changes in microbial community composition resulting from the different treatments, 16S rRNA profiling was employed on 8 of the 14 time points. These represented two sample points (T0 and T2) from the pre-flood phase, three sample points (T3, T5 and T7) from the flooded phase and finally three sample points (T8, T11 and T13) from the recovery phase. These correspond to the first and last sample point of each phase and a middle sample point for the flood and recovery phases.

4.3.9.1 Alpha diversity analysis of soil microbial communities

Rarefaction curves were used to visually assess the species richness per sample as a function of the number of sequences (Figure 4.14). The difference in sequencing depth between samples is evident from the rarefaction curves, and provides clear justification for subsampling the samples to even depth of sequences prior to undertaking beta diversity analyses (Figure 4.14). While the samples do not fully plateau (a

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demonstration of ample sampling depth) they do begin to plateau and thus sampling

depth was determined to be sufficient for downstream analyses.
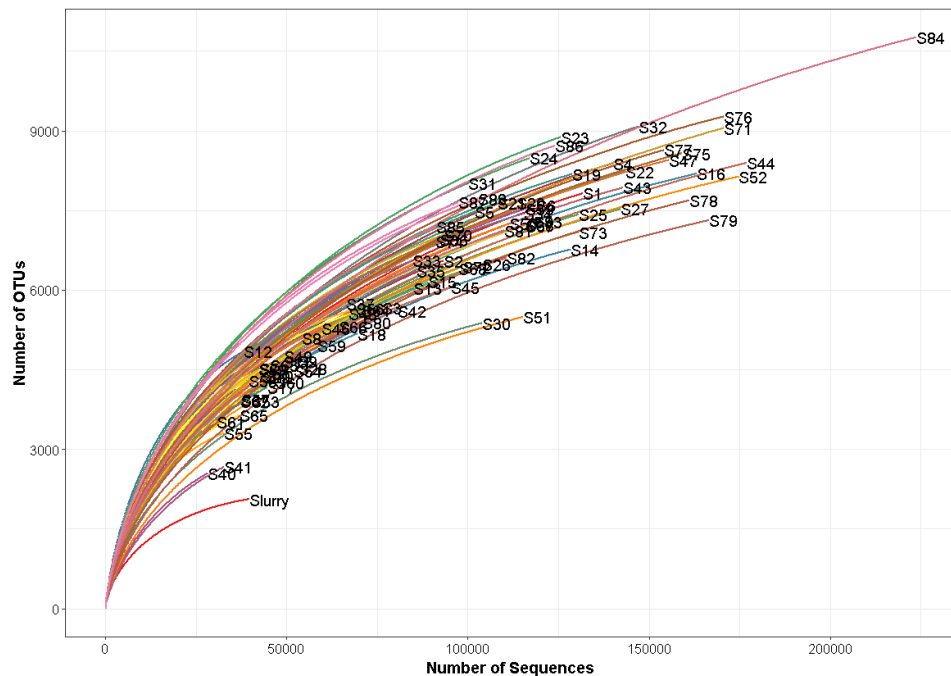
Figure 4.14. Rarefaction curves for all soil samples and the slurry sample. The number of OTUs detected as a function of sequences allows for the comparison of richness of the un-subsampled dataset, from all samples, as a measure of the sampling intensity. Each sample is represented by a single line labelled with 'Slurry' for the cattle slurry sample used to amend the soils, and all other soils microcosm samples are labelled with the prefix S-.

It is also evident from the rarefaction curve of the slurry sample that the microbial

community therein was likely not especially rich. This is evident when comparing the

richness of soil samples, with an average number of 3800 OTUs observed (Figure

4.15B), to the 1872 OTUs seen in the slurry sample (Table 4.5).

Table 4.5. Alpha diversity metrics of the microbial community isolated from slurry used to amend 'slurry' and 'slurry+flood' microcosms on day 0 of the experiment. The number of OTUs observed was used for observed richness while the chao1 estimator (Chao 1984) was used for estimated richness.

Sequence coverage	OTUs observed	Estimated richness	Simpson's evenness	Simpson's diversity
98 %	1872	2281	0.02	44.5

While percentage sequence coverage was exceptionally high in the slurry samples (98 %) it was deemed acceptable for all samples, as coverage was in excess of 90 % (Figure 4.15A) (Lemos *et al.* 2011). As evenness is scaled between 0 and 1, with 1 being a community containing numerically equitable members, the low evenness 0.02 calculated for the slurry sample suggests the OTUs were present in very different abundances. In order to account for the fact that most mixed microbial communities are not sampled to 100 % depth, we can use estimated richness to account for this (Hughes *et al.* 2001). In the slurry sample, the estimated richness was much closer to the observed richness (Table 4.5) than in the soil samples, where estimated richness was roughly 30 % more that the observed richness (Figure 4.15).
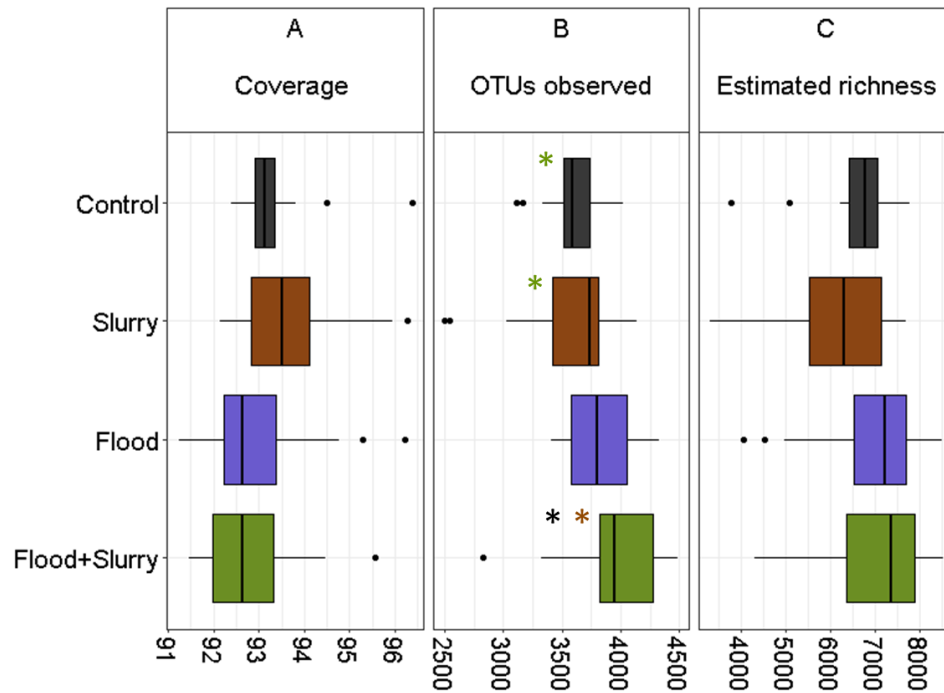
Figure 4.15. Alpha diversity metrics of microbial communities associated with the three disturbance treatments, and the untreated control. Included is A) Percentage 16S rRNA library coverage (with > 90 % being acceptable) B) the OTU count per sample C) The estimated richness of OTUs in the community using the chao1 estimator (Chao 1984). Boxes consist of 3 biological replicate samples for all time points for control slurry amended samples (n=24) and flood and slurry+flood treated samples (n=18). Any statistically significant differences in mean (adjusted $p < 0.01$) within each of the three metrics, are denoted by asterisks coloured according to treatment for which the difference is observed.

There were statistically significant differences in observed species richness between the treatments (Figure 4.15). The number of OTUs observed was higher in the 'slurry+flood' samples than what was seen in both the un-flooded treatments. Microbial community evenness, diversity and richness were investigated as a function of treatment, for each of the sample days (Figure 4.16).
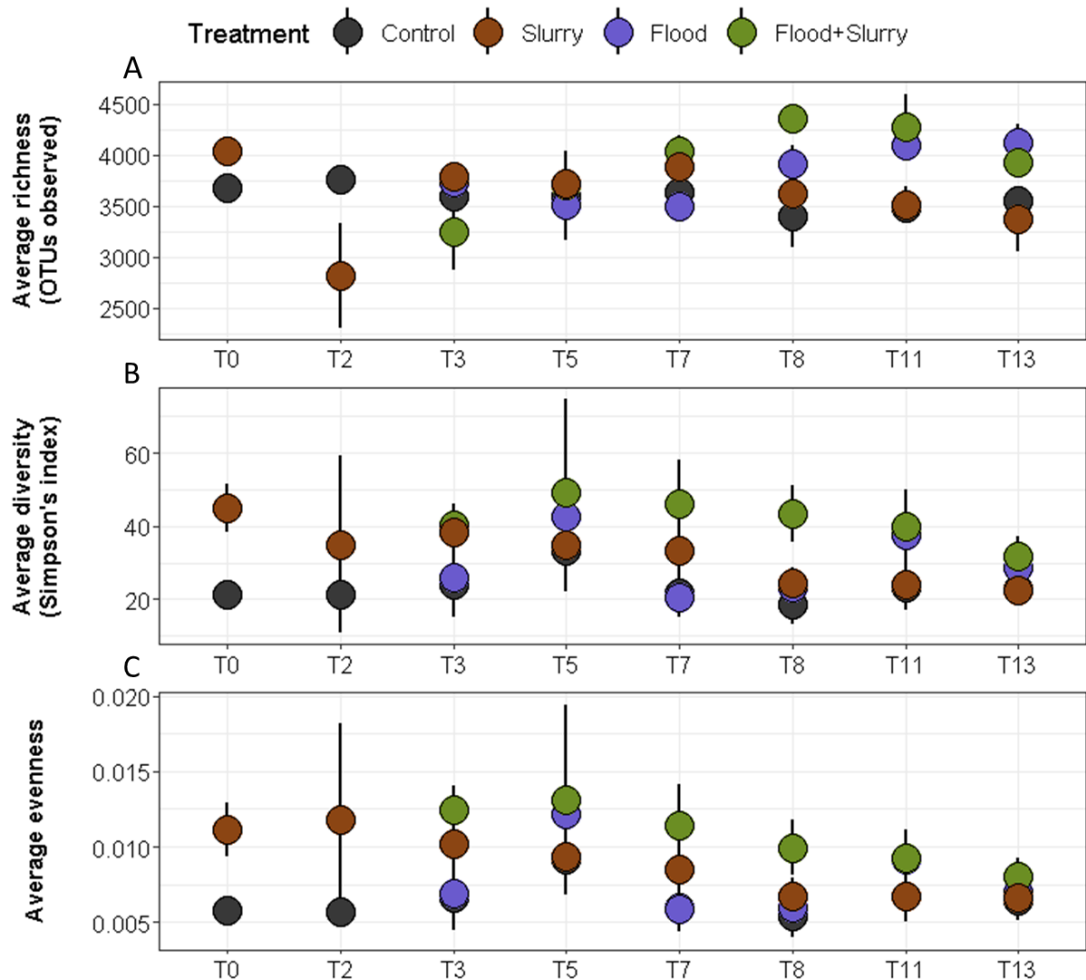
Figure 4.16. Measures of alpha diversity of soil microbial communities for each of the sample days, from the pre-flood phase (T0 and T2), the flooded phase (T3 − 7) and the recovery phase (T8 − 13). Each point represents the average A) number of OTUs observed, B) diversity based on the inverse of the diversity estimator introduced by Simpson (1949) and C) Simpson's evenness for defining numerical equality of the community, for 3 biological replicates. Standard deviation of the mean is represented by the whiskers.

Slurry amendment appeared to increase the evenness of the community, an effect that was observed in 'slurry' treated samples until T7, and in 'slurry+flood' treated samples until T11 (Figure 4.16C). By the final timepoint, T13, community evenness was similar in all soils. As diversity is a measure of richness and evenness, the differences in evenness between treatments were mirrored in the diversity measures (Figure 4.16B). In terms of microbial richness, there was an initial increase in observed richness immediately following slurry application, likely resulting from the influx of

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slurry-associated microbiota (Figure 4.16A). However, 72 hours later (T2) there was a dramatic reduction in richness in slurry-amended samples. This effect was relatively short lived, and observed only at this timepoint (T2) for 'slurry' treated samples and slightly longer in 'slurry+flood' samples; until T3. By the end of the flooded phase (T7) richness had fallen in the 'flood' samples while rising in the 'slurry+flood samples', indeed by this timepoint, the richness, evenness and diversity was higher in the 'slurry+flood' samples compared to all the other treatments (Figure 4.16A, B and C). Once in the recovery phase, an increase in species richness was seen in both treatments that had been waterlogged, and this trend persisted for the remainder of the experiment. Microbial community composition, depicted as bar plots of relative abundance for each sample, can be seen in Appendix V.

In order to further analyse these differences and compare microbial community structure between samples as a result of the treatments, beta diversity was analysed.

4.3.9.2   Beta diversity analysis of soil microbial communities

Sample dissimilarities calculated using the Bray Curtis index were ordinated using non-metric multidimensional scaling, as performed for each of the three phases; pre-flood, flood and recovery. Clustering of samples as a function of treatments was tested for statistical significant using analysis of molecular variance as implemented in Mothur. Included in the dissimilarity matrix for the pre-flood phase was the microbial community isolated from the slurry applied as amendment, referred to as the 'inoculant' (Figure 4.17A).
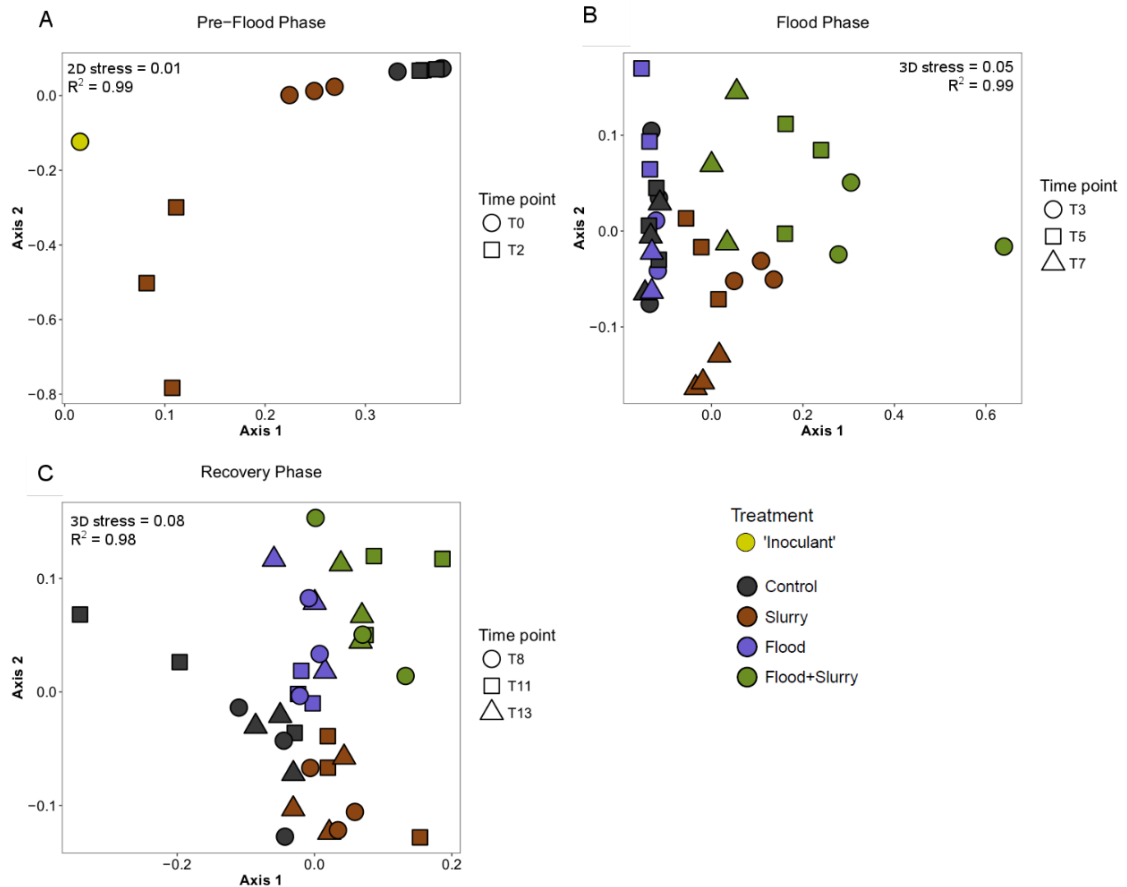
Figure 4.17. Non-metric multidimensional ordination of microbial community dissimilarity based on 16S rRNA profiles, as calculated with the Bray Curtis index. Dissimilarities are represented for A) the pre-flood phase, B) the flooded phase and C) the recovery phase where soil moisture had returned to that of the unflooded control.

The untreated controls during the pre-flood phase group very closely together, and a clear time-dependent divergence of slurry treated samples from the controls is seen between T0 and T2 (Figure 4.17A), and this was deemed statistically significant ($p < 0.01$). Biological replicates from slurry amended samples appear to ordinate further apart, notably at T2, suggesting increased sample heterogeneity as a result of the slurry amendment.

In the flooded phase (Figure 4.17B) there is an evident divide in sample groupings, with non-slurry amended samples grouping closely together, while 'slurry' and

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'slurry+flood' samples form distinct, treatment-specific clusters. While the physiological profiles of the microbial communities during the flooded phase resulted in clear grouping of samples according the water-status of the soils (Figure 4.13B), differences in the community composition during this phase however are driven more by the presence or absence of the prior slurry amendment (Figure 4.17B).

Once in the recovery phase, the dissimilarity of samples as a function of the treatment to which they were subjected is still evident (Figure 4.17C). However, it should be noted that of the three ordination plots shown (Figure 4.17A, B and C), the axes scales during this phase are the smallest, therefore a smaller area was needed to accurately ordinate the samples based on their dissimilarity implying smaller differences were observed.

To test the statistical significance of the observed clustering of samples as a function of treatment, ANOSIM analysis was performed (Table 4.6). Treatments were statistically dissimilar from each other at all phases, with the exception of control and flooded samples during the flooded phase.

Table 4.6. Results of ANOSIM analysis, for comparing the similarity of groups of samples based on their treatment.

Phase	Comparison	R value	*p* value
Pre - flood	Control vs Slurry	0.61	0.002
	Control v Slurry v Flood v Slurry+Flood	0.50	<0.001*
	Control vs Slurry	0.69	<0.001*
	Control vs Flood	-0.05	0.637
Flooded	Control vs Slurry+Flood	0.71	<0.001*
	Slurry vs Flood	0.65	<0.001*
	Slurry vs Slurry+ Flood	0.48	<0.001*
	Flood vs Slurry+Flood	0.61	<0.001*
	Control v Slurry v Flood v Slurry+Flood	0.53	<0.001*
	Control vs Slurry	0.31	<0.001*
	Control vs Flood	0.37	<0.001*
Recovery	Control vs Slurry+Flood	0.72	0.001*
	Slurry vs Flood	0.63	<0.001*
	Slurry vs Slurry+ Flood	0.82	<0.001*
	Flood vs Slurry+Flood	0.53	<0.001*

Ordination based analysis of sample dissimilarity, supported by ANOSIM analyses, revealed a strong effect of each treatment upon microbial community structure. We therefore aimed to elucidate which microbial taxa were contributing to the observed differences.

4.3.9.3   Differential abundance analysis for each experimental phase

The linear discriminative analysis (LDA) effect size (LEfSe) method (Segata *et al.* 2011) was used to identify OTUs that were differentially abundant between the four treatments, at each phase; pre-flood (Figure 4.18A), flooded (Figure 4.18B) and recovery (Figure 4.18C). This analysis aims to determine which OTUs are responsible

for the distinct sample groupings. Only statistically significant differential abundances ($p < 0.01$) are shown and, due to a large number of discriminatory taxa, only those with an LDA score of larger than 3 are presented. OTUs are identified at the taxonomic level of order and class. OTUs assigned to the same taxonomic rank were grouped together, and their mean LDA score is presented with the number of OTUs included in the group shown in the corresponding data point.
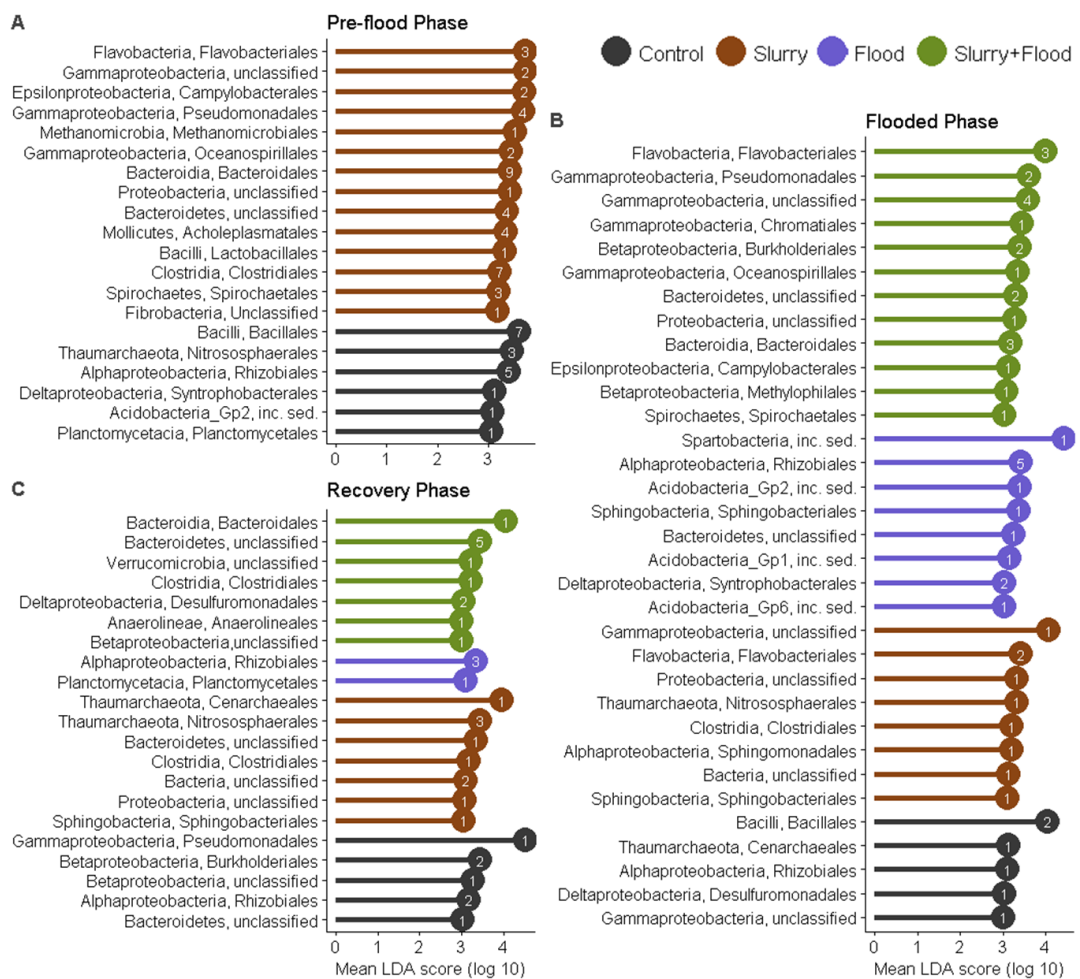
Figure 4.18. Linear discriminative analysis (LDA) effect size (LEfSe) analysis showing differentially abundant OTUs between the four treatments, 'Control' (black), 'Slurry' (brown), 'Flood' (blue) and 'Slurry+Flood' (green). Included are differentially abundant OTUs from A) the pre-flood phase, B) the flooded phase and C) the recovery phase. OTUs with an LDA score of > 3 are plotted and are coloured according to the treatment for which they provide discriminatory power, due to markedly increased abundances. OTUs are labelled at the level of class and order, and only statistically significant discriminatory OTUs are represented ($p < 0.01$). OTUs

assigned to the same taxonomic order had their LDA score averaged and the number of OTUS in each taxonomic group is shown within the corresponding data points.

In the pre-flood phase, a larger proportion of OTUs were identified as differentially abundant in the 'slurry' treated soils compared to the controls (Figure 4.18A). Of the 44 slurry-discriminative OTUs in this phase, 40 could be attributed to the slurry 'inoculum' (i.e. were absent from the T0 soil-only samples). The significant proportion of the OTUs that were differentially abundant in pre-flood, slurry amended soils were of the phylum *Bacteroidetes,* (34 % of OTUs) and were from the orders *Flavobacteriales* and *Bacteroidales* (Figure 4.18A). The phylum *Firmicutes* was also well represented with 7 members of the order *Clostridiales* being discriminatory for slurry treated soils. Seven OTUs from this phylum were also discriminatory for the untreated controls, however, were members of a different order; the spore-forming *Bacillales* (De Vos *et al.* 2015) and represented a diverse range of families. OTUs from the orders *Flavobacteriales* and *Bacteroidales* (both of the *Bacteroidetes* phylum) and *Clostridiales* continued to be increased in their abundance in slurry amended soils during the flooding phase, and indeed into the recovery phase (Figure 4.18B and C). The influence of the anoxic conditions prevailing in the flooded, slurry amended soils is also evidenced by the increased abundance of members of the *Methylophiliales,* the typically-anaerobic *Spirochaetales* and the micro-aerophilic *Campylobacterales.* As in the pre-flood phase, un-amended soils (control and waterlogged) from this phase harboured increased relative abundances of *Rhizobiales spp.* This trend continued throughout the experiment, and some of the same *Rhizobiales* OTUs, (OTUs 4, 10 and 15), were still discriminatory for the un-amended flood treated samples during the recovery phase (Figure 4.18C).

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In the recovery phase, of the 22 OTUs (with LDA > 3) identified as discriminatory for either of the slurry-amended treatments, 'slurry' or 'slurry+flood', two were 'colonisers' originating from the slurry applied on T0 (i.e. were absent from T0 soil). These were OTU 68 (discriminatory for 'slurry' samples) and OTU 201 (discriminatory for 'slurry+flood') and both OTUs were members of the *Clostridiales,* with OTU 68 assigned to the family *Peptostreptococcaceae* and OTU 201 to *Ruminococcus.*

To determine, in a more systematic manner, the contribution of slurry-derived microbiota to the assemblages extracted from slurry-amended microcosms, the software SourceTracker was employed.

4.3.9.4 *Tracking survival of slurry-derived taxa in the soil microcosms*

The tool SourceTracker (Knights *et al.* 2011) was used to determine what proportion of the OTUs detected in each sample could be attributed to the slurry applied at the start of the experiment. When running the software, samples are identified as either a 'source' or a 'sink' of taxa. The 'signature' of each source, based on microbial community structure, is then detected in each of the sink samples chosen. For this analysis, the slurry 'inoculum' microbial community as well as the T0, untreated soil microbial community were included as sources (referred to as 'slurry' and 'soil' respectively for these analyses). Any OTUs not attributable to either of these sources were labelled as 'unknown', and this occurred as a result of that particular OTU being absent (i.e. a count of a 0) from all the source samples provided. Additionally, a fraction of OTUs could not be reliably assigned to a particular source as their relative abundance was similar in all the source samples provided. In an effort to account for this, SourceTracker analysis was also performed on non-slurry amended soils to obtain

a base line for the 'unknown' fraction above which differences could be investigated. The mean proportion of 'unknown' taxa in all non-amended samples was 20 ± 7 % and is represented by a dashed line on each of the graphical representations of the SourceTracker data. Data acquired was in proportion per sample. As proportions were similar between biological replicates, in the interests of concise presentation, data from biological replicates were averaged. Raw data output can be seen in Appendix VI. For ease of interpretation regarding persistence of slurry-derived microbiota as a function of the duration since slurry was applied, sample days are denoted not by the customary T_n format, but with 'days post slurry-amendment'.

In slurry amended samples (no flooding) the contribution of slurry-derived OTUs to those detected in the entire sample is approximately 25 % for the first three days post slurry application (Figure 4.19). This proportion falls rapidly, and 6 days post application has dropped to just 2.8 %. The fraction continues to gradually drop until the final sample days of the experiment, where slurry-derived OTUs constitute around 0.2 % of the total. In the trimmed data set used for analyses, a total of 1717 OTUs remained, thus 0.2 % represents approximately 3 OTUs.

Figure 4.19. Relative contributions of slurry (green) and soil (brown) derived taxa to the microbial communities extracted from slurry amended soils. OTUs that cannot not definitively be tracked to either of these sources are labelled as 'Unknown' in source. The black dashed line represents a baseline of 'unknown' OTUs that were detected independent of slurry amendment. Labels at the end of each bar detail the percentage of taxa that are derived from the slurry 'inoculum' source as identified using SourceTracker (Knights *et al.* 2011).

Of note is the significant increase in contribution of 'unknown' microbial taxa to the relative abundance of the slurry amended samples 3 days post application (Figure 4.19). As seen when analysing the alpha diversity of this 16S rRNA dataset, (Figure 4.14 and Figure 4.15), a fraction of the total microbial community present in each sample was not accessed and could partially account for why the source of these OTUs is not the slurry inoculum or the T0 soil. However, the marked increase seen in the proportion of 'unknown' source taxa appears to have some relation to slurry amendment, as there is a step wise decrease in these taxa with time since the amendment progresses. The slurry amended microcosms that underwent flooding were

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then analysed to investigate this phenomenon, using SourceTracker (Figure 4.20). As flooding did not commence until after the sampling performed on day 3, data for this analysis does not include day 0 or 3. Days 6, 15 and 29 represent the flooded period while days 92, 114 and 140 are from the recovery period.
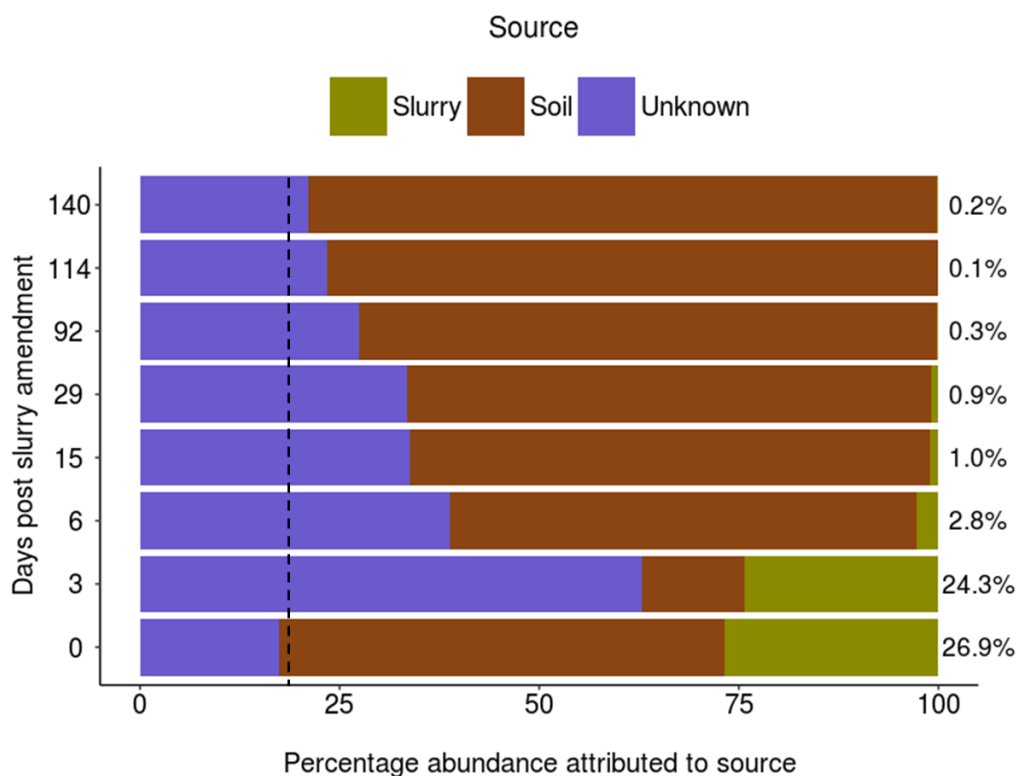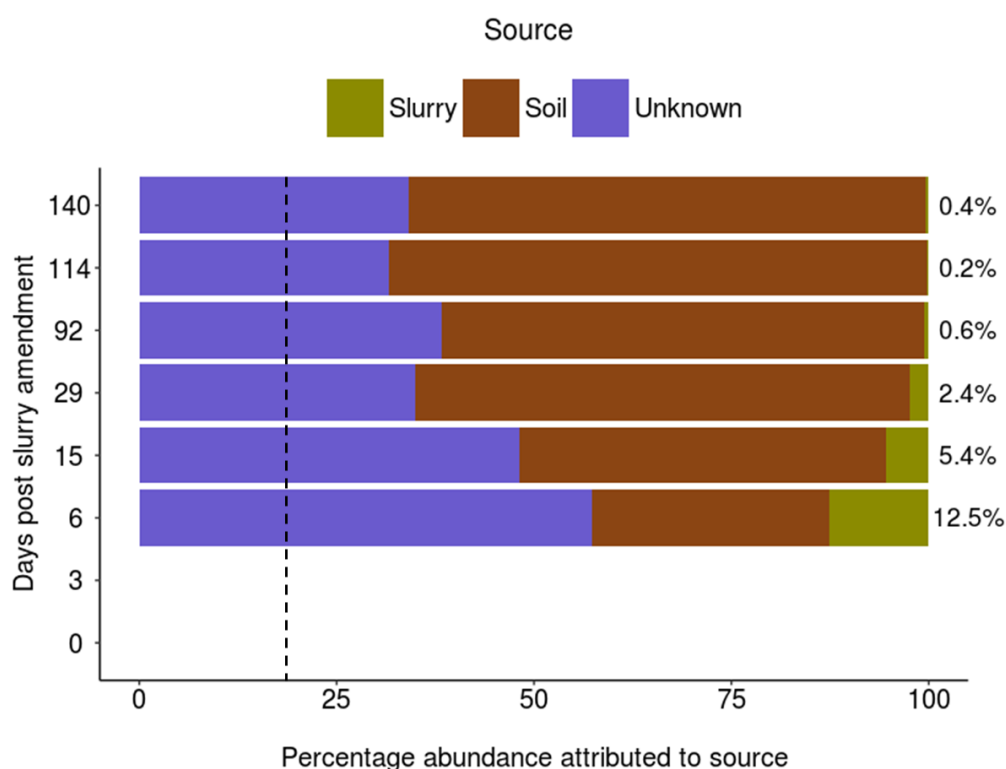

Figure 4.20. Relative contributions of slurry (green) and soil (brown) derived taxa to the microbial communities extracted from slurry amended soils that underwent flooding, where days 6 - 29 represent the flooded phase and days 92 – 140 the recovery phase. OTUs that cannot not definitively be tracked to either of these sources are labelled as 'Unknown' in source. The black dashed line represents a baseline of 'unknown' OTUs that were detected independent of slurry amendment. The labels at the end of each bar detail the percentage of taxa that are derived from the slurry 'inoculum' source, as identified using SourceTracker.

There appears to be increased survival of slurry-derived taxa in the flooded conditions, as at 6 days post slurry amendment 12.5 % of the OTUs could be attributed to the slurry inoculum (Figure 4.20), whereas at the same sample time in non-flooded systems, only 2.8 % of the taxa were slurry derived (Figure 4.19). Again, 15 days after

amendment there were approximately five times more OTUs attributed to the slurry in the flooded versus non-flooded microcosms. Once in the recovery phase, the proportion is similar to that observed in their non-flooded counterparts, at less than 1 %. Again, the same trend is observed whereby a high proportion of OTUs 'unknown' in source are found in the initial time points, and while this does exhibit a decrease, it is less marked than in the non-flooded systems and remains around 35 % for the duration of the recovery phase (Figure 4.20). Taxa within the 'unknown' group that were absent from both the slurry inoculum and the T0 soil communities were extracted from the OTU table (Figure 4.21).
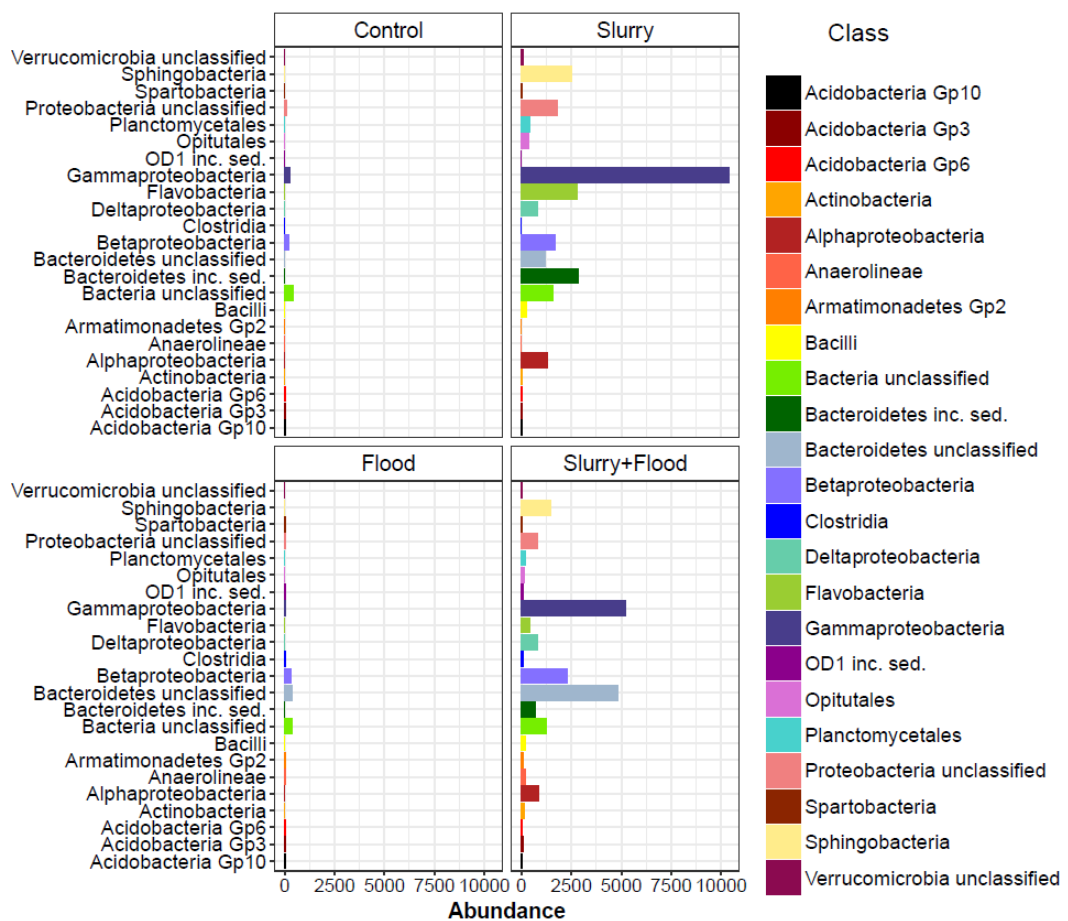

Figure 4.21. Abundances of OTUs absent from the slurry 'inoculum' and the T0 soil microbial community profiles and therefore grouped into the 'unknown' category of source tracker. OTUs are grouped into the phylogenetic group of Class.

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The largest proportion of these OTUs were assigned to the *Gammaproteobacteria*, the majority of which were unidentified at lower taxonomic levels. The two OTUs from which constituted the majority of the counts within this phylogenetic group were OTUs 36 and 82. Using the SILVA database, the closest relative to both OTUs was identified as the heterotrophic *Oceanospirillales*, at approximately 92 % similarity. Members of the *Betaproteobacteria* and the *Bacteroidetes* also contributed significantly to these 'unknown' source category (Figure 4.21). These two bacterial groups have been proposed to be typically copiotrophic in nature (Fierer *et al.* 2007). One of our initial hypotheses was that slurry addition would impact the ratio of copiotrophs to oligotrophs. Either as a result from the large input of rumen-associated biomass, likely copiotrophs by nature, or from the influx of slurry-associated nutrients. While there is some evidence from the differential abundance analysis that some of these groups (for example *Bacteroidetes*) were affected as a function of the treatment, further analysis on the full taxonomic dataset was required.

4.3.9.5 *Relative abundances of copiotrophic and oligotrophic bacterial groups*

The classifications proposed by Fierer *et al.* (2007) were followed, whereby the phylotypes *Betaproteobacteria* and *Bacteroidetes* were taken to represent copiotrophs, while the *Acidobacteria* represented the oligotrophs.

When observing the changes in relative abundance of *Betaproteobacteria* as a function of time, a steady increase is observed from T2 until the final day of the flooded phase, T7, in both 'slurry' and 'slurry+flood' treatments (Figure 4.22). As it is evident that the slurry used for amendment harboured very few *Betaproteobacteria* (total relative abundance of 0.2 %) it is likely therefore that this increased abundance in the slurry treated samples is a consequence of this increased resource availability (Figure 4.22).

Of course, as is always the case with such data, a caveat exists whereby only relative abundances can be reported.

Figure 4.22. Change in relative abundances of *Betaproteobacteria* throughout the experiment, as represented in each of the three phases.

By the recovery phase, the relative abundances in slurry amended soils that had not undergone flooding have fallen, but remained elevated in corresponding microcosms that had been waterlogged. Also seen during this phase is a significant increase in the relative abundances of *Betaproteobacteria* in the control soils, principally attributed to two OTUs both from the family *Oxalobacteraceae* (Figure 4.22).

The next group of proposed copiotrophs to be considered, the *Bacteroidetes*, were present in high relative abundances in the slurry 'inoculum' where they represented approximately 25 % of the total community composition, versus around 8 % in the non-amended soil (Figure 4.23). This therefore contributes to the marked increase in relative abundance seen in slurry amended soils at T0. The *Bacteroidetes* continue to

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increase in abundance in the slurry amended samples and by T2 (3 days after slurry application) had more than doubled to approximately 35 %. After this time however, the relative abundance *Bacteroidetes* steadily decreased in 'slurry' treated samples (Figure 4.23).
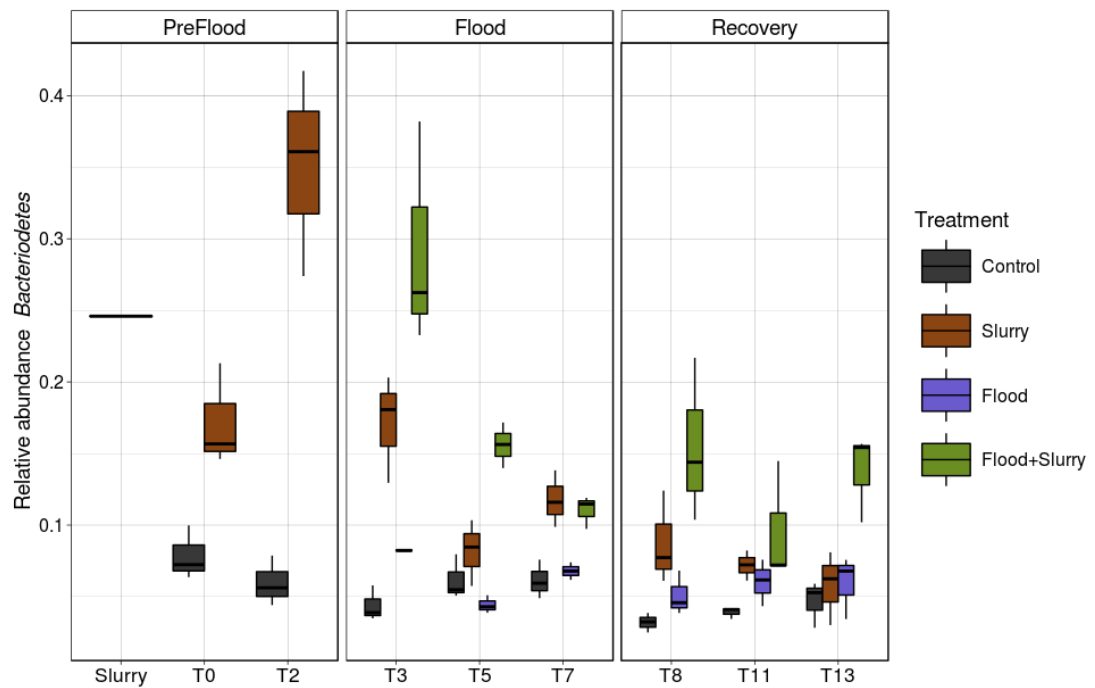
Figure 4.23. Change in relative abundances of *Bacteroidetes* throughout the experiment, as represented in each of the three phases.

Bacteroidetes in the 'slurry+flood' samples initially persisted in higher abundances than in their un-flooded counterparts during the flooded phase T13 (Figure 4.23). In fact, this groups continued to be present in excess of the other treatments until the final sample point, T13 (Figure 4.23). In total, there appeared to be a strong relationship between slurry amendment and *Bacteroidetes* abundance.

The *Acidobacteria* were a dominant phylum in the untreated soils, where they represented close to 10 % of the microbial consortia (Figure 4.24). Conversely, no

Acidobacteria were present in the slurry 'inoculum'. During the pre-flood and flooded phase, this phylotype remained lower in samples from both slurry treatments compared to the control and 'flood' treatments, until T7.
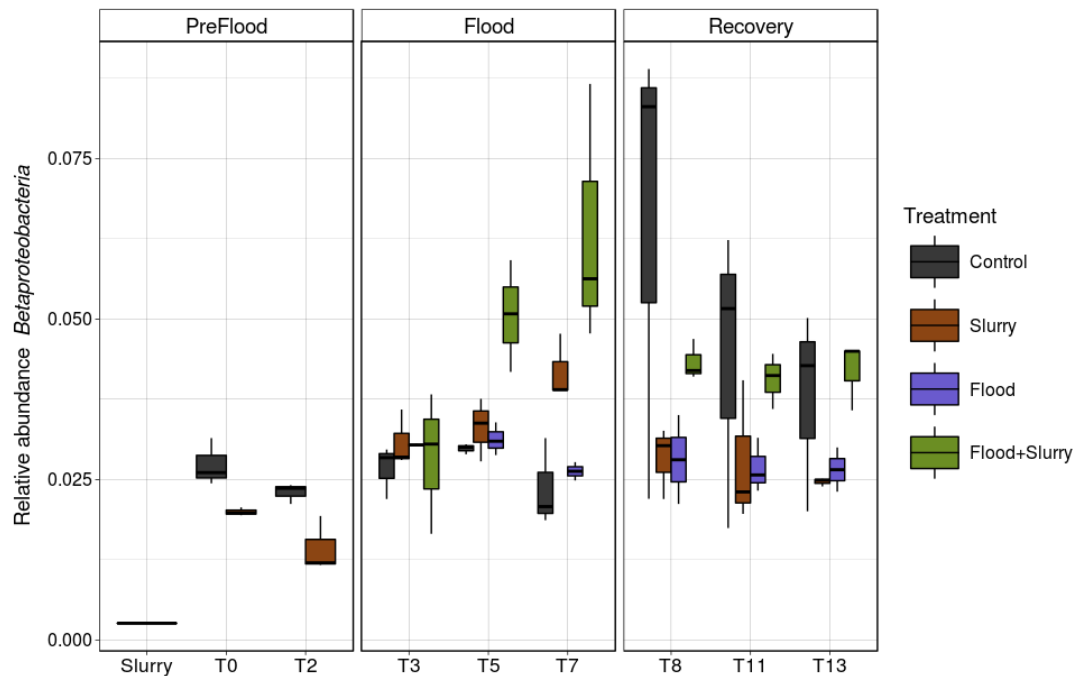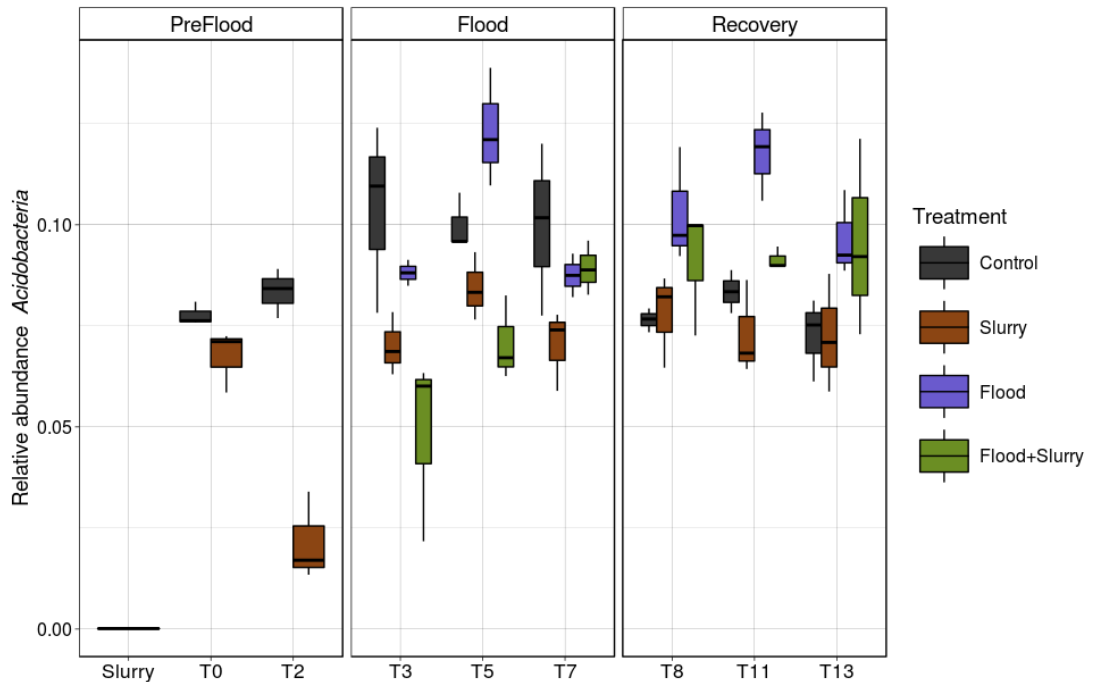

Figure 4.24. Change in relative abundances of *Acidobacteria* throughout the experiment, as represented in each of the three phases.

Finally, we wished to examine microbial groups associated with nitrogen cycling. As a diverse consortium of soil dwelling microorganisms are capable of denitrification, those involved in nitrification, namely the ammonia oxidising archaea (AOA) and bacteria (AOB) were investigated. This was in an effort to elucidate possible microbial groups contributing to the increased rates of potential nitrification still observed 140 days after slurry amendment (Figure 4.6).

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4.3.9.6   Relative abundances of nitrifying archaea and bacteria

The genera of nitrifying bacteria analysed included the ammonia oxidising *Nitrosospira,* within the phylum *Betaproteobacteria* and the nitrite oxidising *Nitrospira* within the phylum *Nitrospirae.* While *Nitrosomonas, Nitrosococcus*, and *Nitrobacter* are also among those also associated with nitrification, no OTUs assigned to these three phylotypes were detected in the dataset trimmed following the recommendation of Bokulich *et al.* (2013).

The archaeal ammonia oxidisers of the class *Thaumarchaeota* (Stieglmeier *et al.* 2014) were the most abundant archaeal group observed, and together comprised approximately 3 % of the total assemblage of T0 control soils, while being absent from the slurry used to amend the soils (Figure 4.25). Their relative abundances remained lower in the soils exposed to flooding, both when these soils were waterlogged and during the recovery phase, perhaps as a result of the higher ammonia concentrations seen in these soils (Figure 4.10; Prosser & Nicol 2012).
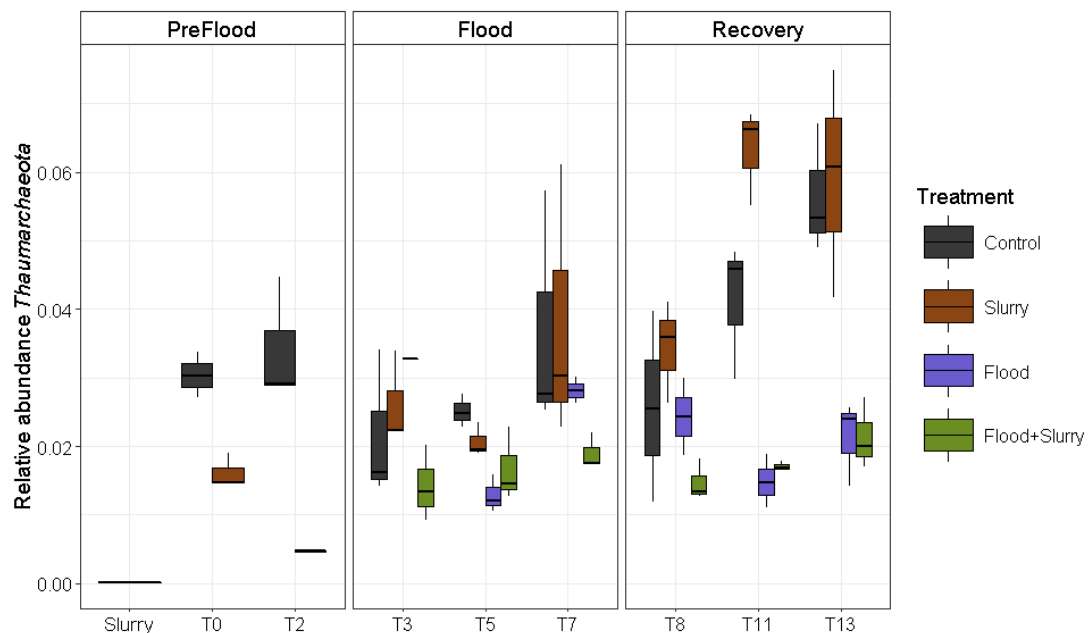
Figure 4.25. Total relative abundance, per treatment and time point, of AOA from the class *Thaumarchaeota*.

While the relative abundance of AOA remained relatively stable in the soils subjected to the flooding disturbance, there was some fluctuation in the control and 'slurry' treated soils, with a notable increase in their relative abundances from T8 to T13 (Figure 4.25). It would therefore appear that, in the long term these ammonia oxidising archaea were impacted more by the flooding event than the slurry application. Conversely, when considering AOB, the relative abundances of both *Nitrospira* (Figure 4.26) and *Nitrosospira* (Figure 4.27) varied more as a function of slurry addition than soil water status. In slurry amended soils, *Nitrospira* abundances remained lower than non-amended soils throughout the experiment (Figure 4.26). The compounded disturbance of the flood introduced some fluctuation in *Nitrospira* abundances in 'slurry+flood' soils, and indeed by the final two sample points, T12 and T13, the same relative abundance was observed as in the non-amended soils (Figure 4.26).
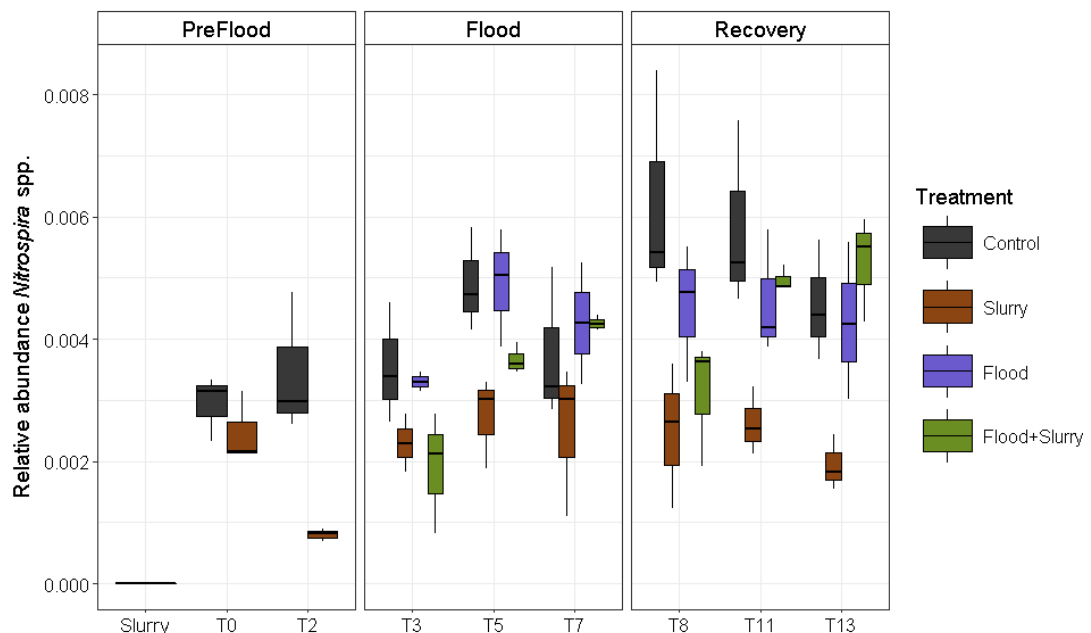
Figure 4.26. Total relative abundance, per treatment and time point, of nitrifying bacteria from the genus *Nitrospira.*

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In the case of the *Nitrosospira,* the opposite trend was seen, whereby slurry amended (without flooding) corresponded with increased relative abundances of this particular group of AOB (Figure 4.27).
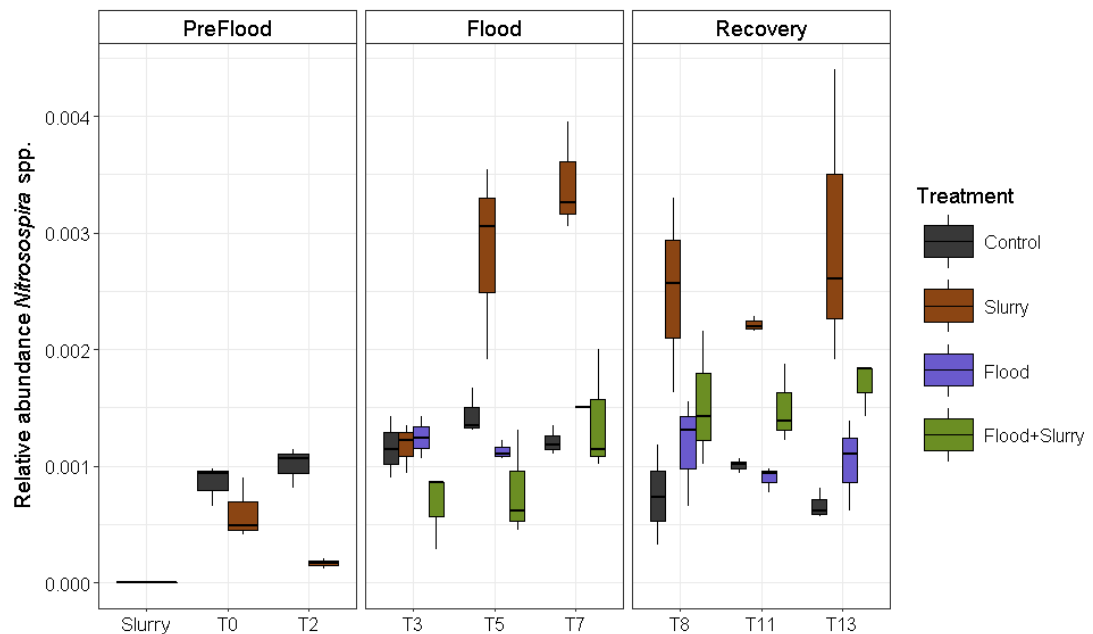
Figure 4.27. Total relative abundance, per treatment and time point, of AOB from the genus *Nitrosospira.*

During the flooded phase, *Nitrosospira* abundances were notably decreased in soils experiencing the compounded disturbance. However, flooding in the absence of slurry did not appear to affect their abundance, perhaps indicating that the increased ammonia concentrations detected were not favourable. As already mentioned, a caveat in these analyses is that it is based on relative abundances alone and as such has the potential to be impacted by large changes in abundance of other, non-related genera. For example, the majority of bacterial groups investigated in this analysis and in section 4.3.9.5 (oligotrophs versus copiotrophs) exhibited a large relative abundance decrease in slurry amended soils at T2. When observing the *Bacteroidetes* however (Figure 4.23), their abundance increases to 35 %, and perhaps contributes significantly to the

reduction seen in other groups. Thus, if time were not a constraint, it would be beneficial to investigate these observations further using a quantitative PCR.

4.4   Discussion

While microbial community stability is a well-researched field, as detailed in the review by Shade *et al.* (2012), there exist a number of avenues within this field that warrant further investigation. Among the research gaps identified were pulse, biological disturbances and combined disturbances. The aim of this experiment was therefore to test a number of posited hypotheses regarding microbial community stability, while addressing these research gaps.

Effect of slurry addition

As we hypothesised, slurry addition indeed caused changes in the structure and functioning of the soil microbiota and so acted as a pulse, biological stress. Slurry amendment has previously been investigated in the context of its effect upon the soil microbiome, with the microbial community structure assessed using a variety of methods including phospholipid fatty analysis (PLFA) (Nyberg *et al.* 2006), fingerprinting methods such as denaturing gradient gel electrophoresis (DGGE) (Stark *et al.* 2007), and more recently 16S rRNA profiling (Suleiman *et al.* 2016). As in the referenced studies, the largest influence of slurry upon the autochthonous microbiota in our study occurred within the first 3 days. While it is evident that the influx of slurry-derived biomass contributed to some of these observed differences, many of the largest differences in community structure and functioning were observed not immediately following slurry application but 24 to 72 hours post amendment. This infers that certain

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bacterial groups, either slurry- or soil-derived, responded positively to the increased nutrient availability resulting from the organic amendment. It has been shown that labile carbon associated with animal wastes is typically consumed by the soil microbial consortia within the first 24 to 48 hours post application, after which more recalcitrant forms of carbon begin to be incorporated into the soil (Bol *et al.* 2003; Sauheitl *et al.* 2005). As we hypothesised that slurry addition selects for native microbiota with a particular trophic strategy, namely copiotrophs, it is likely this period would be the most favourable to such groups. Within this 48-hour time frame, the phylum *Bacteroidetes* exhibited the most notable increase in relative abundance in slurry treated soils. Significant increases in this phylum have also been seen following soil amendment with cattle slurry (Faissal *et al.* 2017; Suleiman *et al.* 2016) and with cattle urea (Samad *et al.* 2017). Additionally, environmental strains of *Bacteroidetes* have consistently been shown to exhibit large increases in abundance following influxes of organic matter (Thomas *et al.* 2011). These findings, in addition to our own, would seem to support the classification of members of the *Bacteroidetes* as copiotrophic in nature (Fierer *et al.* 2007). Therefore, as we hypothesised, it would appear that in our study, slurry amendment provided conditions ideal for copiotrophs, and thus lead to alterations in the relative abundances of copiotrophs to oligotrophs. The quantitative PCR assays developed by Fierer *et al.* (2007) would be useful for investigating this finding in a more quantitative manner.

A shared finding from previously mentioned studies of Nyberg *et al.* (2006), Stark *et al.* (2007) and Suleiman *et al.* (2016) was the relatively short-lived effect of slurry amendment upon the soil microbial community, ranging from just 10 days (Stark *et al.* 2007) to 50 days (Suleiman *et al.* 2016) after which no distinguishable differences remained between the community structure of slurry amended and control samples. In

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accordance with these findings, we hypothesised the effect of slurry addition would be relatively short term, however distance-based analyses of microbial community structure suggest a distinct community remained in slurry treated soils 140 days post application. It is possible that the methods employed in the aforementioned studies did not allow for sufficient resolution to determine such changes

Resistance and resilience as affected by alterations in nutrient availability

Among the hypotheses we aimed to test, was whether increased nutrient availability, resulting from slurry addition, would increase the resistance and/or resilience of the soil microbial community when exposed to a disturbance. This will be discussed first in terms of functional potential and then in terms of microbial community composition. Upon exposure to the compounded flood disturbance, we observed some evidence, based on the functional assays that the prior slurry amendment improved resistance. The duration of this effect varied depending on the function in question, as detailed in the appropriate sections below.

i) Resistance as affected by nutrient availability: respiration rates and litter decomposition

Slurry amendment alleviated the drop in basal respiration measured in the flooded soils. This effect was seen throughout the flooded period where basal respiration remained elevated in samples receiving slurry amendment versus those without. The litter decomposition rates in soils treated with slurry also demonstrated improved resistance to the flooding event. Indeed, despite being waterlogged these rates were in excess of those seen in the untreated control during initial weeks of the flooded period. Typically, increased soil water content has negative impacts on litter decomposition (Schuur 2001) thus it would appear that in our study increased resource availability

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associated with slurry application conferred an initial benefit to the litter decomposing consortia upon flooding. Research into the effects of nitrogen deposition on organic carbon decomposition rates in soil has produced contrasting results, with evidence for all possible outcomes; no effect (Prescott 1995), reduced rates (Thirukkumaran & Parkinson 2000) and increased rates (Ågren *et al*. 2001; Fog 1988; Wang *et al*. 2011). The form of nitrogen introduced into the soil ecosystem may account for some of these differences. For example, Du *et al*. (2014) observed a decrease in soil carbon cycling in soils exposed to inorganic nitrogen amendments, while organic inputs (urea and glycine) induced the opposite trend. The decomposition of litter and other forms of organic matter is a multistep process involving the conversion of larger, recalcitrant forms of carbon by extracellular enzymes into labile monomers, which can then be hydrolysed intracellularly (Blagodatskaya *et al.* 2016). The production of such extracellular enzymes is resource intensive (Frankena *et al.* 1988) and often limited by available nitrogen (Treseder *et al.* 2011). However, the suite of extracellular enzymes present in soil are differentially affected by the addition of nitrogen, for example cellulases and phosphatases tend to exhibit a positive response to nitrogen amendment, meanwhile those involved in lignin degradation are often negatively affected (Allison *et al.* 2010; Keeler *et al.* 2009). Grass clippings such those employed in our decomposition assays are typically low in lignin and consist mostly of cellulose (30 %) and hemicellulose (20 %) (Triolo *et al.* 2012). As such, the increase in litter decomposition rates following slurry amendment supports findings of improved activity of cellulolytic enzymes in response to increased nitrogen (Keeler *et al.* 2009).

ii) Resistance as affected by nutrient availability: nitrogen cycling

In situ nitrification appeared to be rapidly impacted by the flooding event, with ammonium accumulation seen after 3 days of waterlogging. As nitrification is typically an oxygen dependent process (Hart *et al.* 1994), this was an expected outcome. Flooded soils consist of two zones; the upper zone is aerobic due to oxygen diffusion from the surface and the lower zone is anoxic (Reddy *et al.* 1984). In a biologically active soil such as the one employed in the present study, this aerobic layer is thought to be just a few millimetres thick (Reddy *et al.* 1984). Nitrification can therefore still occur in the oxic layer of flood waters, after which the resulting nitrate can diffuse down into anoxic soil layer where it is denitrified, resulting in coupled nitrification-denitrification (Nicolaisen *et al.* 2004; Reddy *et al.* 1984). Thus, it is likely that while some nitrification was still carried out in the oxic layer of our soil (as evidenced by the slow accumulation of nitrate in the system), it still represents a significant reduction when compared to the aerobic (un-flooded) soils. Despite the evident reduction of *in situ* nitrification, when potential nitrification rates (PNR) were assessed, there appeared to be little effect of flooding on the potential of the ammonia oxidising consortia, even after 26 days of waterlogging. The accumulation of relatively high ammonium concentrations was seen in the slurry amended, flooded microcosms yet it appeared not to reach a level inhibitory to the system as PNR remained stable. It would therefore appear that, despite extended exposure to unfavourable, anoxic conditions, the AOA and/or AOB retained the ability to oxidise ammonia to the same extent as unflooded controls *in vitro*. Similarly, Pett-Ridge *et al.* (2006) observed that nitrification was still taking place in a forest soil, despite 6 weeks incubation in micro-aerobic conditions. Contrary to conditions typically associated with nitrification, high *in situ*, gross nitrification rates have been observed in a waterlogged, peat soil (Alves

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et al. 2013), and the ability of the ammonia-oxidising consortia to adapt to alterations in the water-status of soils has been demonstrated elsewhere (Pett-Ridge *et al.* 2013). Together, this seems to demonstrate that nitrifiers perhaps have a few tricks up their sleeve when it comes to persisting, and even performing ammonia oxidation, in sub-optimal redox conditions.

The process of dissimilatory reduction of nitrate to ammonium (DNRA) is often overlooked in soils, with nitrate reduction in soils more typically attributed to just the denitrifiers (Cole 1990). However, Tiedje (1988) noted that while the process of denitrification may be more energy efficient, DNRA represents a more valuable sink for electrons. Therefore, under highly reductive conditions such as anoxic soils, where electron acceptors are lacking, DNRA may be favoured over denitrification (Tiedje 1988; Matheson *et al.* (2002). As well as redox conditions, it is theorised that the partitioning of nitrate to either denitrification or DNRA is also determined by the ratio of electron donor (carbon sources) to elector acceptor (nitrate) (Tiedje 1988). When the $C : NO_3^-$ ratio is low, carbon as opposed to electron donors, will be limiting and thus denitrification is favoured, while when the ratio is high, electron donors are in excess while acceptors are limiting so DNRA is favoured (Tiedje 1988). While this seems to hold true in some systems, research has produced some contrasting results, as reviewed by Rutting *et al.* (2011). While it is not possible to ascertain from our experimental set-up, it is possible therefore that some of accumulated ammonium we observed was produced by DNRA, as well as through anaerobic nitrogen mineralisation.

In addition to the accumulation of ammonium during flooding, nitrate concentrations also increased as a function of flood duration. Through the process of denitrification, nitrate is typically converted under anoxic conditions to dinitrogen gas, or if the

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reaction is incomplete, to nitrous oxide (Smith & Tiedje 1979). The conditions in the flooded soils, particularly those with slurry amendment, would appear to be ideal for denitrification, with limited oxygen and surplus nitrogen (Knowles 1982). However, the nitrate accumulation observed here suggests that it was being produced at a faster rate than it could be consumed, in other words; the rates of nitrification in the aerobic water layer exceeded the rates of denitrification in anoxic layer. Reddy *et al.* (1978) demonstrated that denitrification proceeded at a slower rate in flooded soils where a surface layer of water was present (as in the 'flooded' microcosm set up used herein), versus those without a surface layer of water. They proposed this to be a consequence of reduced diffusion of nitrate, which may also have contributed to the accumulation of nitrate in our flooded soils. Alternatively, as this accumulation is only seen after 19 and 26 days of flooding, perhaps it was the availability of carbon that was limiting denitrification by the end of the flooded phase (Firestone & Davidson 1989; Weier *et al.* 1993). When considering *potential* denitrification, evidence of increased denitrifying enzyme activity was seen in un-amended, flooded soils. However, the rates were significantly higher in samples exposed to the compounded slurry and flood disturbance. In unflooded systems, slurry addition also allowed for increased rates of denitrification; while no effect was seen immediately after amendment, 24 hours later the potential denitrification rate had doubled. As soils were provided with excess nitrate for this assay, the increased carbon availability is likely accounting for this increase. The effect of slurry amendment on the potential denitrification rate of un-flooded soils is seen until 22 days after amendment. This could also indicate that by this sample point there is no longer an effect of carbon from the slurry, and would concur with the accumulation of nitrate (i.e. reduced *in situ* denitrification) seen at this point of the flooded phase.

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iii) Resilience as affected by nutrient availability

As well as affecting resistance, resource availability has also been proposed to affect the resilience of soil microbial communities (Wallenstein & Hall 2012). We observed that the recovery of *in situ* rates of nitrification were indeed improved in slurry amended soils that had undergone flooding versus the un-amended soils. However, no improved resilience as a result of the prior slurry amendment was seen in the other functional assays performed (PNR, PDR, litter decomposition and basal respiration). It should be noted however, that the soil used in this experiment would not be classed as nutrient poor, with organic carbon levels of approximately 4.5 % (where 2 - 6 % represents 'medium' SOC levels; Rusco *et al.* 2001), therefore if indeed resilience is limited by labile carbon availability as proposed by Wallenstein & Hall (2012), perhaps this soil did not allow for an accurate assessment of this hypothesis. Additionally, as detailed by Evans & Wallenstein (2012), the precipitation regime that soils are exposed to affects how their resident microbiota respond to moisture-related disturbances, such as drying-rewetting cycles. The soil chosen for this work is a moderately draining sandy loam, whose mean water filled pore space in the field, when assessed over two consecutive years was 51 % and 59 %, with some extended periods of circa 75 % WFPS (Harty *et al.* 2016). Thus, any resident microbiota with an adaptive response to increased soil moisture content likely had an advantage in this soil.

iv) Resistance and resilience: microbial community composition and physiological profiles

Most of the assays we performed to investigate microbial functioning suggested the microbial community was resilient as by the final time point the disturbed soils were

able to perform ecosystems service-related functions to the same capacity as the undisturbed controls. Meanwhile, in terms of substrate induced respiration profiles and microbial community composition, slurry amended samples and flood affected samples remained distinct from the untreated controls for the duration of the recovery phase. When investigating the taxa contributing to these differences using LEfSe analysis, it was revealed that no slurry-derived prokaryotes were persisting in sufficient abundances to still be affecting the microbial community structure during the recovery phase. Increased abundance of members of the spore-forming, typically anaerobic *Clostridiales* phylotype (Rainey *et al.* 2015) were seen in slurry amended soils, both with and without flooding at all three experimental phases. As already referred to, various members of the phylum *Bacteroidetes,* typically thought of as copiotrophs (Fierer *et al.* 2007) were observed in higher abundances in slurry treated samples throughout all three phases, including the classes of *Sphingobacteria, Bacteroidia* and *Flavobacteria*. A clear trend of increased abundance of numerous members of the *Gammaproteobacteria* was also observed in slurry treated soils. Approximately 40 % of the more abundant OTUs from 'slurry+flood' treatment in the recovery phase were from this phylum. Three days after amending soils with pig slurry, Suleiman *et al.* (2016) also noted an increased representation of members of the *Gammaproteobacteria* in amended vs un-amended soils, further supporting findings of the preference of this class for nutrient rich environs (Lladó & Baldrian 2017; Ramirez *et al.* 2010).

When considering the oligotrophs, namely the *Acidobacteria,* whose abundance typically falls with increasing nutrient availability (Fierer *et al.* 2007; Ramirez *et al.* 2012), these were only detected as significantly differentially abundant in non-slurry amended samples, and most notably in flooded soils. During a soil re-wetting event,

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Barnard *et al.* (2013) also observed an increased relative abundance of this phylotype with increasing soil moisture. Perhaps the ability of this phylotype to produce exopolysaccharides (Kielak *et al.* 2016) allows for improved survival of adverse osmotic conditions resulting from the flood.

The relative abundances of known ammonia oxidisers were also investigated in an effort to explain changes in nitrogen cycling functions seen in response to our disturbances. The phylum *Thaumarchaeota*, known to be involved in ammonia oxidation (Stieglmeier *et al.* 2014), exhibited significant changes in abundance as a function of treatment. There was a clear increase in their abundance with time and this was seen only in unflooded soils (both with and without slurry amendment. Thus, it would appear these AOA were more differentially affected by the legacy of soil moisture status than by the organic nutrient amendment. The AOA have been shown to be more adapted to performing ammonia oxidation in acidic soils (pH < 5.5) than their bacterial counterparts (Prosser & Nicol 2012). Therefore, perhaps the acidic soil used to construct the soil microcosms (pH 5.69) was more selective for AOA than AOB. Waterlogged soils however, tend towards neutrality (Parent *et al.* 2007) suggesting those soils exposed to the flooding event would no longer have been selective for more acid tolerant species, as above pH 5.5, AOA and AOB are regarded as being equally adaptable (Prosser & Nicol 2012). This negative response of AOA to waterlogged soil conditions was also seen by Szukics *et al.* (2010) using quantitative measures of AOA and AOB abundance. The treatment-independent alterations in the relative abundance of *Thaumarchaeota* in the undisturbed controls could therefore imply that flooding interfered with the stochastic community succession of these AOA, perhaps through the change in pH or due to sensitivity to anaerobic conditions.

The ammonia oxidising bacteria (*Nitrosospira*) and the nitrate oxidising bacteria (NOB) *Nitrospira* responded differently to the treatments, based on changes in their relative abundances. Thus, we were unable to associate the increase in PN rates seen in slurry amended soils with differences in the relative abundances of either *Nitrospira* or *Nitrosospira*. Additionally, there appeared to be no consistent trend of flooding upon these bacteria, perhaps as a consequence of their ability to perform well in low oxygen environments (Le Roux *et al.* 2016). Their survival in anoxic conditions has been hypothesised to be a result either of slowing their metabolism dramatically or their ability to switch to denitrification (Geets *et al.* 2006; Kim *et al.* 2010), as evidenced by the presences of denitrification genes harboured within the genomes of several AOB of the *Nitrosospira* (Garbeva *et al.* 2007; Shaw *et al.* 2006).

It would be of interest to determine if any significant changes in the copy numbers of genes such as the ammonia monooxygenase occurred with treatment. While it would appear ammonium and nitrate concentrations of amended soils were the same as the control, perhaps the recalcitrant forms of carbon in the slurry were steadily being broken down and made available for the nitrifiers, thus explaining the increased PN rates.

Conclusions

The aim of this work was to assess microbial community stability in the face of an ecologically relevant disturbance, with the intent of testing some proposed hypotheses on theme of community stability. In terms of ecosystems functions, the microbial community was resilient to extended waterlogging, with basal respiration, litter decomposition, potential denitrification and *in-situ* nitrogen cycling returning to that seen in the undisturbed control. The effect of the prior slurry amendment was most

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evident on potential nitrification rates which remained significantly enhanced for the duration of the experiment. We hypothesised this organic amendment would increase resource availability in the system thereby improving resilience, yet we saw no evidence of this in terms of the assays of functional potential we performed. In contrast to the rapid resilience seen in functioning, the microbial community structure and physiological profiles were affected significantly by each of the treatments, and even by the end of the recovery phase, remained distinct from each other. This lack of resilience in terms of community composition suggests a potential regime shift resulting from our treatments. Additionally, these differences in community profiles, despite recovery of ecosystems functions suggests there was redundancy in these functional groups present in our soil and would be worthy of further research, for example by employing metaproteomics.

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--------- Chapter V ---------

Conclusions and future research directions

The aim of the work completed for this thesis was to investigate the stability of soil microbial communities subjected to a selection of perturbations relevant in the context of agriculture and climate change. The perturbations we investigated included the application of lime and soil amendment with cattle slurry in the presence and absence of a compounded flood event. These disturbances were chosen as particularly relevant in the Irish agricultural context. Additionally, with the global need to improve soil quality in order to increase crop yields, liming to optimise soil pH and slurry application as a sustainable means of improving soil nutrient status, are ever-increasing agricultural practices.

The first phase of this project aimed to develop a method that would allow for the co-extraction of DNA, RNA and proteins from the same soil sample. This was to enable the utilisation of cutting-edge molecular techniques for investigating the effects of lime application, slurry amendment and flooding on the autochthonous, soil microbial community. In particular we hoped to employ metaproteomics to elucidate the effects of such disturbances on the functioning of the microbial ecosystem, as it is valuable tool for investigating microbial community functioning through its ability to inform on both microbial diversity and functionality (Becher *et al.* 2013). For example, metaproteomics has been used to elucidate the succession of microbial groups during litter decomposition while also revealing the major pathways involved in this process and the taxa contributing therein (Schneider *et al.* 2012). Metaproteomics therefore has significant potential as a tool for investigating how soil processes associated with ecosystems services are impacted by perturbations while connecting phylogeny with function. A significant drawback in the field of environmental metaproteomics and soil in particular, is the lack of sufficient databases for relevant microbial proteins. This significantly impairs the protein identification rate; as succinctly noted by Becher *et*

al. (2013), a metaproteomic dataset can only ever be as good as the database used. As the vast majority of soil microbial members are uncultured and/or lacking in annotated genomes, using a matched metagenome as a database, an experimental approach referred to as community proteogenomics (VerBerkmoes *et al.* 2009), is a valuable tool for increasing protein identification. For example, Delmotte *et al.* (2009) were able to increase the number of protein identifications of leaf-associated microbiota by as much as 87 % through the use of a matched metagenome. Thus, given the heterogeneity of soil, we believe that to maximise on the outputs of a combined omics approach, biomolecules should be co-extracted from the same biological sample. As an appropriate method to achieve this was not available for soil samples, the first aim of this PhD was to develop such a tool. We were successful in this objective, and the co-extraction method was in fact highly adaptable to other sample types, including cattle slurry; anaerobic digester sludge, digestate and leachate; and pure culture. It has since been employed in a number of research projects both within and outside of our research group. It is our hope that this method, easily adoptable in most laboratory set-ups, will serve to be a valuable tool for microbial ecologists, allowing for more widespread use of metaproteomics, in conjunction with other omics tools, to address ecological hypotheses.

A comprehensive survey of the literature revealed that liming of agricultural soils has significant effects on microbial community structure. However, we identified a research gap whereby deeper characterisation of these compositional changes, using culture-independent techniques is required. Such a characterisation needed to include how any resulting changes in microbial community structure might relate to plant productivity and ecosystems processes, thus presenting real-world applicability of

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such research. Assessing the impacts of this agricultural activities, such as liming, on the soil microbiota which modulate crop health and productivity becomes increasingly important as the need for more sustainable agricultural practices grows. In an effort to address the identified research gap, we investigated liming in the context of a field trial of Spring Barley, analysed at three distinct crop growth stages. While DNA and RNA were used for profiling of the rhizosphere microbial community, it was our initial hope to employ our co-extraction method and thus perform metaproteomics to investigate the functional aspects associated with pH changes induced by lime application. As the quicklime product did not alter soil pH sufficiently to induce any phenotypic changes in the barley crop, in particular no yield improvement was seen, we did not employ metaproteomics. However, as sequencing data had already been acquired, these were analysed to determine if the small changes in pH (albeit statistically insignificant) had induced any change in the microbial community. Through analysis of alpha and beta diversity, it was revealed that indeed there were no effects on the microbial community as a function of lime application, at any barley growth stage. However, the community showed temporal shifts, with distinct communities present during the stem elongation, heading and ripening phases of the barley development. This finding is consistent with other temporal studies regarding microbial interactions with plants including grasses (Shi *et al.* 2015), rice (Breidenbach *et al.* 2016) and soybean (Sugiyama *et al.* 2014), a relationship strongly driven by alterations in root exudates as a function plant development (Aulakh *et al.* 2001; Chaparro *et al.* 2013). Indeed, while soil type is a significant factor explaining divergence between different soil microbial communities, Wieland *et al.* (2001) identified plant type as the ultimate driver. Furthermore, it has been shown that plants appear to select for assemblages of microbiota distinct not only from the bulk soil, but also from that seen in the rhizosphere of other plant types

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(Beauregard 2015). This has led to the idea of the 'core microbiome', exemplified in the work of Lundberg *et al.* (2012) who, using data from the rhizosphere of 600 plants, described the core microbiome associated with *Arabidopsis thaliana*. Such endeavours to define the core microbiota of different crops will be vital as it becomes increasingly evident how plant health and productivity are inextricably linked to their associated microbiota, leading to their consideration as a 'holobiont' (Vandenkoornhuyse *et al.* 2015). In practical terms, information on the core microbiome associated with a particular crop could be used to inform on microbial interactions and thus potential antagonisms that may result, particularly with regards to species introduced as bio-fertilisers. Defining such interactions could be aided by investigating plant-associated core microbiomes in terms of networks.

Network analysis has been used as a tool by microbial ecologists to infer interactions between species (OTUs) based on their co-occurrences, i.e. repeatedly observed occurrence of two OTUs, across a number or replicates, indicates their possible interaction, or at least a shared niche preference (Barberán *et al.* 2012). This tool has also been used in the context of rhizosphere microbial communities, as reviewed by van der Heijden & Hartmann (2016), in an effort to elucidate how microbial interactions affect plant health. For example, Agler *et al.* (2016) used network analysis to reveal 'hubs' of highly connected species associated with *A. thaliana*; they then manipulated abundance of these hub species and were able to demonstrate their role as keystone species determining the microbial network structure. Microbial networks can also be investigated to determine the effect of a species' removal, for example after a perturbation (Faust & Raes 2012). As such they hold promise for modelling the persistence of non-native species, for example biofertilisers in the context of plant microbiomes. Such analysis should also consider temporal variation of these networks

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as a function of plant developmental stage; Shi *et al*. (2015), demonstrated that rhizobacterial networks showed a clear increase in complexity with development of wild oats plants. It would therefore be of interest to determine how this relates to 'invasibility' of allochthonous microbiota (van Elsas *et al*. 2012) such as biofertilisers.

While several studies have investigated the root associated microbiota of barley (Bulgarelli *et al.* 2015; Garbeva *et al.* 2007), a conclusive study, using a large set of data from different soil types and plant grow stages, as in Lundberg *et al.* (2012), has yet to be done in the context of barley. Thus, we propose this would be a valuable advance in the field and our data would contribute significantly. The recent introduction of a tool for mining core microbiomes from 16S rRNA datasets, COREMIC (Rodrigues *et al.* 2017), will aid in this effort, and has the added benefit of including by default an 'out group' of samples (i.e. non-barley associated microbial communities) to isolate associations which are indeed specific to the crop of interest, a feature lacking from the approach of Lundberg *et al.* (2012). Using the described network analysis methods, such data could then be analysed in terms of potential interactions or antagonisms seen between microbial taxa at different stages of plant development. This could be used to inform on the most appropriate crop developmental stage at which to apply biofertilisers to maximise their chance of persisting long-term in their new environs and inducing phenotypic changes in the crop of interest.

Subsequent to investigating the microbiome responses to alterations in soil pH bought about by the application of lime, we aimed to investigate the effect of amending soil with cattle slurry upon the native microbiota in the presence of a compounded flooding event. Inputs of nutrients represent yet another factor mediating soil microbial

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community composition and function, demonstrated herein and in other work including that of Bol *et al.* (2003); Suleiman *et al.* (2016) and Stark *et al.* (2007). In terms of microbial community composition, while the aforementioned studies demonstrated a return of the microbial communities in amended soils to that of the un-amended control by the end of the experiment, we did not observe this, despite sampling for a longer time post-amendment. We attribute this to differences in sample depth, for example Stark *et al.* (2007) analysed microbial community shifts using DGGE, which does not provide the same resolution as high-throughput sequencing techniques (Hamady & Knight 2009). While Suleiman *et al.* (2016) did employ 16S rRNA sequencing, they rarefied to a depth of 1836 sequences per sample, while our dataset was rarefied to 24540 sequences per sample which likely provided significantly higher resolution, especially of low abundance taxa (Hamady & Knight 2009). It should be noted that these previous works regarding the effect of slurry application upon the soil microbiota did not investigate the fate of the microbiota associated with the slurry itself. Using SourceTracker software we were able to determine how long the slurry-derived persisted for, and this allowed for the observation that a compounded flooding event extended the survival time of slurry OTUs within the soil microcosms. Whether this enhanced persistence was a result of the lower oxygen concentration in flooded soils or the increased resource availability (for example from accumulating ammonium or slower decomposition of labile carbon) or a combination of both remains uncertain and worthy of investigation. In conjunction with the findings of Cools *et al.* (2001) who observed improved faecal indicator bacteria survival under waterlogged soils, this further demonstrates that appropriate timing of slurry application, in relation to predicted heavy rainfall, is important not only for minimising

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losses of nutrients into waterways (under the EU Nitrates directive), but also in terms of enhanced potential for pathogen survival.

The most significant phenotypic effect of slurry amendment on our system was the marked, and prolonged, increase in potential nitrification rates that it induced. While initially this may have been a consequence of improved resource availability in slurry amended soils, notably labile carbon forms, we imagine that by the end of the experiment, 140 days post-slurry application, such resources would have been consumed by the resident microbiota. Analysis of changes in the relative abundances of ammonia oxidising consortia as a function of treatment also did not reveal any obvious explanation for the increased potential nitrification rates. As such, the use of quantitative tools, such as qPCR targeting the ammonia mono-oxygenase genes of archaea and bacteria would be of use to determine if these increases can be explained by increased abundance or activity of these groups.

The compounded flooding disturbance we applied was intended to test whether the increase in resource availability resulting from slurry amendment affected the resistance and or resilience of the microbial community. Indeed, slurry application appeared to confer improved resistance to the flooding event in terms of functional capacity, including nitrification, litter decomposition and basal respiration rates. Resilience after the flooding event however, was not so obviously affected, perhaps as more of the resources had been consumed during the course of the experiment.

As already noted, potential nitrification rates in slurry amended soils remained high throughout the experiment and this occurred regardless of the compounded flooding event. Processes such as nitrification are viewed as narrow-scale and therefore more vulnerable in the face of disturbances, due to less redundancy across taxa. The flooding

event we chose was perhaps not a particularly 'stressful' event for the nitrifying community as our results, in conjunction with other research, for example Pett-Ridge *et al.* (2006), suggests they may indeed be well adapted to fluctuations in redox conditions. It has been well demonstrated that across soil aggregates there exist gradients of oxygen, with the centre of aggregates being relatively anaerobic (Sexstone *et al*. 1985). These aggregates represent a relatively stable niche for soil microbial communities in terms of moisture status for example, with the added benefit of reduced chances of predation by larger organisms, as reviewed by Rillig *et al*. (2017). Thus, it is likely that the majority of soil microbiota are relatively frequently exposed to varying redox conditions and have likely developed a number of strategies for persisting in unfavourable redox conditions (DeAngelis *et al*. 2010; Pett-Ridge *et al*. 2006). It would be of interest to determine if the same effect on potential nitrification rates we observed herein would be seen in different soil types, for example those with lower soil organic matter and/or soils that typically do not experience high moisture content for extended periods. The previous would help to further test hypotheses regarding resource availability and resilience (Griffiths & Philippot 2013; Wallenstein & Hall 2012), while the latter would be particularly relevant to test hypotheses regarding the role of historic precipitation regimes in modulating the response of soil microbiota to changes in moisture status, as in Evans & Wallenstein (2012).

In closing, much of the current literature demonstrates an urgent need to marry up microbial taxonomy and functioning and go beyond simply cataloguing microbial communities present in various ecosystems (summarised in Jansson & Prosser (2013)) and we believe that, metaproteomics (more specifically proteogenomics) represents a valuable tool to achieve this. As such, the co-extraction method developed herein will

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hopefully provide a useful platform from which to achieve this goal. This can then allow for the testing of hypotheses regarding microbial community stability in the face of perturbations. Additionally, we believe this work advances current knowledge in this field of microbial community stability after exposure to stresses that were carefully selected to be of relevant both within and outside of the field of microbial ecology. As well as testing hypotheses in this field, detailed by de Vries and Shade (2013), this research also revealed some potentially valuable research avenues to be investigated.

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--------- Appendices ---------

Appendix I: Extra data from co-extraction method

Table S.I. Yields per extraction of DNA, RNA and protein from all sample types tested, as well as quality ratios, as assessed prior to purification.

Sample type	DNA yield µg [1]	RNA yield µg [2]	A260/A280 [3]	A260/A230 [3]	Protein yield µg [4]
Soil 1	7.99 ± 1.21	4.27 ± 0.75	1.69 ± 0.02	0.82 ± 0.06	96.63 ± 4.33
Soil 2	13.80 ± 3.70	3.56 ± 1.10	1.58 ± 0.02	0.79 ± 0.01	54.68 ± 6.76
Soil 3	11.37 ± 1.85	2.85 ± 0.48	1.74 ± 0.05	1.11 ± 0.11	96.00 ± 8.90
Soil 4	28.60 ± 0.20	7.07 ± 1.68	1.68 ± 0.07	0.98 ± 0.03	82.36 ± 11.08
Soil 5	5.27 ± 1.36	2.56 ± 0.38	1.87 ± 0.17	1.32 ± 0.35	67.01 ± 12.56
Soil 6	14.17 ± 4.80	3.47 ± 1.30	1.79 ± 0.03	1.23 ± 0.10	51.54 ± 18.07
Soil 7	11.40 ± 3.18	3.40 ± 0.70	1.71 ± 0.05	1.06 ± 0.06	68.76 ± 20.34
Soil 8	12.13 ± 0.50	3.43 ± 0.18	1.78 ± 0.03	1.07 ± 0.09	87.64 ± 15.85
Anaerobic granules	18.03 ± 5.05	11.31 ± 2.10	1.93 ± 0.01	1.97 ± 0.03	142.53 ± 13.42
Food waste (digestate)	5.13 ± 0.15	8.70 ± 1.82	1.94 ± 0.05	1.83 ± 0.13	440.83 ± 10.10
Food waste (leachate)	4.74 ± 0.03	6.83 ± 2.64	1. 98 ± 0.01	2.06 ± 0.01	466.67 ± 57.52
Cattle slurry	9.93 ± 0.72	3.99 ± 0.23	1.81 ± 0.01	1.22 ± 0.10	140.70 ± 2.52
Gram positive cells	2.34 ± 0.57	4.15 ± 0.80	1.96 ± 0.05	2.00 ± 0.42	216.8 ± 56.60
Gram negative cells	3.14 ± 0.57	31.12 ± 0.57	1.95 ± 0.10	1.98 ± 0.05	113.95 ± 25.01

[1] DNA yields were determined using the broad range, ds DNA Qubit (Invitrogen); [2] RNA yields were established using the broad range, RNA Qubit (Invitrogen); [3] Nucleic acid absorbance ratios were determined using a NanoDrop spectrophotometer. [4] The Amido Black assay was employed for protein quantification (Standard deviation of mean calculated from three replicate samples).
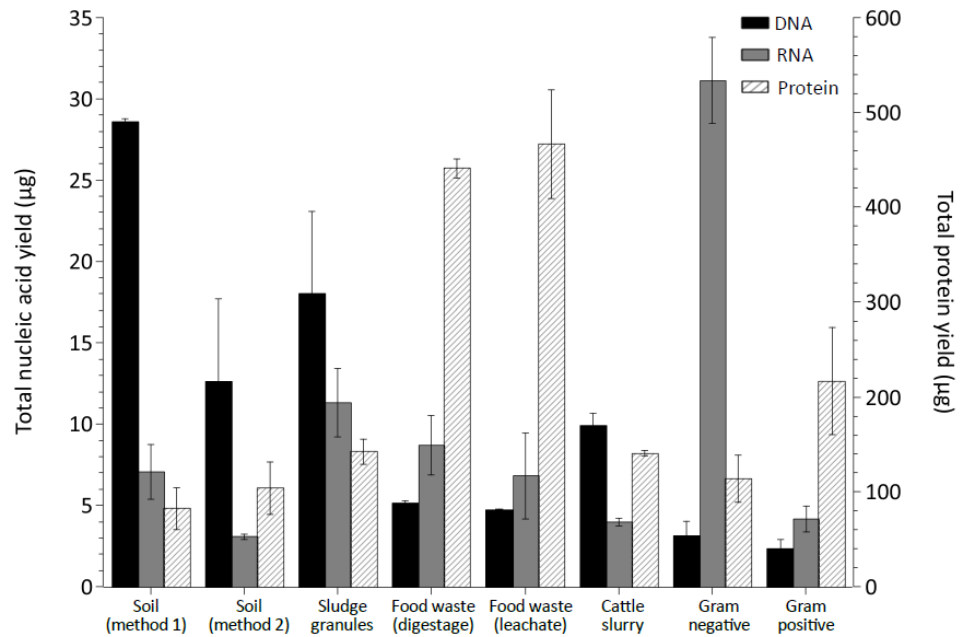
Figure S.Ia: Yields of DNA (black) RNA (grey) and protein (shaded) from all samples tested. Soil (method 1) refers to Soil 2 with desorption pre-treatment applied while Soil (method 2) refers to Soil 2 without desorption pre-treatment. Error bars show standard variation (n = 3).
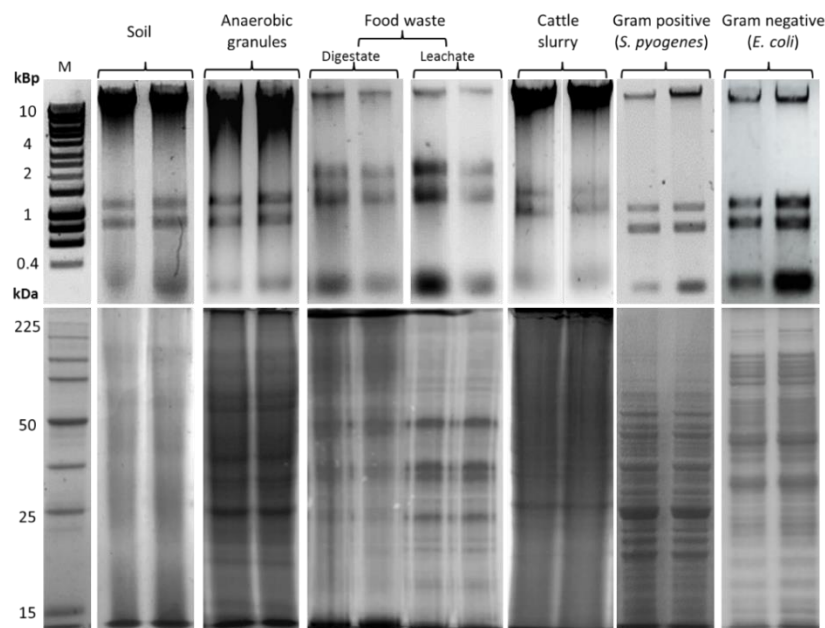

Figure S.Ib. Representative agarose gels and their corresponding SDS-page gel images from the three biomolecules recovered from the diverse range of sample types, with duplicate samples shown for each. For all samples, excluding cattle slurry, raw nucleic acid extracts are shown. Purification using the OneStep kit (Zymo) was necessary for cattle slurry samples prior imaging. The soil sample shown is Soil 2.

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Appendix II: Metaprotein list from Soil 2; co-extraction method

In the interests of space and paper, please find the tables of complete Metaprotein data on the Github page of Dr Florence Abram's research group, by following the link provided:

https://github.com/FEMLab/DNA-RNA-Protein-Coextraction-Method-Soil/blob/master/AllMetaproteinResultsData_ThesisAppendixII.xlsx

Appendix III: Details of fertiliser and herbicide in Lime Trial.

Table S.IIIa. N, P and K Fertiliser Programme

Application	Grower/Trials		Grower/Trials
	Fertiliser Type		Rate (kg/ha product)
	N	N-P-K	
Seedbed (16th April 2014)		11-7-24	500 kg/ha
Top-dress	CAN		100 kg/ha
Total	CAN		100 kg/ha

Table S.IIIb. Herbicide, Aphicide and Plant Growth Regulator Programme

Date	Growth Stage	Herbicide & Rate
09/05/14	Emergence	IPU @ 1.5 l/ha
		Headland Nova @ 1.0 l/ha
Date	Growth Stage	Aphicide & Rate
09/05/14	Emergence	Euro Lambda @0.05 l/ha
Date	Growth Stage	Herbicide & Rate
09/05/14	Tillering	Boudha @ 20 g/ha
		Flurox @ 0.75 l/ha
Date	Growth Stage	Aphicide & Rate
15/05/14	Tillering	Cypersect @ 0.25 l/ha
Date	Growth Stage	PGR & Rate
15/05/14	Tillering	CCC @ 0.50 l/ha
Date	Growth Stage	Wild Oats & Rate
11/06/2014	Flag Leaf	Acute @ 0.25 l/ha
Date	Growth Stage	Aphicide & Rate
01/07/2014	Ear Emergence	0.25 l/ha

Appendix IV: Dendrograms representing microbial community dissimilarity from the cDNA fraction (Chapter 3)
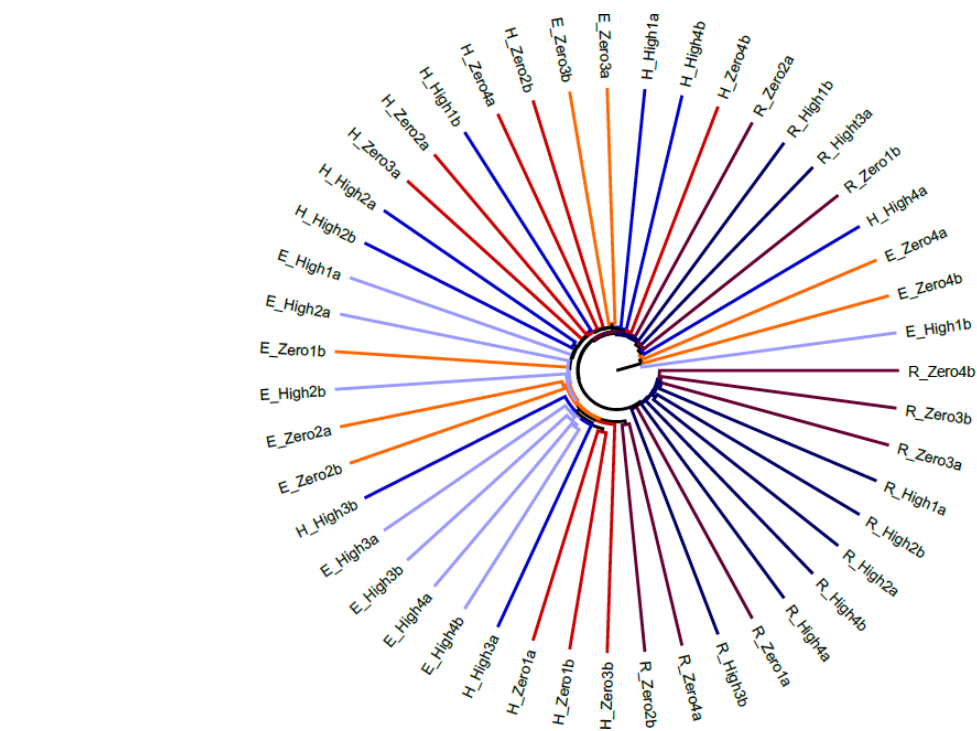

Figure S.IVa: Dendrogram of hierarchal clustering of cDNA samples, as calculated with the Jaccard dissimilarity index (presence/absence)
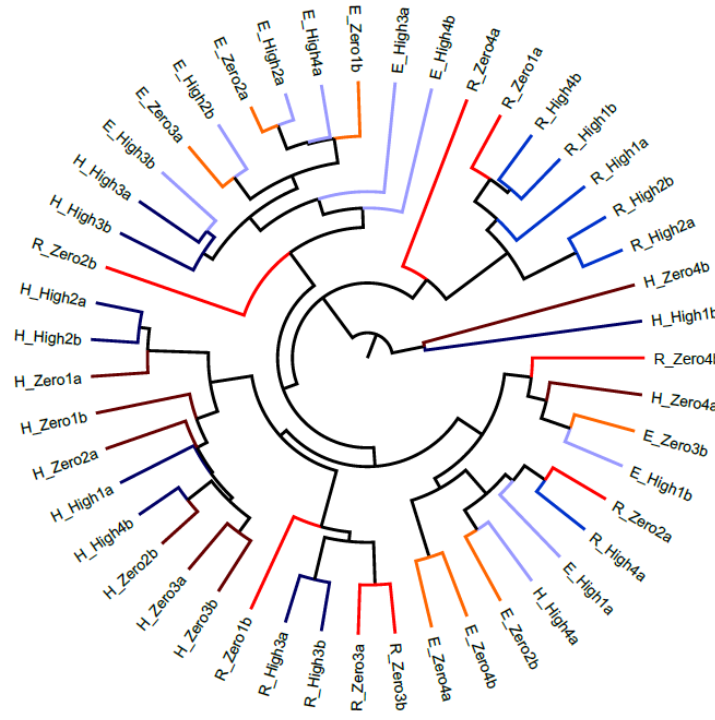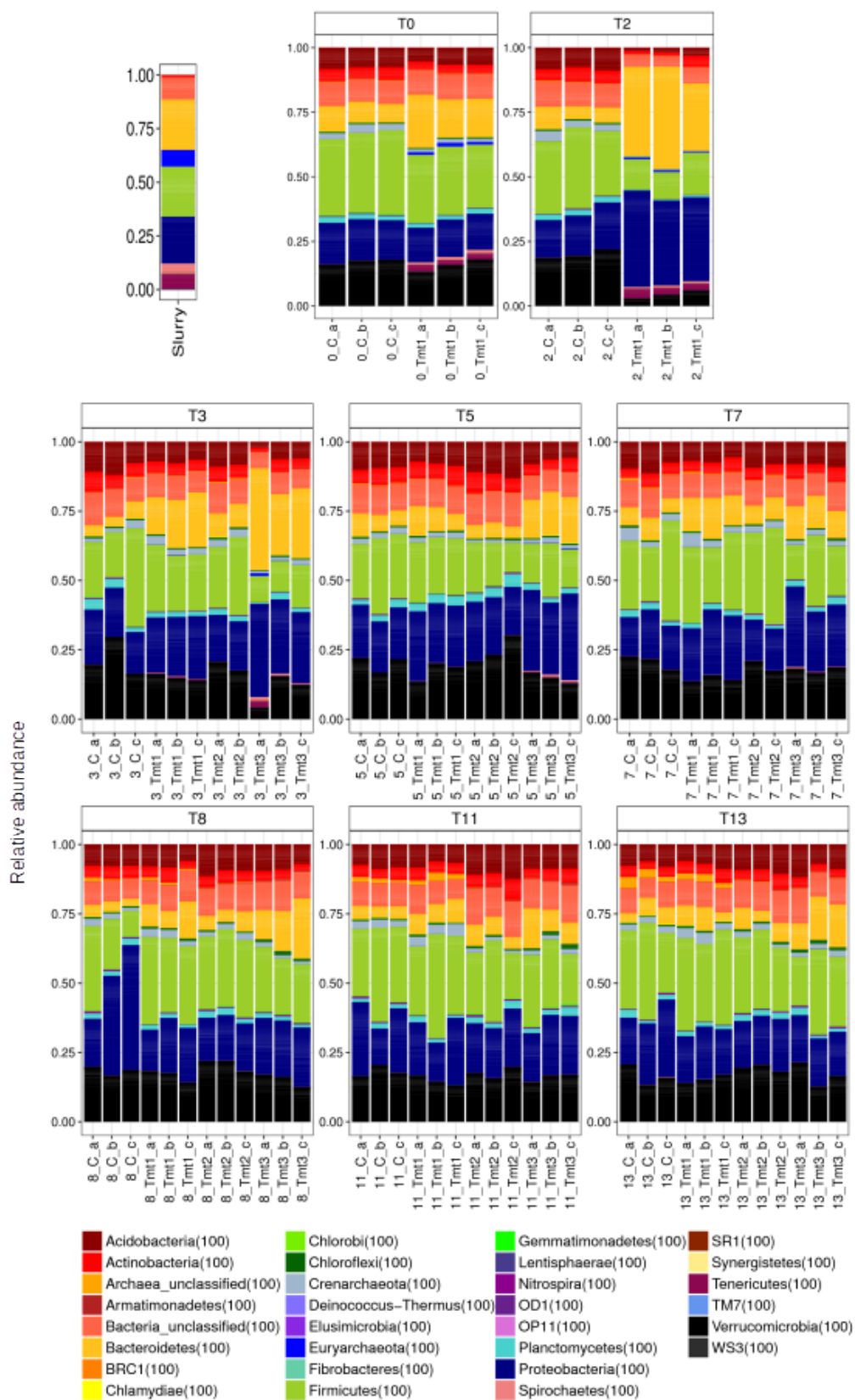

Figure S.IVb: Dendrogram of hierarchal clustering of cDNA samples, as calculated with the theta Yue & Clayton index (relative abundance).

Appendix V: SourceTracker output: proportions per biological replicate

For descriptor of sample IDs please see footnote.

Table S.IV

| Sample ID | Proportion of OTU from each source | | |
	Slurry	Soil	Unknown
T0_Tmt2_a	0.3277	0.5112	0.1611
T0_Tmt2_b	0.2168	0.5933	0.1899
T0_Tmt2_c	0.2615	0.5652	0.1733
T2_Tmt2_a	0.2644	0.0898	0.6458
T2_Tmt2_b	0.1795	0.1055	0.715
T2_Tmt2_c	0.2861	0.1908	0.5231
T3_Tmt2_a	0.0208	0.6691	0.3101
T3_Tmt2_b	0.0307	0.5615	0.4078
T3_Tmt2_c	0.0326	0.5206	0.4468
T3_Tmt4_a	0.2774	0.1057	0.6169
T3_Tmt4_b	0.0659	0.3537	0.5804
T3_Tmt4_c	0.0341	0.4405	0.5254
T5_Tmt2_a	0.0102	0.5553	0.4345
T5_Tmt2_b	0.0134	0.6877	0.2989
T5_Tmt2_c	0.006	0.7135	0.2805
T5_Tmt4_a	0.0655	0.5127	0.4218
T5_Tmt4_b	0.0316	0.5064	0.462
T5_Tmt4_c	0.0648	0.3739	0.5613
T7_Tmt2_a	0.0084	0.7593	0.2323
T7_Tmt2_b	0.0059	0.5816	0.4125
T7_Tmt2_c	0.0113	0.6314	0.3573
T7_Tmt4_a	0.0201	0.5571	0.4228
T7_Tmt4_b	0.0375	0.6577	0.3048
T7_Tmt4_c	0.0133	0.666	0.3207
T8_Tmt2_a	0.0007	0.7834	0.2159
T8_Tmt2_b	0.0019	0.7692	0.2289
T8_Tmt2_c	0.0058	0.6182	0.376
T8_Tmt4_a	0.0042	0.6828	0.313
T8_Tmt4_b	0.0071	0.6207	0.3722
T8_Tmt4_c	0.006	0.5309	0.4631
T11_Tmt2_a	0.0019	0.7358	0.2623
T11_Tmt2_b	0.0017	0.8136	0.1847
T11_Tmt2_c	0.0006	0.7417	0.2577

T11_Tmt4_a	0.0008	0.6781	0.3211
T11_Tmt4_b	0.0028	0.6895	0.3077
T11_Tmt4_c	0.0026	0.6759	0.3215
T13_Tmt2_a	0.0029	0.7835	0.2136
T13_Tmt2_b	0.0034	0.7482	0.2484
T13_Tmt2_c	0.0005	0.8296	0.1699
T13_Tmt4_a	0.0005	0.6289	0.3706
T13_Tmt4_b	0.0085	0.6396	0.3519
T13_Tmt4_c	0.0017	0.6971	0.3012

Where T- prefix denotes timepoint; Tmt1 = control; Tmt2 = 'slurry'; Tmt3 = 'flood' and Tmt4 = 'slurry+flood'; biological replicates per timepoint are denoted using a, b and c.