Disentangling the effects of long-term fertilisation on soil carbon dynamics

By

Jonathan David Kidd

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Abstract

The application of fertilisers has greatly increased agricultural yields. However, it has also had wide ranging consequences for ecosystem properties and processes, generating concern over the long-term sustainability of fertiliser management. Despite this, there is considerable uncertainty regarding the long-term impacts of fertilisation, especially on soil carbon, and this is largely due to the paucity of published findings from long-term experiments. In this project very long-term field experiments and a short-term microcosm trial were used to investigate the effects of fertilisation on agroecosystem properties with a particular focus on soil carbon dynamics. The effects of long-term fertiliser addition on key ecosystem properties in the Palace Leas Hay Meadow Experiment was determined and compared with those observed at other long-term and with short-term experiments. Farmyard manure addition was found to have many benefits over the use of inorganic fertilisers for improving agricultural production including higher nutrient availability, hay yields and the prevention of soil acidification. The magnitude of short- and long-term effects of fertilisation differed markedly, underlining the value of using long-term experiments to realise the true ecosystem response. The relative importance of three major classes of fertiliser-mediated mechanism was also evaluated using structural equation modelling and a complementary ley-arable rotation experiment. These studies found that soil C dynamics were altered by both direct (nutrient) and indirect (plant community- and soil pH-change) effects of fertilisation. Soil pH was the primary regulator of the microbial response to fertilisation, with soil acidification effects strongly negatively affecting microbial functioning when it reduced soil pH below 5. A microcosm study indicated that these effects of acidification on the microbial community cannot easily be reversed. As microorganisms regulate biogeochemical cycles which are critical for food production, fertiliser-induced acidification could therefore hinder long-term agricultural sustainability. Accordingly, soil pH in fertilised systems must be managed vigilantly.

Declaration

This thesis has been composed by myself, unless otherwise stated, and has not been submitted as part of any previous application for a degree. All sources of information have been specifically acknowledged by referencing.

Jonathan Kidd

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Chapter 1: Introduction

1.1 The importance of soil carbon

The capacity of the world's soils to store substantial quantities of carbon (C) is a critical ecosystem function (FAO, 2015). The terrestrial ecosystem holds approximately 3150 Pg C, 2500 Pg of which is stored within the soil (Lal, 2004). The soil contains more C than that stored in both terrestrial vegetation and the atmosphere (Batjes, 1996; Jobbagy and Jackson, 2000) and of the global pools, only the Ocean (38,400 Pg C) is a greater C store (Houghton, 2007). To 1 m soil depth, approximately 1550 Pg of the total soil C stock is soil organic C (SOC) and 950 GT is inorganic C (SIC) (Trumper *et al.*, 2009), however including estimates >1 m the global SOC pool may be as great as 3000 Pg C (Köchy *et al.*, 2015). Soil organic matter (SOM) is the complete organic fraction of the soil (Hayes and Swift, 1978), of which SOC is the greatest contributor (Dungait *et al.*, 2012). SOM is made up of a heterogeneous mixture of plant litter, exudates and microbial products which are at various stages of decomposition and differ in terms of their soil residence time (von Lützow *et al.*, 2007). There is increased interest in preserving and, in severely degraded soils (i.e. areas of agricultural intensification), increasing SOC stocks due to the range of benefits it can confer, including; climate change mitigation, enhanced soil quality, greater retention of water and growth limiting nutrients, reduced rates of soil erosion and increased food security (Powlson *et al.*, 2011; Lal *et al.*, 2015).

1.2 Soil carbon dynamics

SOC stocks are not perpetually stable, rather they are in dynamic equilibrium with vegetation and the atmosphere, primarily as a result of photosynthetic input and CO_2 output via soil respiration (Amundson, 2001; Jastrow *et al.*, 2007). The majority of SOC is plant-derived (Lal, 2008) with generally greater input from root than shoot litter (Rasse *et al.*, 2005). Following the fixation of CO_2 during photosynthesis, C enters the soil directly as decaying root litter and as organic compounds from root exudation, whilst shoot litter resides on the soil's surface and is reliant on faunal and microbial action to be incorporated into the mineral soil. Non-plant-derived pathways also increase the input of C to the soil, including the deposition of animal waste and additions through farming practice e.g. manures, composts and paper waste (Bardgett and Wardle, 2010).

Soil C compounds are subject to breakdown and assimilation by the living fraction of the SOM; the microbial biomass. The soil microbial biomass is largely made up of fungal and

bacterial decomposer groups. Fungi tend to be more abundant than bacteria, however, ratios of fungi to bacteria (F/B) have been shown to vary (0.9 and 4.5) depending on climatic conditions and land management (Khan *et al.*, 2016). Fungi and bacteria are functionally distinct, soils with increased abundance of bacteria (low F/B) are generally regarded to be 'fast cycling' systems, exhibiting high rates of nutrient and labile C mineralisation and low C-use efficiency (Bardgett, 2005; Strickland and Rousk, 2010). In contrast, 'slow cycling' fungal-dominated communities (high F/B) are thought to be functionally conservative, display higher efficiency of C utilisation and lower C turnover and nutrient release (Bardgett and Wardle, 2010; De Vries *et al.*, 2012a). However, others have shown that the dominance of a fungal or bacterial food web does not necessarily determine microbial C-use efficiency (Six *et al.*, 2006; Thiet *et al.*, 2006) or the rate at which belowground processes such as C mineralisation occur (Rousk and Frey, 2015).

In acknowledgement of the role of the microbial community, it has been suggested that a greater understanding of microbial functioning and community composition at a finer level of resolution is required (Rousk *et al.*, 2015). Several methods have been employed to measure microbial function and activity including; respiration (CO_2 production) (Liu *et al.*, 2014; Chen *et al.*, 2015b), extracellular enzyme activities (Ramirez *et al.*, 2012; Fanin *et al.*, 2016) and nitrification potential (Grigulis *et al.*, 2013; Carey *et al.*, 2015). However, one of the most widely used approaches is community level physiological profiling (CLPP), and in particular the BIOLOG™ (Garland and Mills, 1991) and MicroResp™ assays (Campbell *et al.*, 2003). To detect differences in the functional capabilities of microbial communities, CLPP assays exploit the fact that soil microbes assimilate C (and N and phosphorus (P)) for growth and activity in the form of soluble low-molecular weight (LMW) substrates (Strickland *et al.*, 2015), by measuring the rate at which they are utilised (through the resulting CO_2 production). LMW substrates consumed by microbes and used in the assays include a mixture of carbohydrates, amino acids, carboxylic acids and phenolic acids, which, in the soil, are mainly derived from root exudates, leaf litter leachate or catalysed (via extracellular enzymes) complex organic matter (Burns *et al.*, 2013; Hobbie and Hobbie, 2013). MicroResp™ is generally recognised as a more suitable assay as it is capable of detecting more subtle differences in microbial function and it is easier to use than BIOLOG™ (Lalor *et al.*, 2007). The BIOLOG™ technique also requires the extraction and growth of soil microbes (*in vitro*) and as a result introduces bias, as not all of the extracted community can grow or metabolise in the experimentally-induced conditions in the BIOLOG™ well plates (Smalla *et al.*, 1998; Blagodatskaya and Kuzyakov, 2013). This methodological constraint does not arise when

using MicroResp^{TM} as substrates are directly applied and respiration is measured from whole soil samples (Campbell *et al.*, 2003; Chapman *et al.*, 2007).

To measure the composition of soil microbial communities, a relatively cheap tool that has been widely used is phospholipid fatty acid (PLFA) analysis (Rousk *et al.*, 2009; De Vries *et al.*, 2012b; Wei *et al.*, 2013). While PLFA provides detail of overall changes in community composition, it cannot reveal how communities differ taxonomically. Recently there have been major methodological developments, including the advent of pyrosequencing approaches (Lauber *et al.*, 2009) and latterly next generation DNA sequencing (NGS) technologies such as Illumina sequencing (Caporaso *et al.*, 2012), capable of comprehensively evaluating microbial communities at a much greater level of resolution. Techniques such as Illumina sequencing (of the 16S rRNA gene for bacteria and 18S rRNA gene for fungi) are cheap relative to other sequencing methods, afford increased accuracy and enable high throughput of samples (Bartram *et al.*, 2011; Fakruddin and Mannan, 2012). The accrual of detailed taxonomic information using sequencing technologies has enabled the identification of rare taxa not discovered previously (Bartram *et al.*, 2011) and also allowed hypotheses related to soil C cycling to be investigated. For example, Fierer *et al.* (2007) proposed that variation in soil bacteria community function and hence microbially-mediated ecological processes can be better understood according to the taxonomy of the soil microbial community. Communities dominated by taxa considered to exhibit copiotrophic traits such as *Betaproteobacteria* and *Bacteroidetes* are adept at decomposing labile C substrates and possess fast turnover rates, while those with greater abundance of oligotrophic taxa, e.g. *Acidobacteria*, prefer to degrade more plant-derived residues (Fierer *et al.*, 2007). Sequencing methods have also been successfully employed using soils from a range of ecosystems to understand how the copiotrophic-oligotrophic concept amongst bacterial communities extends to soils that differ in terms of pH (Lauber *et al.*, 2009; Rousk *et al.*, 2010a), nutrient availability (Ramirez *et al.*, 2010b; Fierer *et al.*, 2012) and plant community composition (Leff *et al.*, 2015).

1.3 Stability of soil carbon

The formation of stabilised SOM (i.e. resistant to microbial decomposition) capable of residing in the soil for extended periods of time was originally attributed to greater biochemical recalcitrance of material (high lignin:N) entering the soil. Increased recalcitrance of substrates was thought to restrict decomposition and caused such organic compounds to be selectively preserved forming humus whilst labile materials (low C:N) were readily

mineralised (Piccolo, 2002; Krull *et al.*, 2003). However, this mechanism is now suggested to be of secondary importance (Amelung *et al.*, 2008; Marschner *et al.*, 2008; Schmidt *et al.*, 2011; Dungait *et al.*, 2012), rather it is more generally accepted that the persistence of SOM and hence the longer term storage of SOC is principally due to the physical and chemical protection of SOM. This occurs via sorption to fine particles (silt and clay) and microbially driven occlusion in stable soil microaggregates, making SOM spatially inaccessible to microbial decomposition (Schmidt *et al.*, 2011; Dungait *et al.*, 2012; Lal *et al.*, 2015). Whilst SOM protection and stabilisation may be predominantly dependent on soil physical properties, microbially-transformed residues make up approximately 50% of SOC in grassland and arable soils (Khan *et al.*, 2016) and microbial processes are a key route through which stabilised SOM is formed (Schmidt *et al.*, 2011; Miltner *et al.*, 2012; Lal *et al.*, 2015). Consistent with this, Cotrufo *et al.* (2013) proposed 'The Microbial Efficiency-Matrix Stabilisation' (MEMS) framework. This suggests that proportionally more high quality labile litter compounds are assimilated to form decomposition products which have the potential to be protected by the soil matrix compared with litter of low quality (Cotrufo *et al.*, 2013).

In recognition of the fact that SOC is composed of a heterogeneous mixture of organic carbon compounds with varying turnover times (minutes to thousands of years) (Trumbore, 2000; von Lützow *et al.*, 2006; Lal *et al.*, 2015), researchers often separate the total SOC stock into functional fractions or 'pools' (Smith *et al.*, 1997). The separation and measurement of C in component soil fractions, commonly known as fractionation, has been essential for dynamic simulation models, e.g. Century (Parton *et al.*, 1993) and RothC (Smith *et al.*, 2005), capable of making large-scale soil C stock predictions and to enable the evaluation of factors that control soil C dynamics at local or landscape scales (Manning *et al.*, 2015a; Riggs *et al.*, 2015; Ward *et al.*, 2016). However, an important barrier preventing a better understanding of soil C dynamics is that there is no single universally accepted fractionation technique (von Lützow *et al.*, 2007; Murphy *et al.*, 2011). The methods used to isolate soil C constituents are numerous and include physical (e.g. aggregate, particle-size and density) and chemical (e.g. hydrolysis and oxidation) fractionation procedures and a combination of the two (von Lützow *et al.*, 2007; Marschner *et al.*, 2008), making it difficult to reliably generalise findings from different studies or conduct meta-analyses of previously published data. Despite methodological differences, SOC is typically (but not always; Zimmerman *et al.*, 2007) split into three conceptual fractions which correspond to their turnover times; 1) active or labile fraction, 2) intermediate or slow fraction and 3) stable or passive fraction (Amundson, 2001; Six *et al.*, 2002; von Lützow *et al.*, 2007). The active or labile fraction is composed of fresh

plant material and low-molecular weight carbon (LMW-C) substrates which turn over rapidly and have a residence time of minutes to a few years. The intermediate or slow fraction is typically microbially-processed material that is partially protected in aggregates or on soil mineral surfaces with residence times of decades. Stable or passive fractions turn over in the range of hundreds to thousands of years and comprise of OM occluded in microaggregates or organo-mineral associations (Kleber *et al.*, 2007; von Lützow *et al.*, 2008; Cotrufo *et al.*, 2013).

1.4 The role of agricultural fertilisers and long-term experiments

The addition of fertilisers has enabled agroecosystems to deliver a key ecosystem service i.e. enhanced plant biomass production through greater availability of growth-limiting nutrients. To the satisfy the food demand for a growing global population, which is expected to reach 9.7 billion by 2050 (United Nations, 2015) and cope with increased need for greater feedstocks for bioenergy production (Fulton *et al.*, 2015), projections forecast an approximate increase in world fertiliser consumption of 60 million tonnes on the present day total (Alexandratos and Bruinsma, 2012). Such changes are expected to initiate a cascade of effects on the world's terrestrial ecosystems beyond increasing primary productivity, including significant alterations to key ecosystem properties and services (Vitousek *et al.*, 1997; Power, 2010; Manning, 2012), leading some to question the sustainability of modern management practices in the long-term.

The long-term impacts of fertilisation on ecosystem properties and services are not fully understood. This is primarily due to the difficulty in obtaining reliable information with regards to management history and dependence on the use of short-term experiments. The findings of short-term studies are useful in demonstrating the initial trajectory of the response (Richter *et al.*, 2007; Knapp *et al.*, 2012), therefore they may be applicable in locations where management practices are frequently changing (e.g. crop rotations) or in areas receiving fertilisers for the first time. However, the results may not be representative of the response at sites remaining under intensive management for long periods. Of the few studies that have evaluated the effects of long-term fertilisation, variation in ecosystem properties have been shown to be driven by both direct (nutrient input) and indirect effects (acidification) of fertilisation (Crawley *et al.*, 2005; Hejcman *et al.*, 2014). Indirect effects of fertilisation may not be immediately apparent and therefore the timeframe for short-term experiments is unlikely to be sufficient to detect the effects (Knapp *et al.*, 2012). Furthermore, evidence from existing long-term experiments has revealed that the response of some ecosystem properties

may be slow (Silvertown *et al.*, 2006), this is particularly true for SOC stocks which have been shown to take several decades to equilibrate to a change in management (Hopkins *et al.*, 2009; Johnston *et al.*, 2009). Similarly, Geisseler *et al.* (2016) established in a review of over 200 grassland and annual cropping systems that the effects of fertilisation on the size of the microbial biomass and rate of respiration were less prominent in short-term experiments (<5 years). Therefore, extrapolating the effects of fertilisation from short-term studies when predicting long-term ecosystem responses may be unreliable and lead to false conclusions. Accordingly, long-term experiments are valuable to understand the relationship between fertilisation and ecosystem properties more accurately, including enabling researchers to elucidate the mechanisms responsible (Silvertown *et al.*, 2010). However, due to the time and expense required to maintain long-term ecological experiments and that research funders endeavour to support innovative but often short-term projects (Silvertown *et al.*, 2010), the number of long-term ecological studies is relatively low (Silvertown *et al.*, 2006). As such, integrating research from existing literature is difficult and to date little has been done to determine the generality of the effects of fertilisation from the long-term study sites.

1.5 Impacts of fertilisation on soil carbon dynamics

As N is one of the most limiting plant growth nutrients (Vitousek and Howarth, 1991; LeBauer and Treseder, 2008) and can have major implications for plant community composition in natural ecosystems (Suding *et al.*, 2005; Clark *et al.*, 2007), the majority of studies that have examined the impacts of fertilisation on soil C dynamics have focused on the influence of N enrichment. Therefore, the effects of other co-limiting nutrients including P and K (Cleland and Harpole, 2010) are less well understood. Generally, the addition of N fertiliser to agricultural soils lowers rates of microbial activities and reduces the decomposition of C (Treseder, 2008; Ramirez *et al.*, 2010a; Ramirez *et al.*, 2012). Several different hypotheses that have been used to explain the negative effects of fertilisation on SOC decomposition commonly attribute the response to the direct effects i.e. nutrient availability. This partially emanates from the fact that some of the earlier studies were very short-term laboratory experiments (≤ 1year) and as such the differences in microbial activities were due to the direct effects of nutrient addition. Ramirez *et al.* (2012) suggested that three mechanisms were likely to explain effects of N amendment of soil C dynamics; 1) the microbial N mining hypothesis, 2) the enzyme inhibition hypothesis and 3) the copiotrophic hypothesis. According to the *microbial N mining hypothesis* (Craine *et al.*, 2007), the input of labile C to the rhizosphere is used by soil microbes as an energy source, stimulating the decomposition of more stable forms of organic matter to acquire N. However, following N

addition, microbial mining of stable organic matter is supressed as soil N availability is increased, reducing microbial respiration. The *enzyme inhibition hypothesis* suggests that N-induced reduction in microbial activities is due to the inhibition of exoenzymes produced to catalyse recalcitrant C (Fog, 1988; Sinsabaugh, 2010). Whilst the two aforementioned mechanisms reflect a fertilisation-induced physiological change in the microbial community, the *copiotrophic hypothesis* proposes the effects of fertilisation are underpinned by a shift in community composition. N addition is suggested to favour an increase in dominance of copiotrophic over oligotrophic taxa (Fontaine *et al.*, 2003; Fierer *et al.*, 2007). A reduction in abundance of oligotrophs, which are said to be adept at decomposing recalcitrant C in favour of copiotrophs that assimilate more labile C substrates (Fierer *et al.*, 2012) may therefore cause a reduction in recalcitrant C decomposition.

A key limitation of the studies by Ramirez *et al.* (2010a) and Ramirez *et al.* (2012) was that they chose to ignore the effects of the plant community by removing vegetation prior to the experiment. Recent work has demonstrated that fertilisation is also likely to influence soil C dynamics indirectly via changes to the plant community (Leff *et al.*, 2015; Geisseler *et al.*, 2016; Zeng *et al.*, 2016) or edaphic factors such as soil pH (Rousk *et al.*, 2011b; Wei *et al.*, 2013; Chen *et al.*, 2015b). Therefore, the response observed in the earlier described studies are not fully representative of processes driving soil C dynamics in the field. Indeed, a global study of field experiments reported that the addition of N and P fertiliser increased the relative abundance of copiotrophic taxa over oligotrophic taxa (Leff *et al.*, 2015), which according to the mechanism above may shift microbial decomposition preference from recalcitrant C to labile C. However, the authors determined that fertiliser-induced changes in plant community composition were of greater importance in shifting bacterial community composition than elevated nutrient availability (Leff *et al.*, 2015). Similarly, a separate fertilisation experiment reported that pH-induced effects coupled with plant community change effects were responsible for alterations to bacterial community composition (Zeng *et al.*, 2016).

In recognition of the fact that in complex ‘real-world’ agricultural systems there are likely to be co-occurring causal pathways through which fertilisation affects soil C dynamics, researchers have recently begun to utilise powerful multivariate analysis techniques including structural equation modelling (SEM) (Grace, 2006) to evaluate the importance of the potential fertilisation pathway effects. The use of such techniques has emphasised the importance of indirect pathway effects on both microbial community composition and functioning. In two separate fertilised grassland experiments, it was demonstrated that N enrichment was associated with a significant shift in microbial community composition and a reduction in

microbial respiration, yet these changes were related to modification of the soil's pH rather than differences in nutrient availability (Wei *et al.*, 2013; Chen *et al.*, 2015b). Whilst the SEM approach used by these studies is undoubtedly a significant step forward, the experiments have thus far been performed in relatively recently established trials and as a result the observed effects may only be intermediate at the point of sampling and the legacies of former management at the site may still be influential. Moreover, the use of a SEM to disentangle to effects of fertilisation of soil C dynamics has not been extensive, rather it has been exclusive to a single grassland ecosystem (semi-arid Mongolian grasslands). As a result, there is a paucity of knowledge whether the relative importance of the pathway effects are context dependent or universal across ecosystems.

On a couple of occasions studies have gone a step further and have attempted to validate the consistency of the effects of fertilisation on soil C dynamics by using complementary trials. In a study by Ramirez *et al.* (2010b), a rotational cropping system was used alongside a long-term grassland system to evaluate the drivers of N fertilisation on bacterial community composition. In an investigation by Chen *et al.* (2015b), an acidity gradient was established to supplement findings from a N addition experiment on microbial respiration. However, in both studies the complementary trials were relatively short-term (acid addition experiment = 4 years, rotational cropping experiment = 8 years), again increasing the possibility that the observed responses were transient. The use of complementary experiments to further examine consistency of the drivers of fertilisation provides great potential, however to minimise the opportunity to detect transient effects and increase the validity of the findings it may be better to utilise experiments that are of a longer duration. Without a better understanding of the relative importance of the pathways through which fertilisation can influence soil C dynamics in contrasting 'real-world' agricultural systems, we are limited in our ability to formulate models robust enough to predict future ecosystem changes.

1.6 Microbial community recovery from the effects of fertiliser-induced soil acidification

A prominent topic within the literature is the consequences long-term fertilisation can have for plant and microbial communities as a result of increased levels of soil acidity. Soil acidification typically occurs following the input of ammonium-containing fertilisers, causing rates of nitrification and the uptake of NH_4^+ by the plant community to be elevated, subsequently increasing the concentration of H^+ ions in the topsoil (Guo *et al.*, 2010). Plant communities have been reported to suffer comprehensive shifts in community composition,

and severe reductions in species diversity and aboveground biomass following increased fertiliser-induced soil acidity (Crawley *et al.*, 2005; Silvertown *et al.*, 2006; Hejcman *et al.*, 2014). Similarly, studies have also described a considerable loss of microbial diversity (Zhalnina *et al.*, 2015), biomass (Geisseler and Scow, 2014) and reductions in activity (Rousk *et al.*, 2011a) which will likely have implications for soil C turnover (Fornara *et al.*, 2011). Recent work has demonstrated that where intense fertiliser-induced acidification has significantly reduced plant diversity, the negative effects may be long lasting and not easily reversed (by the cessation of fertilisation and twice-yearly hay cutting) (Storkey *et al.*, 2015). Despite the crucial role belowground communities play in multiple key ecosystem processes, microbial restoration ecology has received considerably less attention than work on plant community recovery (Calderon *et al.*, 2016). Studies in the past have established that microorganisms are very adaptable to changes in to abiotic and biotic properties (Schmidt *et al.*, 2007), with clear microbial community composition shifts to following alteration to soil conditions (Eilers *et al.*, 2010; Männistö *et al.*, 2016), the ability to adapt their stoichiometry ('non-homeostasis') to that of the available resources (Fanin *et al.*, 2013; Mooshammer *et al.*, 2014) and possess functional plasticity (Carey *et al.*, 2015). Critically though, no studies to date have explicitly tested the consequences of long-term fertiliser-induced acidity for the recovery of microbial communities. Therefore, the extent and speed to which microbial communities respond following the detrimental perturbation of acidity is unclear. Greater understanding of the legacy effect fertilisation leaves behind is likely to be required to aid the development of effective management strategies to mitigate the potentially adverse effects to microbial communities and the biogeochemical process they regulate.

1.7 Research objectives and experimental approach

The principal aim of this thesis is to investigate the effects of long-term fertilisation on agricultural ecosystem properties with a particular focus on the impacts on soil carbon dynamics. In addition, this work aims to further examine the specific pathway effects of fertilisation and the consequences on microbial community composition and potential functioning. The objectives of the thesis are:

1) To characterise the responses of a wide range of ecosystem properties to long-term management and compare ecosystem responses with those observed at other long-term fertilised sites and with results from short-term studies (Chapters 2 and 4).

2) To investigate the relative importance of direct nutrient and indirect soil pH-, and plant community-mediated effects of fertilisation upon agroecosystem C and soil function. (Chapters 3 and 4).

3) To test the effects of an increase in soil pH on the recovery of microbial communities subject to different degrees of fertiliser-induced acidification (Chapter 5)

The experimental approach that will be employed to address the objectives outlined above will include the use of long-term field experiments and a manipulative microcosm study:

In Chapter 2, a comprehensive evaluation of the effects of long-term fertilisation on grassland soil and plant properties will be undertaken using the Palace Leas Hay Meadow Experiment (est. 1896); the second oldest permanent grassland experiment in the world (Hopkins et al., 2009). All 14 fertiliser treatment plots will be sampled, which include plots amended with manure only, mineral fertiliser only (all combinations of N, P and K) and manure and mineral fertiliser applied together.

In Chapter 3, a structural equation modelling framework will be applied to plant and soil data collected from Palace Leas. The data to be used will include plant and soil property measurements also used for univariate statistical analysis in Chapter 2 and a soil microbial dataset including measures of active microbial biomass and microbial functioning.

In Chapter 4, I will sample a long-term ley-arable crop rotation with an experimentally generated pH gradient (pH 4.5-7.5 at 0.5 intervals) at Woodland's Field, Craibstone Estate, Aberdeen (est. 1961). Soils will be collected from plots maintained at pH 4.5, 5.0, 5.5 and 6.0 in three of the eight courses of the crop rotation. By sampling at pH 4.5-6.0 it will provide a pH range similar to that reported at Palace Leas, whilst the 3 courses of the rotation will broadly represent different stages of the nutrient build-up and depletion cycle (Chapter 4).

In Chapter 5, a glasshouse experiment will be set up using soil collected from Palace Leas. Soil will be collected from several of the long-term fertiliser treatment plots. The selected treatment plots will provide a range of soils exposed to different degrees of fertiliser-induced acidification. Soils will be amended with lime to modify the pH, transferred into pots and seeded with a grass species native to Palace Leas and which grows well across the anticipated pH range. Soils will be destructively sampled at the end of the experiment and soil chemical and biological properties will be analysed (Chapter 5).

Chapter 2: Impacts of 120 Years of Fertiliser Addition on a Temperate Grassland Ecosystem

Jonathan Kidd, Peter Manning, Janet Simkin, Simon Peacock and Elizabeth Stockdale. *Plos One.* Under review.

Contribution statement: Jonathan Kidd, Elizabeth Stockdale and Peter Manning conceived the study. Janet Simkin, Simon Peacock and Jonathan Kidd performed the botanical survey. The National Vegetation Classification was calculated by Janet Simkin. Soil sampling, laboratory analysis and analysis of the soil and plant data was carried out by Jonathan Kidd. Jonathan Kidd wrote the paper, all authors contributed to revisions.

2.1 Abstract

The widespread application of fertilisers has greatly influenced many processes and properties of agroecosystems, and agricultural fertilisation is expected to increase even further in the future. To date, most research on fertiliser impacts has used short-term studies, which may be unrepresentative of long-term responses, thus hindering our capacity to predict long-term impacts. Here we examined the effects of long-term fertiliser addition on key ecosystem properties in a long-term grassland experiment (Palace Leas Hay Meadow) in which farmyard manure (FYM) and inorganic fertiliser treatments have been applied consistently for 120 years in order to characterise the experimental site more fully and compare ecosystem responses with those observed at other long-term and short-term experiments. FYM inputs increased soil organic carbon (SOC) stocks, hay yield, nutrient availability and acted as a buffer against soil acidification (>pH 5). In contrast, N-containing inorganic fertilisers strongly acidified the soil (<pH 4.5) and increased surface SOC stocks by increasing the C stored in the coarse (2.8 mm-200 μm) and fine (200-50 μm) fractions. Application of N fertilisers also reduced plant species richness and the abundance of forbs and legumes. Overall, our results were broadly consistent with those observed in other very long-term studies (the Park Grass and Steinach Grassland experiments) in that fertilisation effects on plant and soil properties appeared to be driven by differences in both nutrient input and changes to soil pH. We also established that the direction of long-term fertilisation effects tended to be comparable with short-term experiments, but that their magnitude differed considerably, particularly where ammonium sulphate-induced acidification had occurred. We therefore conclude that short-term studies are unlikely to possess the required timeframe to accurately predict long-term responses, thus necessitating the use of long-term study sites.

Such experiments should be strategically established in regions where future fertiliser use is expected to increase rapidly.

2.2 Introduction

Over the previous century, agricultural production and a growing human population have become heavily dependent on the use of fertilisers. Key technological developments at the turn of the 20th century supported this, most notably the Haber-Bosch process, resulting in >10 fold increase in the use of reactive nitrogen (N) over the past 150 years (Galloway *et al.*, 2008). World fertiliser consumption projections suggest this pattern in fertiliser use is unlikely to diminish, with 263 million tonnes of fertiliser expected to be used annually by 2050, an approximate increase of 60 million tonnes on the present day total (Alexandratos and Bruinsma, 2012).

The critical contribution that fertiliser application has made in increasing plant productivity, and consequently agricultural yields, has long been recognised (Gough *et al.*, 2000; Lee *et al.*, 2010). However, the provision of plant-growth limiting nutrients has had wide ranging consequences for many key ecosystem processes and services beyond simply increasing aboveground biomass, including dramatic alterations of plant and soil communities and the processes they control (Knorr *et al.*, 2005; Elser *et al.*, 2007; LeBauer and Treseder, 2008; Chen *et al.*, 2015a). Fertiliser-induced changes to soil properties are numerous and include changes in soil nitrogen cycling (Mack *et al.*, 2004) and the stocks of soil organic carbon (SOC) found in a range of size and density fractions (Neff *et al.*, 2002; van Groenigen *et al.*, 2006). Fertiliser additions also often result in a decline in plant species richness (Tilman, 1987; Suding *et al.*, 2005) and changes in community structure and functional composition (Suding *et al.*, 2005; Allan *et al.*, 2015). These aboveground changes are accompanied by changes to the microbial community, with effects on soil enzyme activity (Ramirez *et al.*, 2012), microbial biomass (Treseder, 2008; Janssens *et al.*, 2010) and microbial composition (Fierer *et al.*, 2012; Leff *et al.*, 2015).

Fertiliser-induced shifts to ecosystem functions and services have led to questions about the sustainability of current and future agricultural fertiliser management (Vitousek *et al.*, 1997; Sutton *et al.*, 2011; Manning, 2012), e.g. due to problems including; widespread nutrient leaching, groundwater contamination, eutrophication, biodiversity declines and soil acidification. Assessing this potential risk is particularly pertinent in developing countries where fertiliser use per hectare is expected to increase in order to provide food for larger and wealthier populations (Galloway *et al.*, 2004). Furthermore, despite the fact that in many parts

of the developed world, fertilisation has been regular practice for many decades, the long-term impacts of this practice have not been fully quantified due to the difficulty in obtaining reliable data on historic rates of addition.

Uncertainty regarding the long-term impacts of fertiliser addition upon ecosystems has partially arisen from a general reliance on relatively short-term experimental studies (typically <10 years) e.g. (De Vries *et al.*, 2006; Bradford *et al.*, 2008; Ramirez *et al.*, 2012). While understanding the short-term impacts of fertilisation is relevant in situations where management practices are changing i.e. in rotational cropping systems or areas of the developing world where fertilisers are only now starting to be applied, these effects may not be representative of the long-term response of sites remaining under intensive management. As an increase in the number of repeatedly fertilised sites is expected in the future, it may therefore be misleading to extrapolate the effects from short-term studies when predicting long-term ecosystem responses. Ecosystem properties have been shown to take decades to stabilise in response to fertilisation (Silvertown *et al.*, 2006; Johnston *et al.*, 2009), meaning that short-term studies can be unrepresentative of long-term responses. While studies that compare differences in ecosystem properties between short- and long-term experiments are scarce, inconsistencies are likely to exist. This may include a failure to detect critical environmental thresholds, beyond which change in ecosystem properties have been shown to be dramatic (Silvertown, 1980) or gradual species adaptations, such as those evidenced in *Anthoxanthum odoratum* species, both of which were identified at the very long-term Park Grass experiment (160 years) (Silvertown *et al.*, 2006). In addition, transient effects of fertilisation on soil C storage may be observed in short-term studies in labile soil C fractions, yet the influence on the more stable C fractions which possess a longer turnover time (Christensen, 2001) may only be detected using long-term experiments. Variation between short- and long-term studies may also occur as the magnitude of fertilisation effects may differ depending on the length of the experiment. For example, the reduction in the microbial biomass abundance was shown to be greater over longer periods of N fertilisation (Treseder, 2008), a pattern which short-term experiments would be unlikely to detect. In addition, observational studies can also deliver potentially misleading results due to confounding management effects, e.g. the strong association between increased fertiliser inputs and other components of agricultural intensification (Blüthgen *et al.*, 2012). The use of long-term field experiments is consequently of great value. However, the number of long-term ecological studies is very low (Silvertown *et al.*, 2006), thus making it hard to draw general conclusions.

In the very few studies that have examined the impacts of long-term fertilisation, strong shifts in ecosystem properties are attributed to differences in both the provision of nutrients and soil pH. For instance, in both the Park Grass and the Steinach Grassland (83 years) experiments, plant species richness is not only negatively affected by increased biomass production in response to increased nutrient supply, but also by intense acidification following the addition of ammonium sulphate fertiliser which causes the exclusion of species unable to tolerate highly acidic conditions (Crawley *et al.*, 2005; Silvertown *et al.*, 2006; Hejcman *et al.*, 2014). In terms of soil properties, concentrations of plant available nutrients are increased by the application of N, P and K containing fertilisers and reduced via uptake by the aboveground biomass (Hejcman *et al.*, 2010). However, nutrient availability is also mediated by differences in soil pH, whereby the nutrient content is reduced in acid soils (Zhalnina *et al.*, 2015). Up to now, it has not been established if these long-term trends are general responses of grasslands to fertilisation.

To address this, we carried out a comprehensive evaluation of the ecosystem responses of grassland to very long-term fertiliser addition using the Palace Leas Hay Meadow; the second oldest continuous grassland experiment in the world (Hopkins *et al.*, 2009). The experiment was established in 1896 to identify means of improving grassland yield and aftermath growth using fertilisers, liming materials and manures. However, numerous other parameters have been measured subsequently (Shiel, 1986; Coleman *et al.*, 1987; Hopkins *et al.*, 2009; Jenkins *et al.*, 2009). Previous research at the site has involved the study of long–term treatment effects on above- and below-ground properties, but studies have tended to report the effects on only a small number of plant and soil properties, and this has been done separately (Shiel and Hopkins, 1991; Hopkins *et al.*, 2011) and from an inconsistent set of available plots. The overarching aim of this study was to characterise the responses of a wide range of soil and plant properties i.e. hay yield, plant community composition and soil chemical properties (pH, available P and K and SOC stocks), to long-term fertiliser application in order to (i) establish the Palace Leas experiment as a platform for more specific future research (e.g. into the mechanisms underlying these responses, and as a data-set to contribute to further meta-analyses) and (ii) to compare these responses to those observed at other long-term fertilised sites and with results from short-term studies. We hypothesised, based on previous findings from long-term experiments (Crawley *et al.*, 2005; Silvertown *et al.*, 2006; Hejcman *et al.*, 2014), that (i) changes to plant and soil properties will be driven by direct nutrient input and via changes in soil pH, and that (ii) responses observed at Palace Leas to fertiliser addition will be consistent with those from other long-term grassland experiments, but differ

considerably from the findings of short-term studies where plant and soil properties are primarily controlled by differences in the provision of nutrients.

2.3 Materials and Methods

2.3.1 *Field site*

Palace Leas Hay Meadow Experiment is located 30 km north of Newcastle upon Tyne, England at Cockle Park Farm, (55˚13' N, 1˚41' W, UK National Grid Reference NZ 202912). The soil was classified as a pelo-stagnogley (Typic Ochraqualf) from the Hallsworth series (Hopkins *et al.*, 2009) and has a clay loam texture (Jarvis *et al.*, 1977) with 41% sand, 29% silt and 30% clay. Prior to the experiment, the vegetation at Palace Leas was permanent pasture for many years (Arnold *et al.*, 1976). Due to its close proximity to the farm, the field would have regularly received farmyard manure (FYM). Using data from a botanical survey carried out in 1897 (year 2 of the experiment), the initial grassland vegetation on all treatment plots was matched to the National Vegetation Classification (Rodwell, 1998) community U4b (*Festuca ovina-Agrostis capillaris-Galium saxatile* grassland, *Holcus lanatus-Trifolium repens* sub-community) (Simkin, 2015). As it can take many years for grassland communities to respond to change in management, it is reasonable to assume that Palace Leas would have been U4b before the experiment was set up. The experiment was established in 1896 in a *c.* 2 ha grassland field and was arranged as 14 parallelogram strips each *c.* 120 x 15 m (Figure 2.1). There was also a guard strip which stretched along the southern edge of the site, parallel to the road. The establishment of the experiment predated the use of replicated experimental designs by several decades (Fisher, 1935), and so at Palace Leas the fertiliser treatments were not replicated or randomised. The 14 fertiliser treatment plots consisted of five applied with varying amounts of cattle FYM, some of which also received N, phosphorus (P) and potassium (K), eight plots that received mineral fertiliser treatments comprising of all combinations of N, P and K fertilisers and an unfertilised control (Table 2.1). FYM was applied in February while mineral fertilisers were applied in late March or early April. In terms of total fertiliser addition, plots treated with FYM generally received higher rates of nutrient application than those treated with mineral fertiliser (Table 2.1). The experiment has remained under constant management since 1896 with the exception of plot 14 ($_{H}$NPK), which was added in 1976 and received a higher rate of N, P and K (Table 2.1), typical of modern fertiliser management. Among the treatment plots that were applied with mineral fertiliser only, those that received N did so in the form of ammonium sulphate ($(NH_4)_2SO_4$), with the exception of $_{H}$NPK which received ammonium nitrate (NH_4NO_3). Where both FYM

and mineral fertiliser was applied, mineral fertiliser N was a 50:50 split of ammonium sulphate and sodium nitrate ($NaNO_3$). The form of K applied throughout the experiment was muriate of potash (KCl). The only change to the existing treatments throughout the history of the experiment was the form of P applied, which was modified in 1976 from basic slag [$(CaO)_5\ P_2O_5\ SiO_2$] to triple superphosphate ($Ca(H_2PO_4)_2{\cdot}H_2O$) as the phosphate content in slag was becoming too variable. An annual hay cut was taken in July since the start of the experiment in order to determine hay yield in each treatment plot. Cattle or sheep were allowed to freely graze the post-cut aftermath growth in late summer and again briefly in winter which allowed for the potential transfer of nutrients via dung across the site. Permission for sampling was granted by the School of Agriculture, Food and Rural Development at Newcastle University. No endangered or protected species were involved in the study.

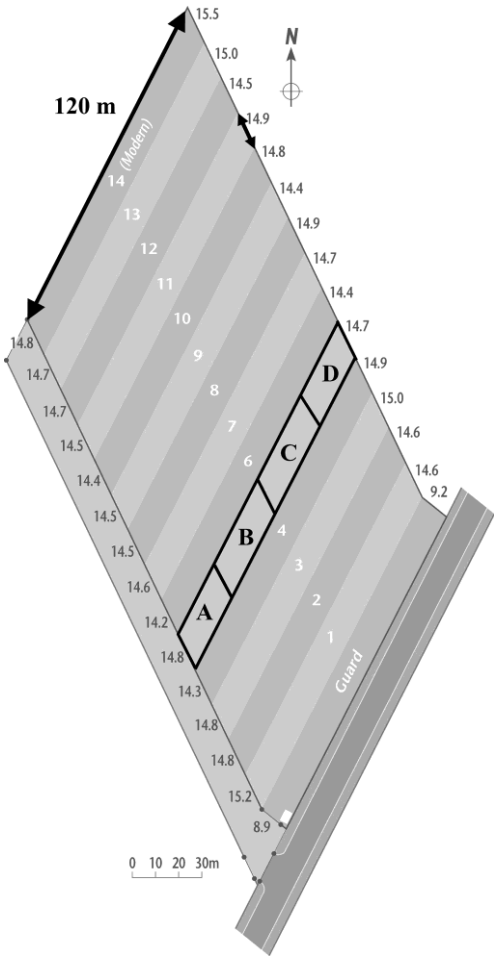

Figure 2.1 Palace Leas Hay Meadow Experiment layout. Illustration in plot 5 of how the treatment plots were divided into the four blocks; A-D. The length (northern edge) and individual treatment plots widths (eastern and western edge) are labelled in the diagram.

Plot number	Treatment	Details	Total nutrient addition (kg ha^{-1} y^{-1})			
			C	N	P_2O_5	K_2O
1	FYM + NPK	20 t ha^{-1} farm-yard manure, 17 kg N ha^{-1}, 30 kg P ha^{-1} and 34 kg K ha^{-1} of inorganic fertiliser applied annually	800	137	94	194
2	FYM	20 t ha^{-1} farm-yard manure applied annually	800	120	64	160
3	FYM + NPK	20 t ha^{-1} farm-yard manure applied in year 1 and 17 kg N ha^{-1}, 30 kg P ha^{-1} and 34 kg K ha^{-1} of inorganic fertiliser applied in year 2	400	69	47	97
4	FYM	20 t ha^{-1} farm-yard manure applied every other year	400	60	32	80
5	FYM + NPK	40 t ha^{-1} farm-yard manure applied in year 1 and 17 kg N ha^{-1}, 30 kg P ha^{-1} and 34 kg K ha^{-1} of inorganic fertiliser applied in years 2, 3 and 4	400	73	55	106
6	Control	No fertiliser applied	0	0	0	0
7	N	35 kg N ha^{-1} of inorganic fertiliser applied annually	0	35	0	0
8	P	60 kg P ha^{-1} of inorganic fertiliser applied annually	0	0	60	0
9	K	67 kg K ha^{-1} of inorganic fertiliser applied annually	0	0	0	67
10	NP	35 kg N ha^{-1} and 60 kg P ha^{-1} of inorganic fertiliser applied annually	0	35	60	0
11	NK	35 kg N $ha^{-1}$and 67 kg K ha^{-1} of inorganic fertiliser applied annually	0	35	0	67
12	PK	60 kg P ha^{-1} and 67 kg K ha^{-1} of inorganic fertiliser applied annually	0	0	60	67
13	NPK	35 kg N ha^{-1}, 60 kg P ha^{-1} and 67 kg K ha^{-1} of inorganic fertiliser applied annually	0	35	60	67
14	NPK	100 kg N ha^{-1}, 66 kg P ha^{-1} and 100 kg K ha^{-1} of inorganic fertiliser applied annually	0	100	66	100

Table 2.1 Details of the fertiliser treatments at Palace Leas Hay Meadow Experiment. Total carbon and nutrient addition was calculated as the average over a 4 year period and included nutrient content in the FYM and inorganic fertiliser. Total nutrient content in the cattle FYM was estimated using the DEFRA Fertiliser Manual (RB209) (Defra, 2010), typical totals for each nutrient = 6 kg N t^{-1}, 3.2 kg P_2O_5 t^{-1} and 8 kg K_2O t^{-1} (fresh weight). The approximate C content of FYM added to plots was estimated assuming FYM is c.40% C when dry (Hopkins et al., 2009).

2.3.2 *Soil sampling*

Soil samples were collected in September 2013 from all 14 plots at four replicate positions across each plot; 15 m (A), 45 m (B), 75 m (C) and 105 m (D) (Figure 2.1). Five cores (5 cm depth, 5 cm diam.) were collected within 30 cm of each replicate sampling point at 0-5 cm and 5-10 cm depth using a manual borer. Soil cores taken from the same depth and sampling point were bulked and homogenised. In plots 7 and 11 the presence of a 6 cm deep organic surface layer (Shiel and Rimmer, 1984), which was absent in other plots, meant that cores were also taken from this O horizon and at 0-5 cm and 5-10 cm from the mineral soil below.

2.3.3 *Soil properties*

Soil pH was determined using a 1:2.5 soil-water suspension. Soil moisture content and bulk density were determined after oven drying 10 g (2.8 mm sieved) of moist soil at 105˚C. Total soil C and N concentrations were determined on a 0.1 g (dry weight equivalent; DW) sample via dry combustion using a Vario Macro Cube (Elementar, Hanau, Germany) and converted to kg C and N per m^2 by multiplying the C and N concentrations by the bulk density and the thickness of the soil layer.

A modified wet sieving method was used to measure C content in the soil particle-size fractions (De Deyn *et al.*, 2011). In brief, 10 g (DW) of soil was mixed with 50 ml of deionised water and agitated for 16 h with glass beads and put through a series of sieves (2.8 mm, 200 µm and 50 µm meshes). Soil samples were sieved for 30 min and a 0.45 µm membrane filter was used to isolate the very fine fraction from dissolved organic C. All fractions were dried at 40˚C until constant weight was obtained. The 2.8 mm-200 µm (coarse carbon fraction) and 200-50 µm (fine carbon fraction) fractions were weighed while the weight of the 50–0.45 µm (very fine carbon fraction) was obtained from the sum of the coarse carbon and fine carbon fractions subtracted from the initial soil mass (10 g). Soil fractions were then ground in a ball mill. The C concentration of each fraction was determined on a 0.1 g dried sample via dry combustion using a Vario Macro Cube and transformed into stocks (kg C per m^2) using the same calculation as used for total C and N stocks.

Available P was extracted using the Olsen-P method (Watanabe and Olsen, 1965) and analysed using molybdate-blue colour determination (Murphy and Riley, 1962). Exchangeable K was extracted using 1 mol L^{-1} ammonium nitrate solution and determined using flame photometry (MAFF, 1986). Exchangeable Al was determined via a 1 mol L^{-1} KCl

extraction, a titration using 0.01 mol L^{-1} NaOH and a back titration using 0.01 mol L^{-1} HCl (Rowell, 1994).

2.3.4 *Plant analysis*

The annual hay cut was taken in early July; a 10 m^2 sample was obtained from the middle of each block (A-D) of each treatment plot, at the same position as where the soil samples were collected. From this a fresh sub sample was taken, dried at 80°C for 24 h and reweighed to determine yield. Hay yield data (recorded from 1896–2015) is stored in the Palace Leas archive, a data repository containing all recorded data from the experiment. Long-term mean hay yield for each of the 14 fertiliser treatment plots was determined using archived data from 1976-2015. For the years 1982, 1985 and 1997 replicate block hay yield values were not recorded, only plot level mean yields were available. An 80 g (DW) sample from the 2015 hay cut from each replicate sampling point was milled to a fine homogenised powder and a 0.1 g subsample was analysed for C and N concentrations using the aforementioned Vario Macro Cube.

On 5th June 2015 a full botanical survey was undertaken at the site. In each treatment plot, quadrats were laid out at four points which approximately corresponded to the same location at which soil samples were obtained (A, B, C and D; Figure 2.1). An additional quadrat was taken in the middle of the plot (between B and C) so that five quadrats were available to allow reliable classification of the vegetation according to the UK National Vegetation Classification (NVC). Percentage cover for all vascular plants and bryophytes was recorded for a 1 m^2 quadrat at each point. Species present at <1% cover were recorded as 0.1%.

2.3.5 *Data analysis*

Measured soil and plant parameters from the four sampling points of each plot were kept separate to give four replicates per plot. One-way ANOVA was then used to compare differences in the measured soil and plant parameters between treatment plots, rather than between replicated treatments, as would be typical in modern replicated and randomised field trials. The statistical approach used here is similar to that previously employed at Palace Leas by Shiel and Rimmer (1984), Hopkins and Shiel (1996) and Hopkins *et al.* (2011). As trends were consistent between the two depths for soil parameters and for reasons of brevity, we present data for the mean or sum of the two depths. Detailed results for the 0-5 cm and 5-10 cm depths can be found in Appendix A. Significant differences between treatment plots were tested using Tukey's post-hoc comparisons of means at significance level $P<0.05$. Before

ANOVA was performed, data was checked for normality and equal variance, data was log-transformed where necessary (Olsen-P and exchangeable K). Where the criteria for ANOVA could not be met by transformation (exchangeable Al, legume cover and bryophyte cover), Kruskal-Wallis tests followed by Dunn's test for multiple comparisons with Bonferroni corrections were performed. O horizon results from plots 7 and 11 were omitted from ANOVA and regression analysis. Pearson correlation coefficient was used to explore relationships between soil and vegetation properties. ANOVA, Kruskal-Wallis tests and Pearson correlation coefficients were computed using R version 3.2.1 (R Core Team, 2015). Regression analysis was performed using SigmaPlot 12.5 (Systat Software, San Jose, CA).

Principal components analysis (PCA) was performed using Canoco version 4.5 and Canodraw (ter Braak and Smilauer, 2002) to identify the major patterns among the plant species. Treatment plot numbers and environmental data, including selected soil and plant parameters, were overlain on the ordination biplot to help explain trends in plant species abundance. Plant species richness was measured as the total number of vascular and non-vascular plant species present in each quadrat. Plant and bryophyte species were grouped into four functional groups; grasses (including sedges and rushes), legumes, forbs and bryophytes. One-way ANOVA was used to assess fertiliser treatment plot effects on the cover of the functional groups and plant species richness.

For the NVC, five quadrats from each treatment plot of the botanical survey were combined for analysis. Doing this accounted for the patchiness of the vegetation due to ridge and furrow originating from historic cultivation at the site. The fit of each plot to defined sub-communities in the NVC was then calculated using TableFit version 2 (Hill, 2015), and published descriptions (Rodwell, 1998).

2.4 Results

2.4.1 *Soil pH*

Soil pH ranged from 3.04 to 5.86 in the soil at 0-10 cm and in the O horizon. Soil pH differed significantly between fertiliser treatment plots; plots applied with FYM (10 and 20 t ha^{-1}) had significantly higher pH (range 4.40-5.86) than those receiving mineral fertiliser (range 3.04-5.09), with the exception of the plots treated with P (plot 8) and PK (plot 12) (Figure 2.2 a). Soil pH decreased with depth in plots where FYM was applied and in the control (plot 6) but increased with depth in plots applied with mineral fertiliser only, with the exception of PK

(Appendix A), thus indicating acidifying effects of inorganic fertilisers and neutralising effects of FYM on topsoil.

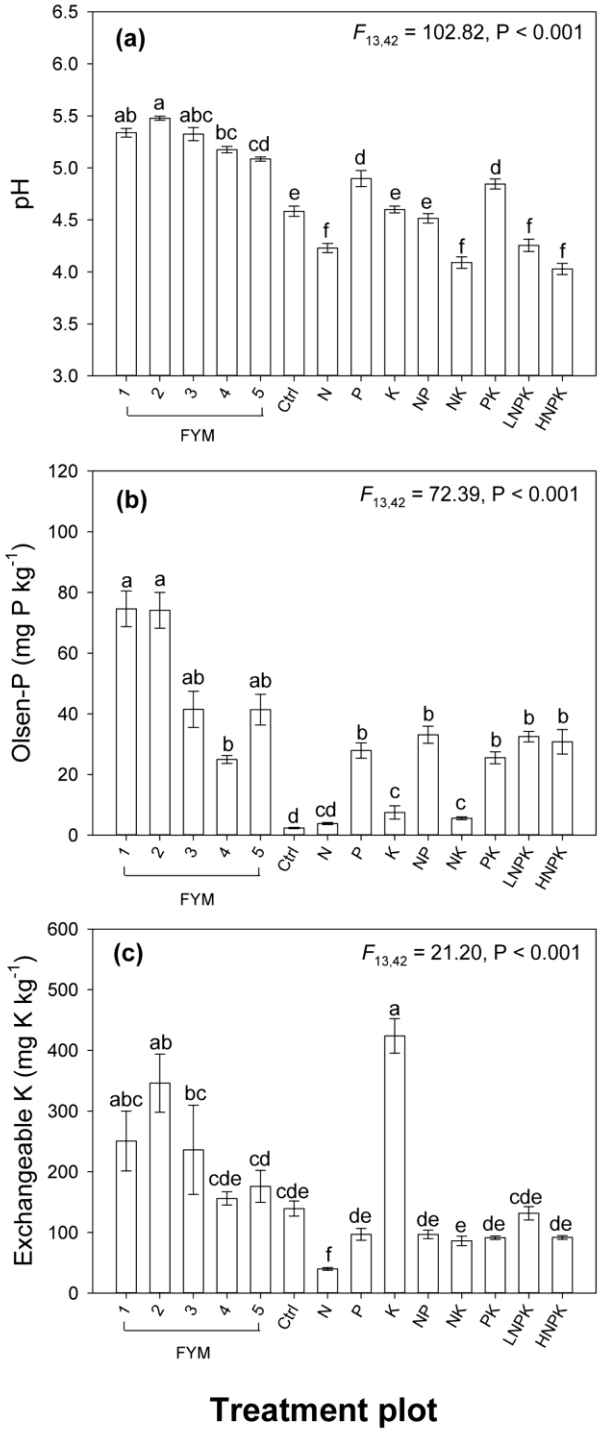

Figure 2.2 Effect of fertiliser treatment plot on (a) soil pH, (b) Olsen-P and (c) exchangeable K. Error bars represent ±1 standard error of the treatment plot mean and contrasting letters denote significant differences between treatment plots. Treatment plot mean is a cumulative 0-10 cm mean.

Plot number	Treatment[a]	Depth (cm)	Total nitrogen content (kg N m^{-2})[b]	Total SOC (kg C m^{-2})[b]	Soil C/N [c]	Coarse fraction carbon stocks (kg C m^{-2})[b]	Fine fraction carbon stocks (kg C m^{-2})[b]	Very fine fraction carbon stocks (kg C m^{-2})[b]	Exchangeable Al (mmol kg^{-1})[c]
1	FYM + NPK	0-10	0.51 (0.03) a	5.88 (0.09) a	11.49 (0.34) d	0.25 (0.03) b	2.39 (0.31) a	2.74 (0.23) abc	0.13 (0.13) c
2	FYM	0-10	0.49 (0.02) ab	5.58 (0.13) ab	12.02 (0.80) cd	0.30 (0.03) b	2.34 (0.31) a	3.17 (0.28) a	0.00 (0.00) c
3	FYM + NPK	0-10	0.42 (0.02) bcd	5.16 (0.18) abc	12.61 (0.53) bcd	0.26 (0.05) b	1.48 (0.27) ab	2.75 (0.12) abc	0.93 (0.71) bc
4	FYM	0-10	0.43 (0.01) abc	5.03 (0.23) bc	12.96 (0.47) abcd	0.27 (0.06) b	1.62 (0.14) ab	2.88 (0.05) ab	0.81 (0.47) bc
5	FYM + NPK	0-10	0.44 (0.01) ab	5.20 (0.05) abc	11.92 (0.32) cd	0.28 (0.05) b	2.14 (0.24) a	2.81 (0.12) ab	0.31 (0.12) bc
6	Control	0-10	0.35 (0.03) cde	4.42 (0.19) cde	13.37 (0.27) abcd	0.23 (0.02) b	1.69 (0.34) ab	2.26 (0.10) bc	8.42 (1.34) abc
7	N	0-10	0.26 (0.01) ef	4.01 (0.24) de	15.21 (0.49) ab	0.29 (0.07) b	0.97 (0.08) b	2.69 (0.21) abc	40.90 (1.34) a
8	P	0-10	0.34 (0.02) de	4.09 (0.16) de	12.77 (0.49) abcd	0.30 (0.11) b	1.63 (0.14) ab	2.47 (0.13) abc	3.86 (0.42) abc
9	K	0-10	0.33 (0.01) def	4.85 (0.10) bcd	14.74 (0.12) abc	0.41 (0.08) ab	1.90 (0.06) ab	2.26 (0.19) bc	14.74 (1.47) abc
10	NP	0-10	0.33 (0.01) def	4.44 (0.10) cde	14.30 (0.82) abcd	0.35 (0.13) b	1.53 (0.14) ab	2.47 (0.07) abc	15.29 (0.71) abc
11	NK	0-10	0.24 (0.01) f	4.00 (0.22) e	15.60 (0.31) a	0.30 (0.07) b	0.98 (0.08) b	2.56 (0.15) abc	36.82 (2.43) a
12	PK	0-10	0.33 (0.02) def	4.59 (0.18) cde	14.64 (0.77) abc	0.30 (0.12) b	1.42 (0.24) ab	2.36 (0.03) bc	4.86 (0.96) abc
13	NPK	0-10	0.31 (0.01) ef	4.18 (0.23) de	14.28 (0.66) abcd	0.35 (0.06) b	1.57 (0.06) ab	2.01 (0.17) c	17.25 (1.83) abc
14	NPK	0-10	0.35 (0.02) cde	4.61 (0.10) cde	14.60 (0.90) abc	0.76 (0.11) a	1.99 (0.18) ab	2.17 (0.11) bc	31.39 (2.09) ab
$F_{13,42}$			19.38		5.43	2.79	4.40	4.38	n/a
H			n/a		n/a	n/a	n/a	n/a	52.47
P			***		***	**	***	***	***

Table 2.2 Total nitrogen content, total soil organic carbon, soil carbon to nitrogen ratio (C/N), coarse fraction carbon stocks, fine fraction carbon stocks, very fine fraction carbon stocks and exchangeable Al for the Palace Leas plots. [a] see Table 2.1 for fertiliser treatment details. [b] values are cumulative means ± 1 s.e (0-5 cm + 5-10 cm), [c] values are means ± 1 s.e (0-5 cm + 5-10 cm / 2). *P* (significance) at $P<0.001$;***, $P<0.01$;**, $P<0.05$;*. Contrasting letters denote significant differences between treatment plots. n/a indicates not applicable.

2.4.2 *Olsen-phosphorus*

Olsen-P was highest in plots applied with FYM, with the exception of the plot where FYM was applied every other year (plot 4), which contained the lowest Olsen-P of the plots receiving P (FYM or mineral fertiliser) (Figure 2.2 b). Olsen-P was significantly higher in plots where FYM was applied at a higher rate (plot 1 and 2; 20 t FYM ha^{-1}) than plots receiving mineral fertiliser only or the control; while it was significantly lower where no P had been applied as FYM or mineral fertiliser. At 0-5 cm in the plot treated with $_{H}$NPK (plot 14), Olsen-P was comparable (49.09 mg P kg^{-1}) to that of soils in plots receiving FYM (range 28.04-116.03 mg P kg^{-1}), however, it decreased 4 fold at 5-10 cm; such decreases with depth were less pronounced in plots receiving FYM (Appendix A).

2.4.3 *Exchangeable potassium*

The treatment plots receiving K only (plot 9) and only FYM but at a high rate (plot 2), had significantly more exchangeable K than all other plots with the exception of the plot treated with FYM at a higher rate with NPK (plot 1) and where FYM was applied in alternate years with NPK (plot 3) (Figure 2.2 c). Plots that received only N (plot 7), had significantly lower exchangeable K than all other treatment plots (Figure 2.2 c). Exchangeable K decreased considerably with depth in the $_{H}$NPK treatment plot at 5-10 cm, only the N treated plot (32.26 kg K ha^{-1}) had lower exchangeable K (Appendix A).

2.4.4 *Exchangeable aluminium*

In plots receiving N only and NK, exchangeable Al was significantly higher than in all plots treated with FYM (range 0-4.49 mmol kg^{-1}; Table 2.2). Soil receiving the higher rate of FYM only had the lowest exchangeable Al and was the only treatment plot in which exchangeable Al was absent at both 0-5 and 5-10 cm depth (Appendix A).

2.4.5 *Soil organic carbon stocks*

Regression analysis revealed that at 0-5 cm there was a quadratic relationship between SOC stocks and pH (R^2=0.43, P<0.001), SOC stocks increased below pH 4 and above pH 5 (Figure 2.3). SOC stocks were highest at low pH in the O horizon of the N and NK treated plots, but high SOC stocks at low pH were also found at 0-5 cm in the $_{H}$NPK. There was also a signficant positive relationship between the rate of FYM applied and SOC stocks at 0-5 cm (R^2 = 0.52, P<0.001), most likely due to direct C application. At the lower 5-10 cm depth there was a significant positive relationship between pH and SOC stocks (R^2=0.45, P<0.001),

which was linear rather than quadratic (Figure 2.4). There was also a significant positive relationship between the rate of FYM application and SOC stocks at 5-10 cm (R^2=0.43, P<0.001). There was a decrease in SOC stocks with depth in all plots except in the PK (+2%) (Appendix A). Between 0-5 cm and 5-10 cm in the mineral soil of the most acidic plots (N, NK and $_H$NPK) the reduction in SOC (mean = 24%) was greater than the reduction observed in other plots (mean = 12%). The reduction in SOC between the O horizon and 0-5 cm in the N only and NK was considerably greater, 36% and 37% respectively (Appendix A).

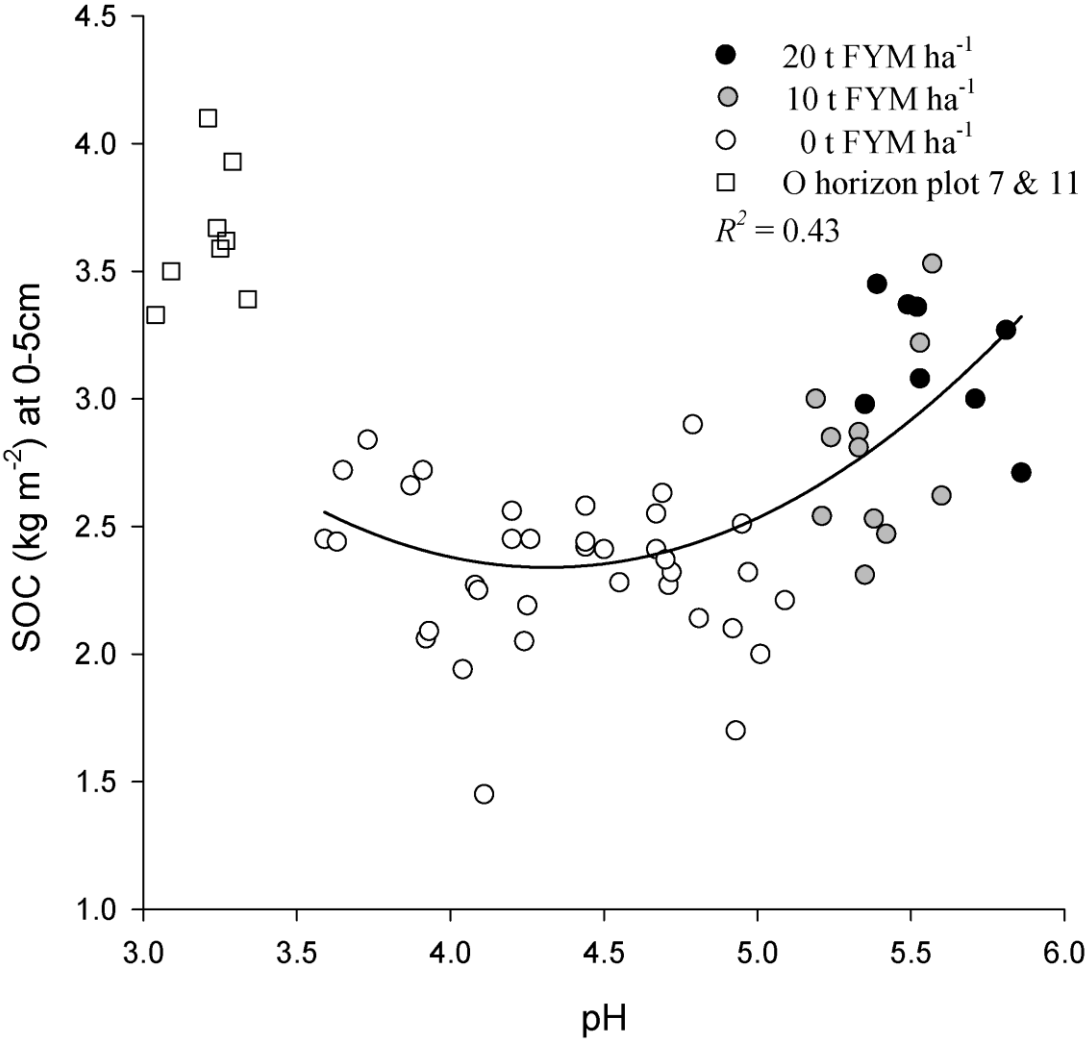

Figure 2.3 Soil organic carbon stocks in relation to pH at 0-5 cm. SOC stocks in the O horizon are presented but not included in the regression. 0 t FYM ha^{-1} (circles with no fill) represents data from plots receiving only mineral fertiliser and the control.

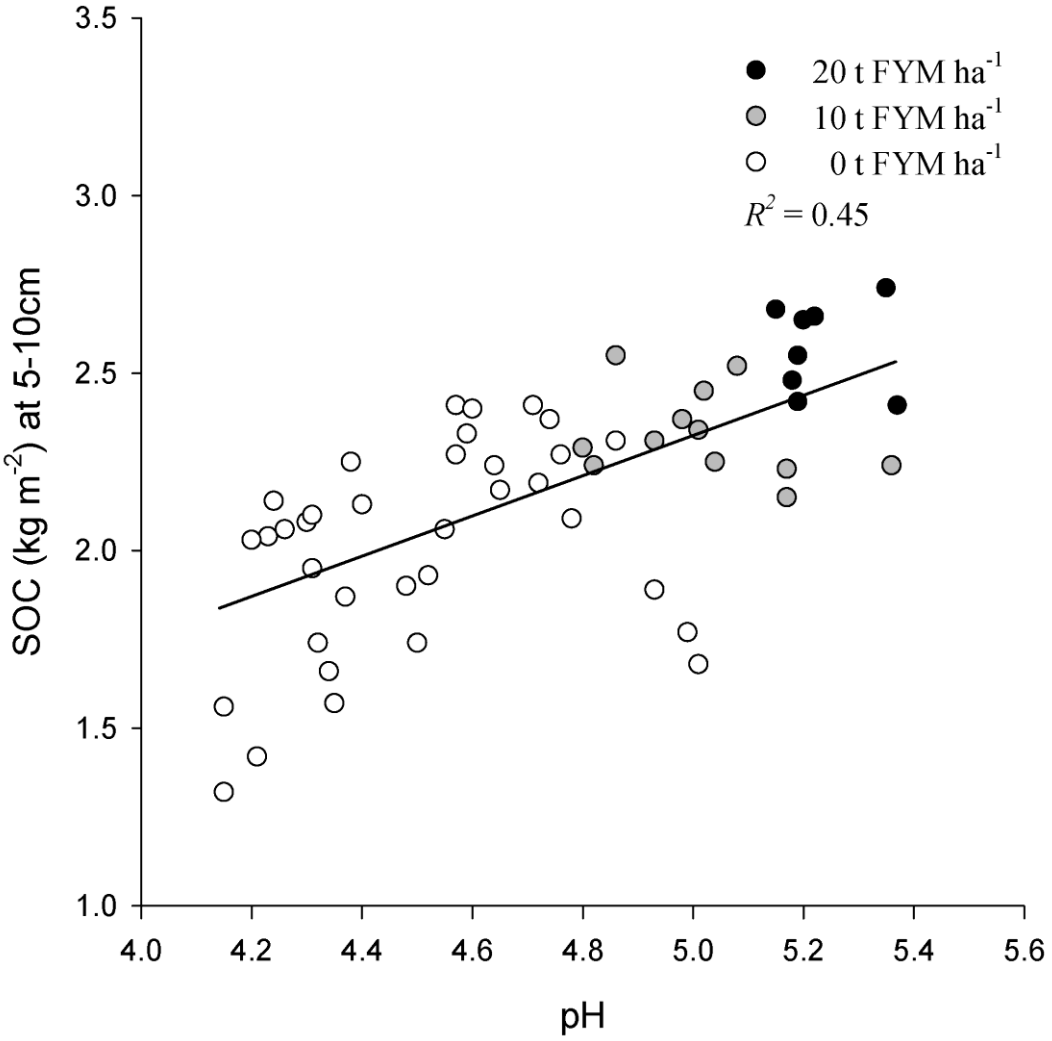

Figure 2.4 Soil organic carbon stocks in relation to pH at 5-10 cm. 0 t FYM ha^{-1} (circles with no fill) represents data from plots receiving only mineral fertiliser and the control.

2.4.6 *Total nitrogen stocks and soil C/N*

Total nitrogen stocks differed strongly between treatment plots (Table 2.2) and followed a very similar pattern to SOC as they were highly positively correlated (r=0.82, P<0.001). Soils with higher rates of FYM application contained significantly more total N than soils with mineral fertiliser applied and the control. The NK treated plot had significantly lower total N than all plots treated with FYM (mean range 0.42-0.51 kg N m^{-2}), the control, P only and the $_{H}$NPK plots (Table 2.2). In $_{H}$NPK, 70% of the total N was found in the top 5 cm; similar to the pattern observed in the N and NK, in which N was considerably higher in the O horizon

(Appendix A). The NK treated plot also had significantly higher soil C/N than those plots receiving FYM, except the plot that received FYM every other year (plot 4) (Table 2.2).

2.4.7 *Coarse, fine and very fine soil carbon fractions*

In total, 97% (±1.51) of SOC was recovered in particle-size fractionation. The very fine carbon fraction (50-0.45 µm) contained 52% of the total C stock with 39% and 9% in the fine carbon fraction (200-50 µm) and coarse carbon fractions (2 mm-200 µm), respectively (Table 2.2). The $_{H}$NPK plot had significantly higher coarse C fraction stocks than all other plots, with the exception of the K treated plot. Similarly, in the other two very acid plots (N only and NK) coarse fraction C stocks were high in the O horizon (Appendix A). Plots treated with the higher rate of FYM and one receiving FYM in the first year followed by three years of NPK (plot 5) had significantly higher fine carbon fraction stocks than plots applied with N and NK (Table 2.2). Soil that received the higher rate of FYM only, contained significantly more very fine carbon than the control, K only, PK, low rate NPK (plot 13, $_{L}$NPK) and $_{H}$NPK treatment plots (Table 2.2). Very fine carbon fraction stocks were on average 20% greater in plots receiving FYM (mean range 2.74-3.17 kg C m^{-2}) than those applied with only mineral fertiliser or the control plot (mean range 2.01-2.69 kg C m^{-2}).

2.4.8 *Hay yield and hay C/N*

Hay yield (1976-2015) and hay C/N ratio differed significantly between fertiliser treatment plots (Figure 2.5 a; Table 2.3). Hay yield was highest in plots applied with FYM and with the exception of $_{H}$NPK (6.69 t ha^{-1}) was significantly higher (range 5.71-8.46 t ha^{-1}) than where only mineral fertiliser was applied (range 2.43-7.22 t ha^{-1}) or in the control plot (3.17 t ha^{-1}). Hay yield was significantly higher in the plot treated with FYM at a higher rate with NPK (plot 1) than all other plots with the exception of the plot that received the high rate of FYM only (plot 2). The K only treated plot had the lowest hay yield, producing less than half of the biomass grown by plots receiving FYM or the $_{H}$NPK (Figure 2.5 a). Despite notable long-term differences in hay yield between plots applied with FYM and those receiving only mineral fertiliser, at the beginning of the experiment there was only relatively small differences between the treatment plots (Figure 2.6). Hay C/N was significantly lower in the $_{H}$NPK than in the NP (plot 10), P only, control, FYM every other year and PK treatment plots (Table 2.3).

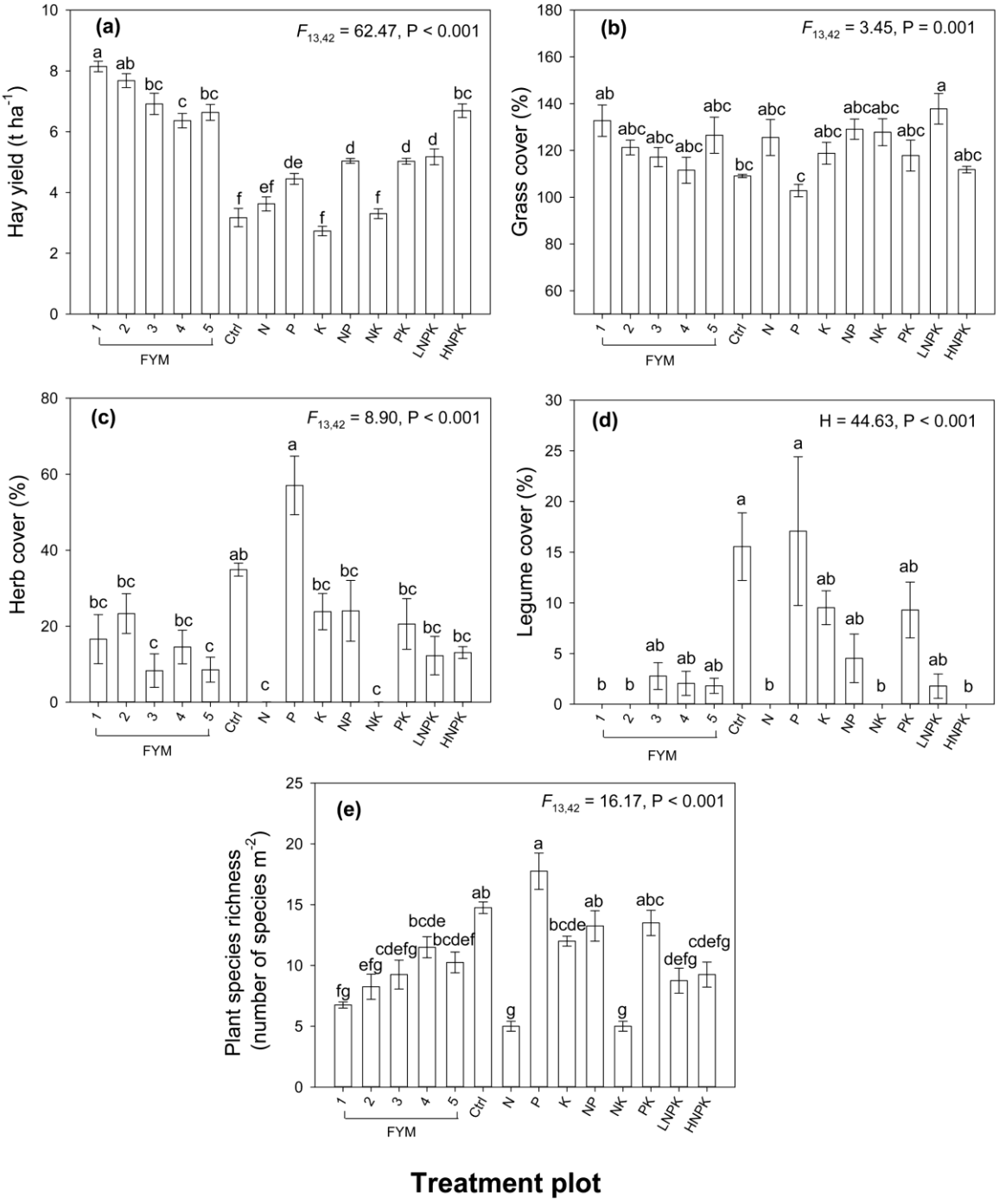

Figure 2.5 Effect of fertiliser treatment plot on (a) hay yield, (b) grass cover, (c) herb cover, (d) legume cover and (e) plant species richness. Error bars represent ±1 standard error of the treatment plot mean and contrasting letters denote significant differences between treatment plots.

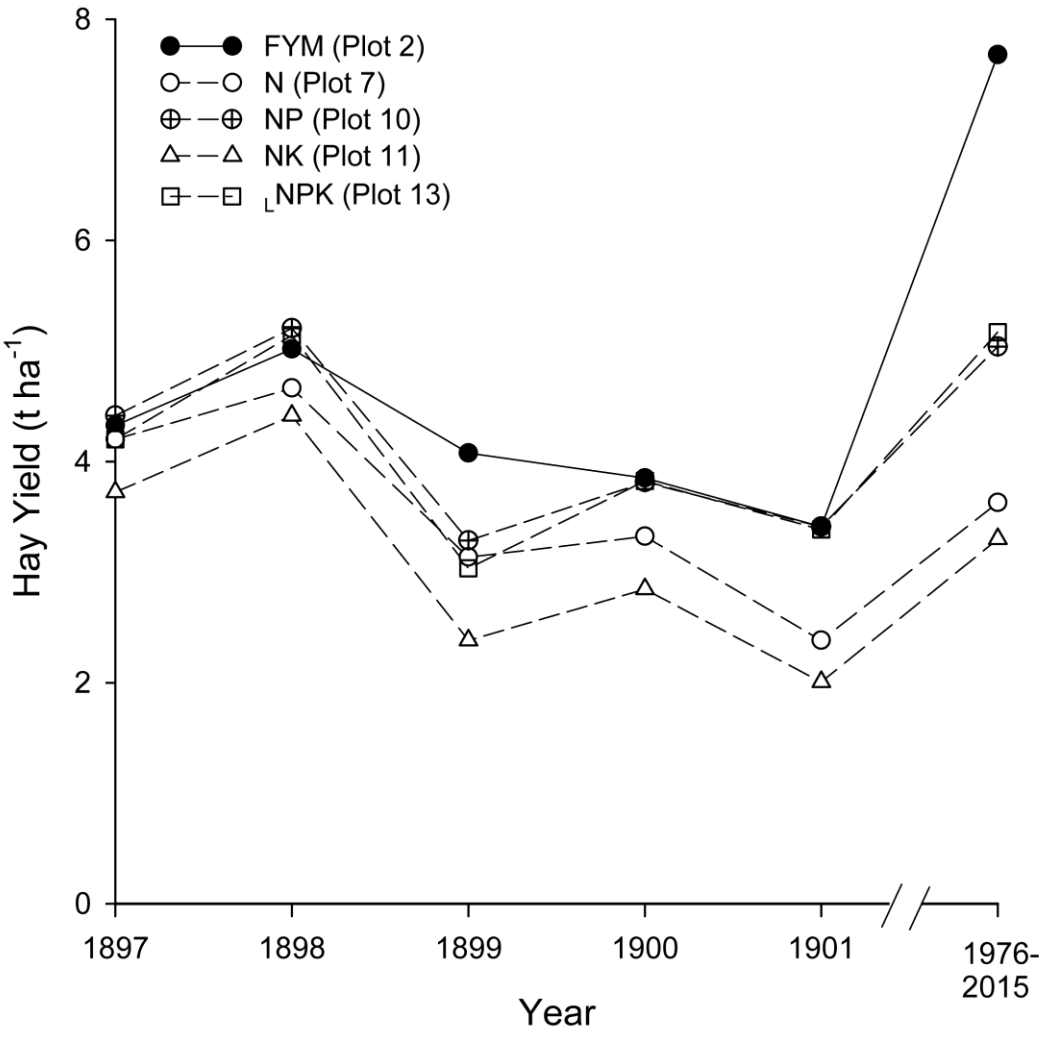

Figure 2.6 Changes in mean hay yield at Palace Leas for the FYM (20 t ha^{-1}) and four ammonium sulphate-containing fertiliser treatments for the first 5 years of the experiment (1897-1901) and the more recent long-term average (1976-2015).

Plot number	Treatment[a]	Hay C/N	Bryophyte cover (%)	NVC
1	FYM + NPK	34.71 (2.09) ab	0.00 (0.00)	MG7d
2	FYM	35.24 (1.62) ab	0.00 (0.00)	MG7d
3	FYM + NPK	34.60 (1.18) ab	0.00 (0.00)	MG7d
4	FYM	36.06 (2.13) a	0.00 (0.00)	MG7d
5	FYM + NPK	34.51 (1.58) ab	0.00 (0.00)	MG7d
6	Control	36.64 (2.14) a	0.03 (0.02)	MG5a
7	N	33.39 (1.47) ab	0.00 (0.00)	U4b
8	P	36.96 (1.50) a	0.00 (0.00)	MG5a
9	K	35.65 (1.14) a	0.25 (0.25)	U4b
10	NP	36.96 (0.64) a	0.03 (0.03)	U4b
11	NK	33.20 (2.22) ab	0.00 (0.00)	U4b
12	PK	35.78 (2.01) a	0.00 (0.00)	U4b
13	NPK	35.15 (2.56) ab	0.00 (0.00)	U4b
14	NPK	26.68 (0.53) b	0.00 (0.00)	U4b
$F_{13,42}$		2.23	n/a	n/a
H		n/a	11.42	n/a
P		*	NS	n/a

Table 2.3 Hay C/N ratio, bryophyte cover and National Vegetation Classification (NVC) in each fertiliser treatment plot. [a] see Table 2.1 for fertiliser treatment details. Values are means ± 1 s.e. *P* (significance) at *P*<0.001;***, *P*<0.01;**, *P*<0.05;*, *P*>0.05; NS. n/a indicates not applicable. Contrasting letters denote significant differences between treatment plots.

2.4.9 *National Vegetation Classification*

Fertiliser treatment plots were matched to one of two broad NVC categories; mesotrophic grasslands (MG) or calcifugous grassland communities (U). Plots where FYM was applied, the control and P only treatment plots were classified as mesotrophic grasslands, but their closest fit was to two different NVC communities. Those plots receiving FYM were closest to MG7d (*Lolium perenne - Alopecurus pratensis* grassland), which is typical of hay meadows on fertile, moist soils. In contrast, the control and the P only treatment plots were closest to MG5a (*Cynosurus cristatus - Centaurea nigra* grassland, *Lathyrus pratensis* sub-community), which is typical of traditionally managed grazed hay meadows (Table 2.3). Where plots were applied with mineral fertilisers, with the exception of the P only plot, the plant community most closely resembled the type initially found at the site U4b (*Festuca ovina-Agrostis capillaris-Galium saxatile* grassland, *Holcus lanatus-Trifolium repens* sub-community), which is typical of relatively fertile but base-poor grasslands (Table 2.3).

2.4.10 *Plant species cover and richness*

Across the site 37 plant species were recorded, 14 grasses, 14 herbs, 7 legumes and 2 bryophytes; this included 7 species which were each only recorded in one plot. *Holcus lanatus* and *Rumex acetosa* were present in all 14 plots, while *Anthoxanthum odoratum* was recorded in 13 (Table 2.4).

The two dominant axes of the PCA for the percentage cover of the plant species explained 76.1% of the total variance. PC1 accounted for 60.5% of the variation in the data and was strongly positively associated with the application of FYM (r = 0.91). The axis PC2 accounted for 15.6% of the variation in the data and was negatively correlated with the application of N (*r*=-0.55) and K fertiliser (*r*=-0.32) and positively correlated with P fertiliser application (*r*=0.30; Figure 2.7). The plant community composition was distinctly different where FYM was applied. The abundance of the grasses *Alopecurus pratensis, Bromus hordeaceus, Holcus lanatus* and *Poa trivialis* was positively associated with the application of FYM (Figure 2.7), while the percentage cover of herbs and legumes decreased, with the exception of the tall herb *Anthriscus sylvestris* (Figure 2.5 c and d).

Where FYM was not applied and hay yield and soil pH was lower; there was a high abundance of several less vigorous grass species; *Anthoxanthum odoratum, Agrostis capillaris* and *Festuca rubra* (Figure 2.7). The acidic and low yielding mineral N fertiliser plots (N and NK) had very low plant species richness (5 species per m^2) (Figure 2.5 e) and herb cover (0.03 %), and legumes and bryophytes were absent (Figure 2.5 c and d; Table 2.3). In plots where neither N nor FYM was applied (6; control, 8; P, 9; K, 12; PK), species richness was higher (range 11-21 species per m^{-2}), as was herb (range 12.0-74.1 %) and legume cover (range 4.0-39.0 %) (Figure 2.5 c-e). The control and P only plots had the highest plant species richness (range 14-21 species per m^2), herb cover (range 30.0-74.1 %), legume cover (range 8.1-39.0 %) and the lowest cover of grass species (range 95.1-110.1 %) (Figure 2.5 b-e). In these plots the cover of herb species *Plantago lanceolata* and *Rhinanthus minor* and legumes including *Trifolium pratense* was high (Table 2.4). Bryophytes were only present in the control, K and NP treatment plots (Table 2.3).

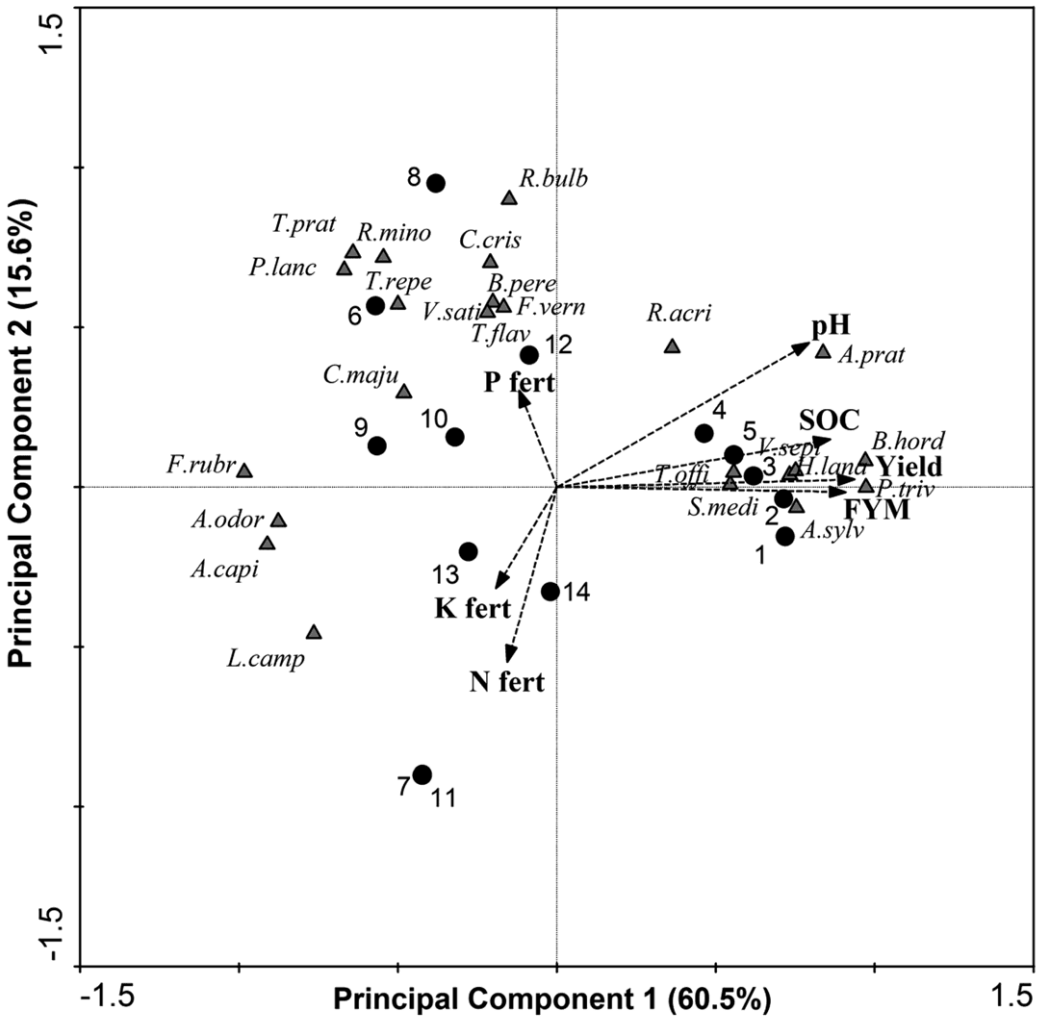

Figure 2.7 Principal components analysis (PCA) illustrating the variation in the plant species (Δ) dataset at Palace Leas. Environmental variables including soil and plant variables (dotted arrow) and treatment plot number (●) are overlain. *A. capi = Agrostis capillaris, A. prat = Alopecurus pratensis, A. odor = Anthoxanthum odoratum, A. sylv = Anthriscus sylvestris, , B. pere = Bellis perennis, B. hord = Bromus hordeaceus, C. maju = Conopodium majus, C. cris = Cynosurus cristatus, F. rubr = Festuca rubra, F. vern = Ficaria verna, , H. lana = Holcus lanatus, L. camp = Luzula campestris, P. triv = Poa trivialis, R. acri = Ranunculus acris, R.bulb = Ranunculus bulbosus, R. mino = Rhinanthus minor, S. medi = Stellaria media, T. offi = Taraxacum officinale, T. prat = Trifolium pratense, , T. repe = Trifolium repens, T. flav = Trisetum flavescens, V. sati = Vicia sativa, V. sepi = Vicia sepium.*

Species	Functional group	Cover (%)													
		Plot number	1	2	3	4	5	6	7	8	9	10	11	12	13	14
		Treatment[a]	FYM +NPK	FYM	FYM +NPK	FYM	FYM +NPK	Ctrl	N	P	K	NP	NK	PK	NPK	NPK
Agrostis capillaris	Grass		0	0	0.1	0	0	13	20	11	13	24	34	17	24	24
Alopecurus pratensis	Grass		51	44	58	44	51	1	0	21	0	8	0	25	11	5
Anthoxanthum odoratum	Grass		0	1	2	13	6	24	64	33	27	38	62	41	27	34
Bromus hordeaceus	Grass		30	28	30	23	33	0.1	0	0	0	1	0	8	2	3
Cynosurus cristatus	Grass		0	0	0.1	0.1	1	1	0	11	0.1	0	0	1	0	0
Dactylis glomerata	Grass		0	0	0	3	7	0.1	0	4	0	1	0	0	2	7
Festuca rubra	Grass		0	0	0	1	0.1	72	38	28	65	39	19	12	46	6
Holcus lanatus	Grass		33	26	25	18	23	2	9	8	7	14	2	27	18	36
Lolium perenne	Grass		0.1	0	1	3	1	0.1	0	3	0.1	1	0	0.1	0	4
Luzula campestris	Grass		0	0	0	0	0	2	23	2	6	3	14	0.1	12	0
Poa trivialis	Grass		26	32	13	9	9	0	0	0.1	0	1	0	0.1	0	2
Trisetum flavescens	Grass		0	0	0	0	0	0	0	0.1	0	0	0	0	0	0
Anthriscus sylvestris	Herb		11	13	1	0.1	1	0	0	0	0	0	0	0	0	0
Bellis perennis	Herb		0	0	0	0	0	0	0	1	0	0.1	0	1	0	0
Cerastium fontanum	Herb		0	0	0.1	2	2	0.1	0	0.1	0	0	0	0	0	0.1
Conopodium majus	Herb		0	0	0	0	0	3	0	0	2	0	0	0	0	0.1
Ficaria verna	Herb		0	0	0	0.1	0	0	0	1	0	0	0	0	0	0
Plantago lanceolata	Herb		0	0	0	0	0	24	0	17	16	10	0	3	1	0
Ranunculus acris	Herb		0	1	2	4	1	0.1	0	1	0.1	0.1	0	5	2	0.1
Ranunculus bulbosus	Herb		0.1	2	2	3	2	8	0	7	3	5	0	4	1	0.1
Rhinanthus minor	Herb		0	0	0	0	0	19	0	19	3	1	0	1	0	0
Rumex acetosa	Herb		0.1	2	2	4	4	2	0.1	3	3	8	0.1	8	8	14
Stellaria media	Herb		0.1	2	0	0	1	0	0	0	0	0	0	0	0	0
Taraxacum officinale	Herb		2	2	0	0	0	0	0	0.1	0	0	0	0.1	0	0
Lathyrus pratensis	Legume		0	0	0.1	0.1	0.1	0	0	0.1	0	0	0	2	0	0
Trifolium pratense	Legume		0	0	0	0.1	0	12	0	17	10	3	0	7	2	0
Trifolium repens	Legume		0	0	0	0	0	3	0	1	0.1	0.1	0	0.1	0.1	0
Vicia sativa	Legume		0	0	0	0.1	0	0	0	2	0	0	0	0	0	0
Vicia sepium	Legume		0	4	2	1	1	0	0	0	0	0	0	0	0	0

Table 2.4 Mean species cover of the most common plant species present in each fertiliser treatment plot. Species with cover <0.5% presented as 0.1%**.** [a] see Table 2.1 for fertiliser treatment details.

2.5 Discussion

2.5.1 *Effects of long-term fertilisation on soil pH and nutrient availability*

Our results clearly show that very long-term fertilisation (120 years) led to marked differences in plant and soil properties between treatment plots in this grassland ecosystem. As hypothesised, many of these differences appear to result from the effects of long-term fertilisation on both soil nutrient content and pH, which differed considerably between the plots. In general, in plots where FYM was applied organic N, Olsen-P, exchangeable K and soil pH was higher than in plots applied with mineral fertiliser, resulting in increased hay yield. Similarly to the very long-term Park Grass experiment, levels of Olsen-P and exchangeable K were low in soils applied with only N or the control treatment plots. Olsen-P in these treatment plots at both sites was approximately 3-4 mg P kg^{-1}, while on average levels of exchangeable K were higher in these treatment plots at Palace Leas (90 mg K kg^{-1}) than Park Grass (60 mg K kg^{-1}) (Silvertown *et al.*, 2006), potentially due to the inherent high illite content of the soil at Palace Leas (Hopkins *et al.*, 2009).

It is thought that the increase in pH in soils receiving FYM was due to the presence of Ca^{2+} and Mg^{+} in the manure and/or the oxidation of organic anions during manure decomposition, which go on to consume H^{+} ions (Haynes and Mokolobate, 2001). Until now, there has been very little evidence of an effect of FYM on soil pH in long-term field trials. This may be due to the fact that many experiments regularly apply lime to counteract acidification (e.g. Broadbalk and Hoosfield Barley Experiments (Rothamsted Research, 2016)), meaning the liming effect of FYM goes undetected.

Among the mineral fertiliser treatment plots, soils receiving P but no N maintained a higher pH than in plots where N fertiliser was applied, most likely due to the residual liming properties of the basic slag (O'Donnell *et al.*, 2001; Jenkins *et al.*, 2009). In contrast, plots receiving mineral N fertiliser had very acidic soils, no doubt due to acidification associated with the long-term application of ammonium-containing fertiliser (Guo *et al.*, 2010). The negative effects of long-term N fertilisation on soil pH have also been established at Park Grass where ammonium sulphate addition caused a *c.* 2 unit reduction in soil pH (0-23 cm) in the most acidic plots (Crawley *et al.*, 2005). The levels of acidity in the N only and NK treated plots at Palace Leas and their equivalent at Park Grass (plot 1d and 18d) were very similar (Palace Leas 0-10 cm range; pH 4.1-4.2, Park Grass 0-23 cm range; pH 3.9-4.0). After 100 years of ammonium sulphate addition (in 1d and 18d) the soil pH at Park Grass has

reached a pH equilibrium (Warren and Johnston, 1964). Based on this we anticipate a similar situation in the N only and NK treated plots at Palace Leas, with a further dramatic decline in soil pH unlikely. While greater availability of P and K and higher pH amounted to higher hay yield in FYM applied soils in the long-term, differences in hay yield between plots treated with FYM and those treated with ammonium sulphate were far less pronounced at Palace Leas in the first 5 years following the establishment of the experiment (Figure 2.6). This was probably due to temporal differences in the drivers of fertilisation. In the short-term, plant growth response to fertilisation was likely to have been mediated by direct nutrient input, while repeated application of $(NH_4)_2SO_4$ progressively acidified the soil, resulting in a reduction in nutrient availability (Haynes and Mokolobate, 2001), and constraining plant growth in the long-term. Consistent with our hypothesis, this demonstrates that there is disparity between short- and long-term ecosystem responses to fertilisation and emphasises the importance of avoiding extrapolating long-term effects from observations made from short-term experiments.

2.5.2 *Effects of long-term fertilisation on SOC stocks and particle-size carbon fractions*

Long-term FYM addition was associated with an increase in total SOC stocks and greater very fine fraction C stocks, indicative of the mineral-associated C pool, which is typically more stable. We cannot be certain of the mechanism responsible for C accrual in these soils as FYM increases C input and pH simultaneously, making it difficult to evaluate their individual contribution. However, findings from a recent extensive landscape study suggests that increased pH does not result in greater SOC stocks within the observed range (Manning *et al.*, 2015a). While an increase in pH has previously been shown to positively influence SOC, including in the Park Grass experiment, results indicate that this is unlikely to occur at pH <6 (Fornara *et al.*, 2011; Manning *et al.*, 2015a). It appears more plausible that the positive effects of FYM addition on SOC stocks were due to increased C input via the direct addition of organic matter in FYM (Table 2.1). Furthermore, because FYM addition also increases plant productivity and hence plant litter residues entering the soil, this could also have increased soil C (Hopkins *et al.*, 2009). C inputs from aboveground litter and root-derived C have been shown to be a key mechanism in building SOC stocks (Tilman *et al.*, 2006; Fornara and Tilman, 2012).

An increase in SOC stocks following FYM addition as reported here is similar to the trend observed globally. A recent meta-analysis demonstrated that average SOC stock difference was significantly higher where manure was applied (+0.94 kg C ha^{-1}) compared to the

unfertilised control (Maillard and Angers, 2014). At Palace Leas, while SOC stock differences between plots treated with manure only (plot 2 and 4) and the control plot was positive, it was considerably lower (+0.44 kg C ha^{-1}) than the global average. Lower soil C accrual may be explained by a number of factors, including differences in soil texture, climatic conditions or land use and management. However, Maillard and Angers (2014) established that with the exception of climate, which would be expected to favour soil C accumulation at Palace Leas due to the cool temperate climate retarding SOC decomposition, the effects of manure addition on SOC stocks were independent of these explanatory factors. Alternatively, and congruent with our hypothesis the discrepancy may have arisen as a consequence of differences in experiment length. SOC stocks at Palace Leas are at or close to equilibrium (Hopkins *et al.,* 2009) due to over a century of continuous management, which may not be the case in shorter term studies included in the meta-analysis (average 18 years) and which may have included studies recovering from tillage. In short-term experiments, C stocks in both manured and control treatments are likely to fluctuate, hence a transient SOC stock difference may be observed. Once stocks have stabilised, the difference between SOC stocks in the FYM applied and the control treatments may be closer to the figure reported at Palace Leas.

The accumulation of SOC at the soil surface where pH was <4 was strongly associated with increased acidity (Figure 2.3). Results indicate that much of this C was stored in the coarse and fine carbon fractions, rather than being protected against microbial decomposition as mineral-associated carbon. Given that pH is a key determinant of microbial activity (Chen *et al.*, 2015b), intense acidification is likely to have reduced rates of litter and SOC decomposition (Janssens *et al.*, 2010) and encouraged acid tolerant but slower growing plants with slowly decomposing tissues, both of which would instigate the build-up of organic matter at the soil surface. In the $_{H}$NPK treated plot high hay yields and therefore litter inputs may have operated additively to acidification effects to increase C stocks at 0-5 cm.

Our findings are broadly consistent with the long-term Park Grass experiment, where organic matter decomposition also appeared to be retarded by N-induced acidification, similarly resulting in an increase in organic matter at the soil's surface (Hopkins *et al.*, 2009). In contrast, other long-term experiments (37 years (Kemmitt *et al.*, 2006), >100 years (Pietri and Brookes, 2008)) have found no effect of acidification on SOC stocks. It may be that as these studies sampled deeper soil (0-10 cm and 0-23 cm, respectively) this potentially diluted the trend found here; higher SOC content in the top few centimetres of the profile. It is clear from results presented here that soil properties differed greatly between depths, even within the same treatment plot. Sampling deeper in the soil profile and treating soil as a homogenous

sample is becoming more common and therefore the question of depth differences has not been addressed in other long-term experiments. Sampling the soil at finer depth resolution (e.g. in 5 cm layers) may unearth fertilisation effects that otherwise could potentially be missed. This is likely to be particularly relevant in grasslands where tillage tends to be less frequent and in acid soils where there is greater vertical stratification (Conyers *et al.*, 1996). To our knowledge there is no evidence of the magnitude of the acidification effect on SOC observed at Palace Leas and Park Grass, broadly equivalent to the early stages of podzol formation, in short-term experiments. However, it is recognised that it can take decades or centuries for SOC to reach an equilibrium following management change (Johnston *et al.*, 2009), which highlights the importance of using long-term experiments to identify the otherwise hidden long-term responses of SOC.

2.5.3 *Effects of long-term fertilisation on plant properties*

In line with our hypothesis long-term fertilisation at Palace Leas resulted in significant variation in botanical composition between the treatment plots, and this is likely to have been caused by the effects of both direct nutrient input and soil pH changes. Both the higher soil nutrient status and also less acidic soil conditions in the plots receiving FYM resulted in enhanced hay yield and shifted the community towards dominance by tall grass species; *Alopecurus pratensis, Bromus hordeaceus* and *Holcus lanatus* which are likely to have outcompeted herb and legume species for light (Hautier *et al.*, 2009).

In contrast, plots that received mineral fertilisers (apart from the P only) have shifted to support communities typical of semi-improved acidic grassland. With the exception of the $_{H}$NPK treated plot these were low yielding and dominated by short, acid tolerant species including *Anthoxanthum odoratum, Agrostis capillaris, Festuca rubra and Luzula campestris*. Among these communities, the N only and NK treated plots had very low species richness, while legumes and bryophytes were absent. Like the Park Grass and Steinach Grassland experiments, where species richness was lowest in plots receiving $(NH_4)_2SO_4$, it is probable that the cause of the extreme decline in species richness at Palace Leas was also primarily due to soil acidification (Crawley *et al.*, 2005; Hejcman *et al.*, 2014), which has been shown previously to reduce the availability of base cations, including K^+ and Mg^{2+} (Marschner and Marschner, 2012), and P availability (Haynes, 1982) and increase toxic metal concentrations.

Despite intense acidification in the plot receiving $_{H}$NPK, this plot produced high hay yield and a sward including potentially tall but acid tolerant grass species e.g. *Holcus lanatus*. Unlike

the other very acidic treatment plots, low species richness here was likely to be the combined result of both light exclusion and soil acidification (Silvertown, 1980). These responses of species abundances to long-term fertilisation are consistent with reports from the Park Grass and Steinach Grassland experiments where in intensely acidic soils very few species (e.g. *Anthoxanthum odoratum* and *Holcus lanatus*) could endure the extreme acidic conditions and toxic concentrations of Al (Crawley *et al.*, 2005).

Unfertilised and P only plots contained plant community types typical of traditionally managed and unfertilised meadows. These were also low yielding plots with a low number of tall grass species but a high abundance of shorter growing herbs such as *Plantago lanceolata* and legumes including *Trifolium pratense.* These plots also contained the keystone hemiparasite *Rhinanthus minor,* which is known to supress the dominance of tall grasses (Pywell *et al.*, 2004), boost plant species diversity and alter rates of nitrogen cycling of the communities it contains (Bardgett *et al.*, 2006). Results from Park Grass support these findings and show that where N was not applied, species richness and the cover of legumes was higher. However, in contrast to Park Grass, species richness was marginally higher in the P only plots at Palace Leas, rather than the control (Crawley *et al.*, 2005; Fornara *et al.*, 2013). This response was unlikely to be due to an increase in P availability as high species richness has often been associated with low levels of soil P (Venterink *et al.*, 2001). It seems more reasonable to suggest that this was due to pH differences as P only plots (pH 4.9) have a higher pH than the control (pH 4.6). Differences in soil pH may also explain why the total species richness in unfertilised control plots at Park Grass (plot 3d; pH 5.2, plot 12d; pH 5.1) were considerably greater (3d; n = 36 species, 12d; n = 42 species) (Crawley *et al.*, 2005) than that observed at Palace Leas (n = 22 species).

The distinct patterns in the plant community dynamics displayed here and at other long-term fertilised sites are unlikely to be accurately predicted in short-term studies (Silvertown *et al.*, 2006). For example, in lowland semi-improved and unimproved grasslands in Wales, which received 24 t FYM ha^{-1} y^{-1} for 7 years, plant species density ((mean response in the fertiliser treatment/ mean response in the unfertilised control)(Gough *et al.*, 2000)) was reduced by 10% and 21%, respectively (Kirkham *et al.*, 2008). Long-term FYM application at approximately the same rate at Palace Leas (plot 2; 20 t FYM ha^{-1} y^{-1}) caused plant species density to be reduced by 43%, a considerably greater loss of plant species compared to this short-term study. Similarly, in a meta-analysis using data compiled from relatively short-term studies (4-15 years), plant species density was reduced on average by 28% in response to N fertilisation (Gough *et al.*, 2000), whereas at Palace Leas in the plot applied with only N

fertiliser ($(NH_4)_2SO_4$) there was a 66% reduction in species density. These findings indicate that while the direction of short- and long-term fertilisation effects may concur, the magnitude of the effects are markedly different, particularly where long-term ammonium sulphate addition caused soil acidification. While the magnitude of the effect of fertilisation may increase with experimental length meaning the pattern observed at Palace Leas may have been more accurate of the long-term response, we acknowledge that the variation in the magnitude of the effect between studies of different duration may also be a result of other factors, such as; differences in experimental design, community-specific mechanisms (Gough *et al.*, 2000) and the species pool able to colonise the treatment plot (Tilman, 1993).

Despite the fact that species loss and compositional change has been reported in nutrient addition experiments of varying duration (Crawley *et al.*, 2005; Suding *et al.*, 2005), the occurrence of a dramatic change in the vegetation community and species loss as observed here and at Park Grass (Silvertown *et al.*, 2010) was potentially a consequence of very long-term fertilisation. A dramatic change in plant community composition, also known as a regime shift, occurs once a critical environmental threshold has been surpassed causing a shift in the community to an alternative stable state. However, the duration of short-term studies may not be sufficient to detect such phenomena, hence without the use of long-term experiments these events are likely to be missed (Schroder *et al.*, 2005; Silvertown *et al.*, 2010).

2.5.4 *Implications of the findings*

While global agricultural productivity is heavily dependent on the use of fertilisers (Erisman *et al.*, 2008) our results demonstrate that their long-term addition can strongly affect several other ecosystem services. In terms of agricultural production, our results suggest that the addition of ammonium sulphate over long periods of time can cause severe acidification and significantly constrain crop productivity compared to where FYM is applied. An increase in soil acidification is likely to reduce rates of nitrification, solubility of P and base cation availability (Haynes and Mokolobate, 2001; Stevens *et al.*, 2011; Chen *et al.*, 2015a), indicating that crop productivity is unlikely to be maintained by mineral fertiliser addition in the long-term and will not be economically viable for the farmer. Our results also suggest that the long-term addition of N-containing fertilisers (both FYM and mineral fertiliser) will strongly reduce plant diversity, such effects may also reduce the diversity of a range of vertebrate and invertebrate taxa not measured here (Manning *et al.*, 2015b). Attempts to remediate these effects could involve ceasing fertiliser applications and in areas of intense

acidity the addition of lime to increase soil pH. Indeed, a recent study at Park Grass reported that by liming and the cessation of nutrient addition, over time grasslands have the capacity to successfully reverse the negative effects of long-term fertilisation on plant species diversity (Storkey *et al.*, 2015). However, the extent of remediation may be compromised where long-term ammonium sulphate addition causes intense acidity as grassland recovery is considerably slower (Storkey *et al.*, 2015). In light of this we suggest that farmers should maximise nutrient use from FYM to reduce detrimental implications to ecosystem services, lower investment in mineral fertilisers and so that soil C gains may be realised. While the use of ammonium sulphate is now less common than in the 20th Century, we suggest even in situations similar to Palace Leas, where N application rate was relatively low, that soil pH is monitored closely from the onset of fertilisation and periodic liming is undertaken to correct a pH decline and avert the impacts of acidification, rather than after the fact, as grassland recovery is likely to be slow.

While findings from long-term experiments clearly still have practical and biological implications for grassland management, the majority of such experiments were established at a time when management methods and the intentions of their creators were very different to the approaches used and the questions posed by the scientific community today. There is a now a need for new long-term nutrient addition experiments to be established, which take into consideration current and expected trends in fertiliser management and existing medium-term experiments to be maintained. Currently, the majority of the world's longest continuously managed trials are situated in the USA and across Europe. However, projections suggest that the greatest increases in fertilisation will occur in China, India and other developing countries (Alexandratos and Bruinsma, 2012) where the impacts of fertilisation are relatively unknown. Therefore, a strategy must be developed to establish an economically secure network of long-term experiments (Silvertown *et al.*, 2010) across a range of environmental and climatic gradients, and particular in areas where plant diversity is high (Phoenix *et al.*, 2006), the provision of other ecosystem service are important and fertiliser use is predicted to be most intense.

2.6 Conclusions

Long-term fertiliser addition has significantly impacted upon a range of soil and plant properties in this grassland ecosystem, dramatically altering soil function and plant community composition. Our results indicate that the addition of FYM has multiple benefits over inorganic fertiliser for improving agricultural production in acid soils, including

increased hay yield, higher SOC and mineral-associated C stocks, greater availability of nutrients and higher soil pH. We were unable to fully disentangle impacts on plant or soil properties resulting from the form and amount of fertiliser applied given the original design of the experiment. From a conservation perspective the application of N-containing fertiliser, whether it be manure or inorganic fertiliser, caused species richness declines and increased grass dominance. Our results also underline the importance of sampling the soil profile with appropriate resolution with depth, in particular in acid grassland soils which are susceptible to vertical stratification. By using this approach, we were able to establish that there were similar patterns in SOC stocks at low pH as those reported at Park Grass, which in other long-term studies may have been missed. As it often requires decades for ecosystem properties to stabilise in response to fertilisation, short-term experiments may only detect transient or intermediate effects of fertilisation upon ecosystem properties. This, coupled with strong consistencies in patterns of soil and plant response between long-term fertiliser studies firmly emphasises the importance of utilising long-term experiments to better understand long-term ecosystem responses. However, approaches such as those used here remain somewhat phenomenological, making our understanding of the relative importance of the pathways and mechanisms that drive ecosystem responses to fertilisation poor. Thus, more attention is required to investigate the relative importance of direct nutrient, pH and plant community composition mediated pathways on grassland ecosystem properties in the future.

Chapter 3: Changes to soil pH and plant traits regulate the impacts of very long-term fertilisation on grassland carbon and soil function

Jonathan Kidd, Elizabeth Stockdale, Janet Simkin, Simon Peacock, Peter Manning. To be submitted to *Functional Ecology*.

Contribution statement: Jonathan Kidd and Peter Manning conceived the study. Janet Simkin, Simon Peacock and Jonathan Kidd performed the botanical survey. Plant trait data was obtained from the TRY database. Soil sampling, laboratory analysis and analysis of the soil and plant data was carried out by Jonathan Kidd. Jonathan Kidd wrote the paper, all authors contributed to revisions.

3.1 Abstract

Fertilisation of the world's terrestrial ecosystems is causing major alterations to a number of key ecosystem properties and services. Grasslands are one of the world's most extensive ecosystems, and as large, unfertilised grassland areas are projected to regularly receive fertiliser in the future, this may influence the ecosystem services they provide, including carbon (C) storage. Whilst many studies report the impacts of fertilisation on grassland C and soil function, a wide range of mechanisms may underlie these changes and attempts to assess the importance of the pathways through which fertilisation effects are mediated (direct vs. indirect) are uncommon. Using a 120-year-old fertilisation experiment, we investigated the relative importance of three classes of fertiliser mechanism on measures of grassland C and soil function by applying a structural equation modelling framework. These were; (1) direct nutrient addition impacts; (2) soil pH decline- and (3) changes to plant community diversity and functional composition. We found that for several measures of grassland C and soil function, including microbial utilisation of multiple carbon substrates and C stored in particle-size fractions, indirect pH-mediated effects were as great, or greater, than the direct effects of fertilisation. Where indirect plant-mediated effects were detected, they were relatively weak and better explained by shifts in plant functional composition (the community abundance weighted mean (CWM) of leaf N and SLA) than biodiversity change. Changes in soil pH, coupled with a weak effect of plant functional composition changes, (CWM leaf N) explained the microbial response (biomass, C use efficiency and catabolic capacity) to fertilisation, rather than direct nutrient effects. Our results demonstrate that long-term fertilisation alters grassland soil function and C stocks via both direct and indirect pathways, and that indirect pathways, particularly those mediated by soil pH, can dominate ecosystem response.

Understanding the relative importance of these pathways provides a more mechanistic understanding of ecosystem responses to fertilisation that may help tailor the management of fertilisation impacts and improve predictions of long-term responses of ecosystems in an increasingly fertilised world.

3.2 Introduction

Total nitrogen (N) and phosphorus (P) inputs to terrestrial ecosystems are projected to increase by ~40% and ~58%, respectively between 2000 and 2050 (Bouwman *et al.*, 2013). Such changes will cause a cascade of effects on the world's terrestrial ecosystems, including significant alterations to key ecosystem properties and services (Vitousek *et al.*, 1997; Power, 2010; Manning, 2012). Much of this fertilisation will occur in grasslands, which are one of the world's most extensive ecosystem types, covering 20 to 40% of the global terrestrial land area (FAO, 2008). Short-term aboveground responses of plants to fertilisation in grassland ecosystems are reasonably well established (Elser *et al.*, 2007; Lee *et al.*, 2010; Fay *et al.*, 2015) and whilst we understand that fertilisation affects many belowground processes including the acceleration or retardation of soil organic matter decomposition (Neff *et al.*, 2002; Manning *et al.*, 2008), long-term responses to fertilisation and the mechanisms that underpin this response are relatively unknown. It may be that over time indirect effects of fertilisation, i.e. via soil acidification and plant community change, become more prominent in driving ecosystem response than direct effects of fertilisation, i.e. those driven by increased nutrient availability and the physiological response of the biota to this. It is also likely that the cumulative effects of very long-term fertilisation differ to the patterns reported in short-term studies (Silvertown *et al.*, 2010). Addressing this knowledge gap is important as grasslands contribute ~23% of the global terrestrial ecosystem C stock (Trumper *et al.*, 2009) and annual world fertiliser consumption is expected to increase ~30% by 2050 (Alexandratos and Bruinsma, 2012), potentially leading to widespread changes in ecosystem service provision as vast areas of unfertilised land become regularly fertilised.

Much of our understanding of how fertilisation influences grassland C and soil function is based on reviews and meta-analyses of findings assembled from short-term and somewhat phenomenological nutrient addition experiments (Treseder, 2008; Lee *et al.*, 2010; Koerschens *et al.*, 2013; Geisseler and Scow, 2014) and as a result we lack understanding of the mechanisms responsible. Exploring the relative contribution of these pathways is of growing importance as a lack of a mechanistic understanding prevents the formulation of reliable models that accurately predict future changes (Goll *et al.*, 2012) and limits our

capacity to effectively manage and mitigate the impacts of fertilisation, e.g. via the addition of carbon (C) (Eschen *et al.*, 2007) or lime (Fornara *et al.*, 2011) or the diversification of plant communities (Lange *et al.*, 2015).

Here we distinguish between three major pathways through which fertilisation effects upon soil processes may be mediated. The first we term direct effects and includes the direct enhancement of nutrient availability to plants and microbes, leading to an increase in aboveground productivity (Elser *et al.*, 2007; LeBauer and Treseder, 2008). In contrast, belowground biomass production has been shown, on average, to decrease or remain relatively unchanged with fertilisation (Janssens *et al.*, 2010; Lee *et al.*, 2010). Furthermore, increases in the quantity and quality (reduced C:N) of plant derived residues (Xia and Wan, 2008) may alter the supply of C and N to the soil biota (Liu and Greaver, 2010). This in turn can alter microbial activity, stimulating the decomposition of C in fresh residues and reducing turnover of the slower-cycling fractions (Craine *et al.*, 2007). This occurs because of the lower necessity for 'microbial nutrient mining' of recalcitrant soil organic matter under high N supply. Accordingly, N fertilisation is thought to shift microbial enzyme production from those needed to metabolise recalcitrant C to those that aid degradation of labile C, thus facilitating the decomposition of C typically stored in sensitive SOM fractions with shorter turnover times (Knorr *et al.*, 2005; Craine *et al.*, 2007).

Our second class of mechanism is all those factors associated with the effects of fertilisation upon soil acidity. Increased nitrification rates and plant uptake of NH_4^+ following N input releases H^+ ions, which in turn leads to increased aluminium solubility (Al^{3+}), the leaching of base cations and a reduction in soil phosphorus availability (Haynes and Mokolobate, 2001). High concentrations of Al^{3+} and low soil nutrient status may reduce primary productivity (Silvertown *et al.*, 2006), potentially limiting the availability of C to the microbial biomass (Kemmitt *et al.*, 2006). These changes, coupled with the negative effects of Al^{3+} toxicity on the microbes can reduce the size and activity of the microbial biomass (Kemmitt *et al.*, 2006; Pietri and Brookes, 2008), and subsequently influencing the biochemical composition of soil C and its rate of turnover (Fornara *et al.*, 2011; Manning *et al.*, 2015a).

A third pathway of influence is via plant community change. Two competing, but non-exclusive hypotheses have been put forward to explain the effects of plant community composition on ecosystem processes: (1) the biomass ratio hypothesis (Grime, 1998), and (2) the diversity hypothesis (Tilman, 1997). According to the biomass ratio hypothesis; the functional traits of the dominant species in a community drive ecosystem processes.

Fertilisation tends to shift plant communities towards dominance by fast-growing species with exploitative growth strategies and the capacity to rapidly acquire C, nutrients and water (De Vries *et al.*, 2012b; Reich, 2014). Such strategies are typically characterised by higher specific leaf area (SLA), leaf nitrogen content (leaf N) and lower leaf dry matter content (LDMC) (Reich, 2014). An increase in 'fast' plant traits have previously been associated with increased litter decomposition rates (Cornwell *et al.*, 2008), reduced fungi:bacteria ratio (Orwin *et al.*, 2010; De Vries *et al.*, 2012b) and a reduction in soil C concentrations (Garnier *et al.*, 2004). In contrast, the diversity hypothesis postulates that ecosystem functioning is higher under greater species diversity, because diverse communities exploit resources more efficiently and/or are more likely to contain high functioning species (Hooper *et al.*, 2005). Fertilisation is known to reduce plant biodiversity via light exclusion (Hautier *et al.*, 2009) and increased levels of soil acidity (Silvertown *et al.*, 2006; Stevens *et al.*, 2010). Consistent with the diversity hypothesis, high species richness in experimental communities has been shown to boost primary productivity (Tilman *et al.*, 2001), promote soil microbial biomass activity and abundance (Zak *et al.*, 2003; Eisenhauer *et al.*, 2010; Thakur *et al.*, 2015) and increase soil C stocks (Fornara and Tilman, 2008; Lange *et al.*, 2015). A fertilisation induced reduction in plant species diversity may therefore have the opposite effect. A number of previous studies have provided support for both plant-mediated mechanisms, whilst some have also compared the explanatory power of the two (Mokany *et al.*, 2008; Schumacher and Roscher, 2009; Allan *et al.*, 2015). However, little has been done to compare the capacity of these hypothetical mechanisms to explain fertiliser-induced changes to ecosystem function, and to investigate their relative importance compared to the aforementioned abiotic pathways.

Together, research has demonstrated that the mechanisms that mediate the effects of fertilisation are complex, concurrent and hard to separate. As a result, attempts to disentangle these effects and estimate their relative importance are uncommon. Those that have, have either used artificially assembled communities (Manning *et al.*, 2006; Isbell *et al.*, 2013a), which may not accurately represent the natural environment, or relatively recently established field experiments (Chen *et al.*, 2015a; Chen *et al.*, 2015b), where transient responses to fertilisation, rather than long-term outcomes, may be detected (Silvertown *et al.*, 2006).

In this study we addressed these knowledge gaps by using a very long-term fertilisation experiment to investigate the relative importance of direct nutrient, soil pH-, and plant community-mediated (biomass ratio vs. diversity) effects of fertilisation upon grassland C and soil function. This was achieved by applying a structural equation modelling (SEM) framework, which allows for the separation of direct effects of fertilisation from indirect

pathways, to one of the world's oldest fertilisation experiments, the Palace Leas plots (est. 1896) (Shiel and Rimmer, 1984; Hopkins *et al.*, 2011).

3.3 Materials and Methods

3.3.1 *Study site and experimental design*

Palace Leas Hay Meadow experiment is located at Cockle Park Farm (55˚13' N, 1˚41' W, UK National Grid Reference NZ 202912), ~30 km north of Newcastle upon Tyne, England. A detailed description of the site is provided in Chapter 2. The experiment was established in 1896, on a soil that is classified as pelo-stagnogley (Typic Ochraqualf) from the Hallsworth series (Hopkins *et al.*, 2009) and has a clay-loam texture (Jarvis *et al.*, 1977); 41% sand, 29% silt and 30% clay.

The experiment consists of 14 non-replicated (the experiment pre-dates modern replicated designs) parallelogram plots, each ~120 x 15 m. To each plot a different fertiliser treatment has been consistently applied, these consists of; five farmyard manure (FYM) treatments receiving varying amounts of FYM (10 and 20 t ha^{-1} y^{-1}) and some receiving N (0 or 17 kg ha^{-1} y^{-1}), P (0 or 30 kg ha^{-1} y^{-1}) and K (0 or 34 kg ha^{-1} y^{-1}) mineral fertilisers, eight mineral fertiliser treatments which include all combinations of N (0 or 35 kg ha^{-1} y^{-1}), P (0 or 60 kg ha^{-1} y^{-1}) and K (0 or 67 kg ha^{-1} y^{-1}), a treatment consisting of a higher rate of NPK (100-66-100 kg ha^{-1} y^{-1}) and an unfertilised control. The FYM is applied in February whilst mineral fertilisers are applied in late March or early April. An annual hay cut is taken in July and the field is freely grazed post-cut in late summer and autumn by cattle or sheep. In response to the treatments, ecosystem properties have diverged greatly between the experimental plots (Chapter 2; Shiel and Rimmer, 1984; Hopkins *et al*., 2011). For example, soil properties measured in the top 10 cm of the mineral soil varied as follows; pH (H_2O; 3.59-5.86), soil C (1.32-3.53 kg C m^{-2}), Olsen P (1.05-116.03 mg P kg^{-1}) and exchangeable K (13.63-607.06 mg K kg^{-1}) (see Chapter 2). The vegetation in the plots matches three grassland communities according to the National Vegetation Classification (Rodwell, 1998): 1) MG5a (*Cynosurus cristatus* - *Centaurea nigra* grassland, *Lathyrus pratensis* sub-community); containing short herbs and legumes such as *Plantago lanceolata* and *Trifolium pretense*, 2) MG7d (*Lolium perenne* - *Alopecurus pratensis* sub-community); dominated by tall grasses including *Alopecurus pratensis*, *Bromus hordeaceus* and *Holcus lanatus* and 3) U4b (*Festuca ovina-Agrostis capillaris-Galium saxatile* grassland, *Holcus lanatus-Trifolium repens* sub-

community); supporting short grasses including *Anthoxanthum odoratum* and *Agrostis capillaris* (see Chapter 2).

3.3.2 *Soil sampling and analysis*

In September 2013, each plot was sampled at four points; at 15 m, 45 m, 75 m and 105 m from the western edge and 7.5 m-in from the southern edge of the plots. At each sampling point five cores (5 cm depth, 5 cm diam.) were collected at 0-5 cm and 5-10 cm depth. Cores taken from the same sampling point and depth were bulked and homogenised. To determine soil bulk density, a separate 'intact' core of the same volume from each sampling point and depth was collected. The presence of an organic surface layer in plots 7 and 11, which has developed as a result of long-term acidification via ammonium sulphate addition (Shiel and Rimmer, 1984), meant that cores were also obtained from this layer (see Chapter 2). Soil collected from each sampling point was split into two halves, one half was sieved (2.8 mm) and air-dried at room temperature, whilst the other 'fresh' half was transferred to the laboratory within 2 h and stored in the dark at 4˚C.

The intact core soil was sieved (2.8 mm), then weighed. A 10 g subsample was oven-dried at 105˚C for 24 h and reweighed to determine bulk density. Soil pH was determined using 10 g of the air-dried soil in a 1:2.5 soil-water supernatant. As soil inorganic C at the site is negligible (Hopkins *et al.*, 2009), total soil C was considered equivalent to SOC. SOC was analysed via dry combustion on ground, air-dried soil (0.1 g) using a Vario Macro Cube (Elementar, Hanau, Germany) and converted to kg C per m^2 by multiplying the %C value by the dry weight of the sieved soil per m^2. The C stored in three soil particle-size fractions (2.8 mm-200 µm, 200-50 µm and 50–0.45 µm) was also measured according to a modified wet sieving method (De Deyn *et al.*, 2011). 10 g (DW) of soil was mixed with 50 ml of deionised water and agitated for 16 h with glass beads and put through a series of sieves (2.8 mm, 200 µm and 50 µm meshes). Soil samples were sieved for 30 min and a 0.45 µm membrane filter was used to isolate the very fine fraction from dissolved organic C. All fractions were dried at 40˚C until constant weight was obtained. The 2.8 mm-200 µm (coarse carbon fraction) and 200-50 µm (fine carbon fraction) fractions were weighed while the weight of the 50–0.45 µm (very fine carbon fraction) was obtained from the sum of the coarse carbon and fine carbon fractions subtracted from the initial soil mass (10 g). Soil fractions were then ground in a ball mill. The C concentration of each fraction was determined on a 0.1 g dried sample via dry combustion using a Vario Macro Cube and transformed into stocks (kg C per m^2) using the same calculation as for total SOC.

3.3.3 *Active microbial biomass, efficiency and catabolic capacity*

The MicroResp™ CO_2 detection system was used to test the potential of the soil microbial community to catabolise a range of ecologically relevant low molecular weight C and N substrates (Campbell *et al.*, 2003). Fresh soil was adjusted to 40% of water holding capacity (WHC) and pre-incubated for 5 days at 25°C to re-establish an active microbial biomass. We then supplied 0.3 g of soil to each well of a deep well plate containing seven C substrates (Campbell *et al.*, 1997). The substrates chosen were a mixture of amino acids (L-asparagine and L-glutamine), carbohydrates (D-glucose and D-mannose), carboxylic acids (oxalic acid and L-malic acid) and a phenolic acid (syringic acid). Substrates were applied in 25 µl aliquots to provide a concentration of 30 mg g^{-1} soil water (Campbell *et al.*, 2003). Due to low solubility in water, amino acids were applied at 7.5 mg g^{-1} soil water and syringic acid at 0.3 mg g^{-1} soil water.

CO_2 evolution from the individual wells was measured using a detection microplate containing an indicator dye (Campbell *et al.*, 2003). Plates were read at 570 nm on a microplate reader (SpectraMax Plus, Molecular Devices) and immediately inverted onto a seal covering the deep well plate. The whole system was then incubated in the dark at 25°C for 4h. Following incubation, the detection plate was detached and read again. A calibration curve for absorbance versus the headspace equilibrium CO_2 concentration was created by applying different concentrations of CO_2 gas to breakable CombiStrips (Thermo LifeSciences) held in 40ml sealed glass vials. Vials were incubated at 25°C for 4 h, reassembled in a plate carriage and re-read. The %CO_2 was calculated from the difference in absorbance at 0 h and 4 h for each well and expressed as µg CO_2-C g^{-1} h^{-1} according to Campbell *et al.* (2003). By applying glucose it was also possible to estimate active soil microbial biomass via the substrate-induced respiration method (Anderson and Domsch, 1978). To estimate microbial C use efficiency we used the inverse of the microbial metabolic quotient (qCO_2), as the ratio between basal respiration and active microbial biomass (Oren and Steinberger, 2008).

In order to summarise the overall catabolic capacity of the microbial community in a single measure, we used the threshold-based multifunctionality index proposed by Byrnes *et al.* (2014) and applied to catabolic response data by Wood *et al.* (2015). This was quantified as the number of C substrates where the rate of respiration was equal to or exceeded a threshold of the maximum rate response (Wood *et al.*, 2015). Maximum respiration rate was the mean of the seven highest measurements for each C substrate. Three thresholds (30%, 50% and

70%) were used in structural equation modelling (SEM) (see below), however, as results were very similar for all three, only results of the 50 % threshold are reported here. Results for 30 % and 70 % thresholds are presented in Appendix B. Threshold measures were calculated using the *multifunc* package (https://github.com/jebyrnes/multifunc).

3.3.4 *Vegetation survey and trait measures*

On 5th June 2015, the percentage cover for all vascular plant and bryophyte species was recorded in 1 m^2 quadrats at each of the four sampling points across each fertiliser treatment. Species diversity was measured as the total number of vascular plant and bryophyte species present in each quadrat.

To represent differences in functional composition of plant communities, we used data for three leaf traits; specific leaf area (SLA), leaf dry matter content (LDMC) and leaf nitrogen content (leaf N) (Garnier *et al.*, 2004). Trait data for each species were obtained from the TRY database (Kattge *et al.*, 2011) and used to produce an abundance (percentage cover) community-weighted mean (CWM) value using the FD package in R (Laliberté *et al.*, 2014). Despite the absense of bryophyte trait data, 99% of the total plant cover was represented by SLA, leaf N and LDMC trait values, respectively. Two plant species that were abundant in heavily fertilised plots possessed high LDMC, therefore CWM LDMC was also high in these plots. This is contrary to patterns at larger scales where LDMC is often reported to be low in fertile environments (Reich, 2014). This may result from the relatively low range of conditions found in our experiment compared to national and international gradients. Previous studies have also shown that the CWM of SLA and leaf N outperform LDMC as descriptors in explaining variation in ecosystem function (De Vries *et al.*, 2012b; Allan *et al.*, 2015; Manning *et al.*, 2015a), therefore LDMC was not used in the analysis.

In July, an annual hay cut is taken to measure aboveground biomass production. At each sampling point a 10 m^2 section of harvested grass is collected and weighed. A fresh subsample is weighed and oven dried at 80 °C, then rewieghed. These two measures are then used to calculate dry mass production. A subsample (0.1 g) of oven dried material is also milled and analysed for %C using the Vario Macro Cube. We used mean aboveground biomass (2000-2015) and plant %C (2010-2015) data from the Palace Leas archive (see Chapter 2) to estimate aboveground biomass carbon stocks (t C ha^{-1}).

3.3.5 *Data analysis*

All statistical analysis was carried out using R version 3.2.1 (R Core Team, 2015). To assess potential causal relationships between the direct and indirect drivers of fertilisation impacts on multiple measures of grassland C and soil function we applied SEM (Grace, 2006) using the lavaan package (Rosseel, 2012). Measures of grassland C and soil function included; total SOC stocks, stocks of coarse, fine and very fine fraction C, active microbial biomass, C use efficiency, microbial catabolic capacity and aboveground biomass C stocks. Models were fitted at 0-5 and 5-10 cm depth due to the fact that in acidic grasslands soil properties between depths can be distinctly different (Chapter 2), and their drivers at these different depths may also differ. When calculating stocks we considered anything above 5 cm depth in the mineral soil as "0-5 cm" and anything below as "5-10 cm". This was done to account for the organic surface layer in treatment plots 7 and 11, which was absent in all other plots (see Chapter 2). To combine data from the organic layer (6 cm) and the 0-5 cm mineral soil, weighted averages (by depth) were calculated for each soil measure.

Based on mechanisms reported in the literature (see introduction) and analysis of the univariate relationships (Chapter 2) an initial hypothetical SEM was created (Figure 3.1). The core structure represents both direct effects of fertilisation (FYM, inorganic N and P) on ecosystem function and indirect effects operating via soil pH, plant functional composition and plant species richness. Soil pH has been shown to affect plant biodiversity (Stevens *et al.*, 2010), CWM SLA and CWM leaf N (De Vries *et al.*, 2012b), therefore pathways from soil pH to plant species richness and plant resource strategy were also included. Covariance terms were included between FYM, inorganic N and inorganic P as they are applied together in several fertiliser treatment plots (Figure 3.1).

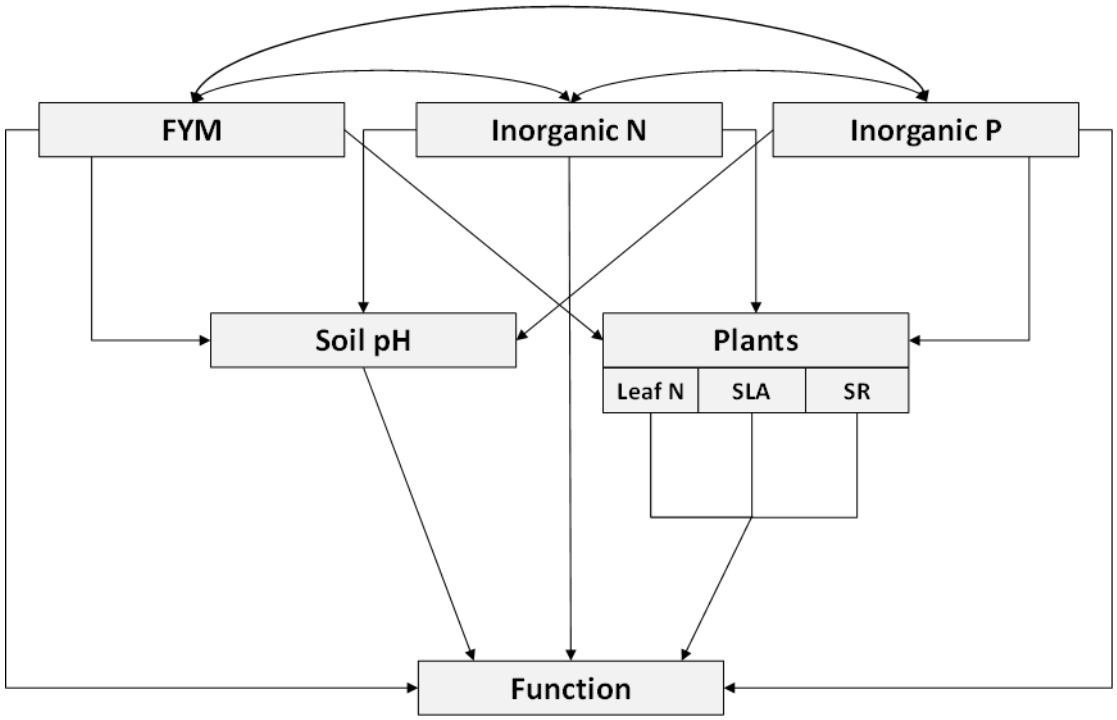

Figure 3.1 Hypothetical diagram of potential causal relationships between the direct (farmyard manure, inorganic N and inorganic P) and indirect (soil pH and plant-mediated; CWM SLA; SLA, CWM leaf N; leaf N or plant species richness; SR) effects of fertilisation and measures of grassland carbon and soil function.

Modelling was comprised of several steps. First we determined if indirect effects of plant community change were better explained by plant species richness ('diversity hypothesis') or the CWM of either SLA or leaf N ('biomass ratio hypothesis') by comparing models containing each of these variables and selecting that with the lowest Akaike's Information Criteria (AIC) (Allan *et al.*, 2015). This variable was used in the modelling approach described hereafter.

In the next step, we fitted the hypothesised model (Figure 3.1) to each soil and microbial measure at two separate depths (0-5 and 5-10 cm). The aboveground biomass C stocks SEM was fitted using soil pH data that was averaged from the 0-5 cm and 5-10 cm depths. Non-significant pathways were removed following a stepwise simplification process; paths with the largest *P* value were deleted until only significant paths remained ($P<0.05$). This enabled a more accurate estimate of the remaining paths as replication was limited by the experimental plot design. Final SEMs were deemed to have adequate fit if the *P* value

associated with the Chi-square statistic (χ^2) was P>0.05. Where there was more than one adequate model, we selected that with the lowest AIC value. Before SEMs were fitted log-transformation was used to correct nonlinearities and non-normality in the data. Bollen-Stine bootstrapping estimations were used to resolve potential problems associated with small sample size (Grace, 2006).

3.4 Results

3.4.1 *Vegetation- and soil pH-mediated effects of fertilisation*

The functions measured in this study were more strongly related to direct fertilisation effects and changes to pH than by the diversity and functional traits of the vegetation. The only significant indirect effects of plant functional composition on soil and microbial properties were those that operated via CWM leaf N at the 0-5 cm depth (Figure 3.2 d-h). Indirect effects operating via CWM SLA were only significant in determining the aboveground biomass C stocks SEM (Figure 3.2 a), whilst plant species richness was not included in any of the final SEMs (Table 3.1).

Vegetation properties were themselves related to direct fertilisation and changes in pH. Leaf N was associated with changes in soil pH and direct fertilisation (FYM and inorganic P), which explained 55 % of its variation. The positive effects of fertilisation (FYM = 0.39, inorganic P = 0.37) (Figure 3.2 d-h) were greater than the indirect pH effects (FYM = 0.28, inorganic N = -0.25, inorganic P = 0.11). In contrast, SLA was only related to fertilisation. The positive direct effects of FYM (SPC = 0.70) were greater than the positive direct effects of inorganic N (SPC = 0.40) or P (SPC = 0.31) on SLA, explaining 54% of its variation (Figure 3.2 a).

At both depths the positive effects of FYM on soil pH (SPC = 0.66 to 0.78) were stronger than the effects of inorganic P (SPC = 0.27 to 0.39) and the negative effects of inorganic N (SPC = -0.45 to -0.60). Fertilisation explained 86 % and 83 % of the variation in soil pH at 0-5 and 5-10 cm, respectively (Figure 3.2 b-h, Table 3.2).

		Indirect plant mediated effects											
		CWM SLA				CWM leaf N				Plant species richness			
Function	Depth (cm)	AIC	Δ AIC	p value	R^2	AIC	Δ AIC	p value	R^2	AIC	Δ AIC	p value	R^2
Total SOC	0-5	762	4	0.53	0.61	**758**	**0**	**0.15**	**0.64**	762	4	0.01	0.64
Total SOC	5-10	786	6	0.26	0.50	**780**	**0**	**0.01**	**0.52**	789	9	0.27	0.50
Coarse fraction C stocks	0-5	778	24	0.62	0.51	774	20	0.28	0.52	**754**	**0**	**0.44**	**0.51**
Coarse fraction C stocks	5-10	819	5	0.28	0.38	**814**	**0**	**0.14**	**0.39**	823	9	0.25	0.42
Fine fraction C stocks	0-5	806	25	0.58	0.39	801	20	0.59	0.43	**781**	**0**	**0.57**	**0.43**
Fine fraction C stocks	5-10	**797**	**0**	**0.15**	**0.35**	801	4	0.63	0.35	801	4	0.01	0.39
Very fine fraction C stocks	0-5	781	1	0.14	0.62	**780**	**0**	**0.46**	**0.58**	784	4	0.56	0.50
Very fine fraction C stocks	5-10	**792**	**0**	**0.26**	**0.56**	793	1	0.14	0.52	797	5	0.27	0.55
Microbial biomass	0-5	732	2	0.59	0.78	**730**	**0**	**0.58**	**0.78**	731	1	0.44	0.77
Microbial biomass	5-10	761	0	0.25	0.68	**761**	**0**	**0.01**	**0.66**	761	0	0.62	0.71
Metabolic quotient (qCO_2)	0-5	755	4	0.65	0.68	**751**	**0**	**0.62**	**0.68**	763	12	0.58	0.66
Metabolic quotient (qCO_2)	5-10	750	4	0.13	0.73	**746**	**0**	**0.14**	**0.74**	756	10	0.14	0.74
MCC30	0-5	744	3	0.42	0.78	**741**	**0**	**0.61**	**0.73**	750	9	0.31	0.73
MCC30	5-10	788	6	0.01	0.48	**782**	**0**	**0.45**	**0.50**	789	7	0.25	0.51
MCC50	0-5	757	1	0.62	0.72	**756**	**0**	**0.62**	**0.65**	758	2	0.57	0.63
MCC50	5-10	794	7	0.48	0.42	**787**	**0**	**0.27**	**0.45**	798	11	0.01	0.42
MCC70	0-5	788	7	0.60	0.51	**781**	**0**	**0.62**	**0.45**	789	8	0.57	0.46
MCC70	5-10	807	11	0.23	0.27	**796**	**0**	**0.26**	**0.36**	811	15	0.25	0.28
Aboveground biomass carbon	0-10	**672**	**0**	**0.01**	**0.91**	675	3	0.77	0.89	675	3	0.42	0.89

Table 3.1 Plant mediated effects of fertilisation could be explained via differences in plant species richness or the CWM of the plant resource strategy (SLA or leaf N). Models were compared using AIC to determine the most parsimonious plant pathway, the pathway judged to be the most appropriate had the lowest AIC (in bold). Values shown for each model are AIC, ΔAIC (difference in AIC between a model and the model with the lowest AIC score), the P value associated with the χ^2 and the R^2. Best fitting plant pathway also displayed for microbial catabolic capacity threshold at 30% and 70% of the maximum as well as the chosen 50% threshold (MCC30, MCC50 and MCC70).

Function	Depth (cm)	Path	Estimate	Standard Error	Path P value	χ^2	Model P value	R^2
Total SOC	5-10	FYM → Total SOC	0.65	0.08	0.00	1.177	0.48	0.43
Coarse fraction C stocks	5-10	FYM ↔ Inorganic P	-0.31	0.10	0.00	4.708	0.15	0.32
		FYM ↔ Inorganic N	-0.30	0.10	0.00			
		Inorganic P ↔ Inorganic N	0.42	0.16	0.01			
		Inorganic N → pH	-0.45	0.06	0.00			0.83
		Inorganic P → pH	0.39	0.06	0.00			
		FYM → pH	0.78	0.06	0.00			
		pH → Coarse fraction C	-0.57	0.11	0.00			
Fine fraction C stocks	5-10	FYM → Fine fraction C	0.49	0.12	0.00	7.385	0.49	0.24
Very fine fraction C stocks	5-10	FYM→ Very fine fraction C	0.66	0.09	0.00	0.432	0.48	0.44
Microbial biomass	5-10	FYM ↔ Inorganic P	-0.31	0.10	0.00	5.914	0.06	0.59
		FYM ↔ Inorganic N	-0.30	0.10	0.00			
		Inorganic P ↔ Inorganic N	0.42	0.16	0.01			
		Inorganic N → pH	-0.45	0.07	0.00			0.83
		Inorganic P → pH	0.39	0.06	0.00			
		FYM → pH	0.78	0.06	0.00			
		pH → Microbial biomass C	0.77	0.09	0.00			
Metabolic quotient (qCO_2)	5-10	FYM ↔ Inorganic P	-0.31	0.10	0.00	12.272	0.07	0.66
		FYM ↔ Inorganic N	-0.30	0.10	0.00			
		Inorganic P ↔ Inorganic N	0.42	0.16	0.01			
		Inorganic N → pH	-0.45	0.06	0.00			0.83
		Inorganic P → pH	0.39	0.06	0.00			
		FYM → pH	0.78	0.06	0.00			
		pH → Metabolic quotient	-0.81	0.07	0.00			

Table 3.2 Individual path coefficients for the structural equation models on measures of grassland carbon and soil function at 5-10 cm.

Single headed arrows signify the direction of the relationship between variables, double headed arrows indicate covariance between variables. Estimates (standardised path coefficients), standard error of the estimates, individual path P value, χ^2, P value associated with χ^2 and the R^2 are presented. Results for microbial catabolic capacity at 5-10 cm presented in Appendix B.

Function	Depth (cm)	Path	Standardized path coefficient
Aboveground biomass C	0-10	FYM → Soil pH →	0.28
		Inorganic N → Soil pH →	-0.22
		Inorganic P → Soil pH →	0.12
		FYM → CWM SLA →	0.14
		Inorganic N → CWM SLA→	0.08
		Inorganic P → CWM SLA →	0.06
Coarse fraction C	0-5	FYM → Soil pH →	-0.21
		Inorganic N → Soil pH →	0.19
		Inorganic P → Soil pH →	-0.08
	5-10	FYM → Soil pH →	-0.44
		Inorganic N → Soil pH →	0.26
		Inorganic P → Soil pH →	-0.22
Fine fraction C	0-5	FYM → Soil pH →	-0.50
		Inorganic N → Soil pH →	0.46
		Inorganic P → Soil pH →	-0.21
Very fine fraction C	0-5	FYM → CWM leaf N →	0.09
		Inorganic P → CWM leaf N →	0.09
		FYM → Soil pH→ CWM leaf N →	0.07
		Inorganic N → Soil pH→ CWM leaf N →	-0.06
		Inorganic P → Soil pH→ CWM leaf N →	0.03
Total SOC	0-5	FYM → Soil pH →	-0.50
		Inorganic N → Soil pH →	0.45
		Inorganic P → Soil pH →	-0.20
		FYM → CWM leaf N →	0.12
		Inorganic P → CWM leaf N →	0.12
		FYM → Soil pH→ CWM leaf N →	0.09
		Inorganic N → Soil pH→ CWM leaf N →	-0.08
		Inorganic P → Soil pH→ CWM leaf N →	0.04

Table 3.3 Standardised path coefficients for the indirect effects of fertilisation on aboveground biomass C, coarse fraction C stocks, fine fraction C stocks, very fine fraction C stocks and total SOC. Indirect effects are determined by multiplying the path coefficient for the effect of fertilisation (FYM, inorganic N or Inorganic P) on the soil pH/SLA/leaf N with the path coefficient for the effect of soil pH/SLA/leaf N on function.

Function	Depth (cm)	Path	Standardized path coefficient
Microbial biomass	0-5	FYM → Soil pH →	0.45
		Inorganic N → Soil pH →	-0.41
		Inorganic P→ Soil pH →	0.25
		FYM → CWM leaf N →	0.08
		Inorganic P → CWM leaf N →	0.07
		FYM→ Soil pH→ CWM leaf N →	0.06
		Inorganic N → Soil pH→ CWM leaf N →	-0.05
		Inorganic P→ Soil pH→ CWM leaf N →	0.02
	5-10	FYM → Soil pH →	0.60
		Inorganic N → Soil pH →	-0.35
		Inorganic P→ Soil pH →	0.30
Metabolic quotient	0-5	FYM → Soil pH →	-0.48
		Inorganic N → Soil pH →	0.43
		Inorganic P→ Soil pH →	-0.19
		FYM → CWM leaf N →	-0.05
		Inorganic P→ CWM leaf N →	-0.05
		FYM → Soil pH→ CWM leaf N	-0.03
		Inorganic N → Soil pH→ CWM leaf N →	0.03
		Inorganic P → Soil pH→ CWM leaf N →	-0.01
	5-10	FYM → Soil pH →	-0.63
		Inorganic N → Soil pH →	0.37
		Inorganic P→ Soil pH →	-0.32
Microbial catabolic capacity	0-5	FYM → Soil pH →	0.39
		Inorganic N → Soil pH →	-0.35
		Inorganic P → Soil pH →	0.16
		FYM → CWM leaf N →	0.11
		Inorganic P → CWM leaf N →	0.11
		FYM → Soil pH→ CWM leaf N →	0.08
		Inorganic N → Soil pH→ CWM leaf N →	-0.07
		Inorganic P→ Soil pH→ CWM leaf N →	0.03
	5-10	FYM → Soil pH →	0.48
		Inorganic N → Soil pH →	-0.28
		Inorganic P→ Soil pH →	0.24

Table 3.4 Standardised path coefficients for the indirect effects of fertilisation on microbial biomass, microbial metabolic quotient and microbial catabolic capacity.

3.4.2 *Total soil organic carbon and particle-size fraction carbon stocks*

Averaged across all treatments the coarse, fine and very fine particle-size fractions represented 11%, 48% and 41% at 0-5 cm and 4%, 25% and 71% at 5-10 cm of the total C stocks, respectively.

Generally, the direct effects of fertilisation on SOC stocks (via FYM, inorganic N and inorganic P) were stronger than those of indirect plant- (leaf N) or pH-mediated effects. The exception to this was coarse fraction C stocks (2.8 mm-200 µm) at 5-10 cm where only the effects of soil pH were significant, and coarse fraction C was higher in more acidic soils (Figure 3.2 b-e, Table 3.2). The addition of FYM increased total SOC, fine fraction C stocks (200-50 µm) and very fine fraction C stocks (50–0.45 µm) at both depths (SPC = 0.49 to 0.91) (Figure 3.2 c-e), whilst the input of inorganic N increased coarse fraction C stocks at 0-5 cm (SPC = 0.44) (Figure 3.2 b).

Indirect pH-mediated effects of fertilisation on total SOC stocks (FYM = -0.50, inorganic N = 0.45, inorganic P = -0.20) were stronger than those regulated by leaf N (both FYM and inorganic P = 0.12) at 0-5 cm. (Table 3.3). Indirect pH-mediated effects were detected on both coarse and fine fraction C stocks (Figure 3.2 b-c), whilst indirect effects operating via leaf N were only detected for very fine fraction C stocks (Figure 3.2 d). An increase in soil pH negatively affected both coarse and fine fraction C stocks (Figure 3.2 b-c) whilst higher leaf N had a weak positive effect on very fine fraction C stocks (Figure 3.2 d).

3.4.3 *Active microbial biomass, efficiency and catabolic capacity*

The direct effects of fertilisation on active microbial biomass, C use efficiency (respiration to biomass ratio; qCO_2) and catabolic capacity were not significant (Figure 3.2 f-h, Table 3.4). In contrast, pH effects were much stronger. The positive indirect effects of soil pH and leaf N increased active microbial biomass, C use efficiency (lower qCO_2) and catabolic capacity. However, the indirect effects of soil pH were more strongly positive than the indirect effects of leaf N (Figure 3.2 f-h, Table 3.4). The indirect effects soil pH and leaf N were significant at 0-5 cm, whilst only soil pH was significant at 5-10 cm (Table 3.2).

3.4.4 *Aboveground biomass carbon stocks*

High aboveground biomass C stocks were associated with positive direct effects of fertilisation (FYM, inorganic N and inorganic P) and positive indirect effects of pH and SLA (Figure 3.2 a). The direct nutrient addition effects were generally greater (FYM = 0.54,

inorganic N = 0.41, inorganic P = 0.26) than the indirect effects via pH (FYM = 0.28, inorganic N = -0.22, inorganic P = 0.12) and SLA (FYM = 0.14, inorganic N = 0.08, inorganic P = 0.06, Table 3.3) on aboveground biomass C stocks. Together, the direct and indirect effects of fertilisation explained 91 % of variation in aboveground biomass C stocks (Figure 3.2 a).

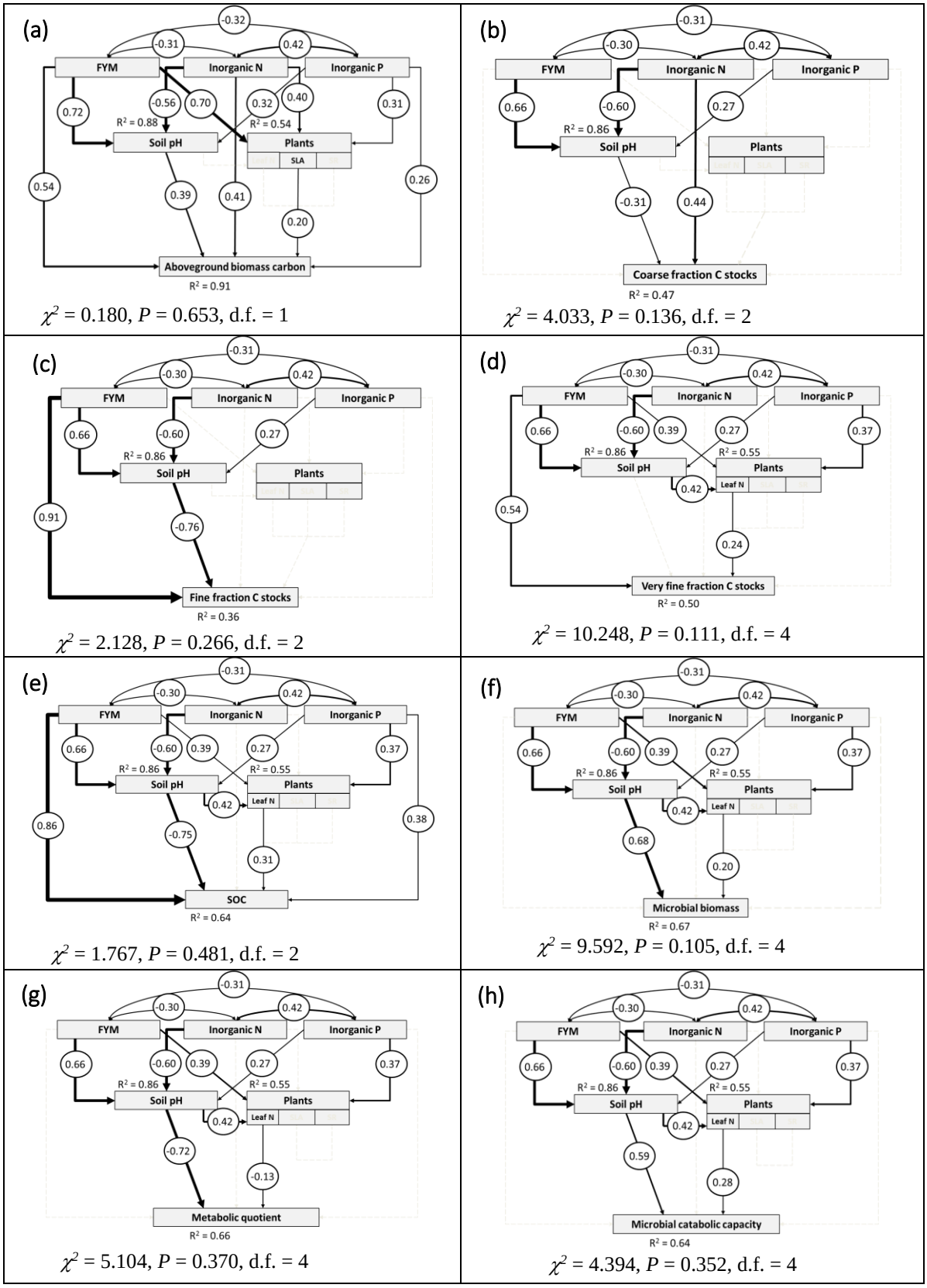

Figure 3.2 Structural equation models showing drivers of eight grassland carbon and soil function measures (a–h) at 0-5 cm. Grey rectangular boxes represent observed variables, solid black single headed arrows represent significant effects (P<0.05), double headed arrows indicate covariance between variables. Non-significant pathways (P<0.05) were removed from the model. Circled numbers on the arrows correspond to standardised

path coefficients and R^2 reflects the variance explained. Model fit results are presented below each model diagram. See Table 3.3 and Table 3.4 for the path coefficients of the indirect effects of fertilisation.

3.5 Discussion

Our results indicate that fertilisation is associated with a change in grassland C and soil function through both the direct effects of nutrient addition and indirectly via changes in soil pH and plant functional composition. In some cases our results also suggest that these indirect effects are of greater importance than the direct effects of fertilisation. In contrast to many experimental studies (Eisenhauer *et al.*, 2013; Lange *et al.*, 2015; Strecker *et al.*, 2015), we were unable to find evidence that biodiversity loss was associated with changes to any measures of grassland C and soil function, despite the very long-term nature of the study.

Where shifts in plant functional composition did alter soil function, such effects were best explained by changes to CWM leaf N. In this study, fertilisation and high soil pH enhanced CWM leaf N, a finding that concurs with national scale patterns of higher CWM leaf N in fertile and less acidic soils (De Vries *et al.*, 2012b). Soil pH was regulated by fertilisation in a number of ways with the addition of inorganic N ($(NH_4)_2SO_4$ and NH_4NO_3) acidifying the soil, whilst FYM and inorganic P acted to buffer the effects of acidification (O'Donnell *et al.*, 2001). However, caution must be taken when comparing the effects of nutrients as the influence of N, P and carbon in the FYM cannot be isolated.

3.5.1 *Effects on total SOC and soil C size fractions*

Disentangling the effects of fertilisation on total SOC stocks and their component size fractions revealed that direct nutrient addition effects were generally greater than the indirect effects of a soil pH change. Indirect effects of plant functional composition (leaf N) were also significant, but relatively weak and confined to the top 5 cm of the soil.

Our results indicate that C stocks in the coarse and fine fractions are promoted by both the direct effects of increasing N fertilisation and the weaker indirect effects of soil acidification. The coarse fraction is a highly dynamic carbon pool which predominantly consists of partially decomposed plant residues (Christensen, 2001). Fertiliser induced increases in coarse fraction C stocks are likely to be due to the direct effect of increased plant C inputs, both above and below-ground, as a result of greater primary productivity (Gough *et al.*, 2000; Lu *et al.*, 2011; Fornara and Tilman, 2012; Fornara *et al.*, 2013). The accumulation of coarse fraction C with

increasing acidity is also consistent with national scale patterns (Manning *et al.*, 2015a). In acidic soils, microbial communities typically express lower rates of respiration (Rousk *et al.*, 2011a), resulting in reduced organic matter turnover and the accumulation of partially decomposed plant C at the soil surface. Similarly, here soil acidity had strong negative effects on the microbial biomass and its function (see below). A growing body of evidence points to the importance of soil microorganisms in the formation of slowly cycling forms of soil C (Kindler *et al.*, 2009; Schmidt *et al.*, 2011; Miltner *et al.*, 2012). The suppression of soil microbial activity at low pH may also explain why C accumulated in the coarse and fine (partially humified) fractions, rather than being transferred to more decomposition resistant pools (Fornara *et al.* 2011).

The indirect effects of fertilisation on very fine fraction C stocks were of less importance than direct effects and were mediated by plant functional composition. Greater stocks of this typically more slowly cycling C, increased as a direct effect of fertilisation, along with the presence of more nutrient rich plant residue inputs in fertilised conditions (CWM leaf N). This may have occurred as a result of reduced 'microbial nutrient mining'. In accordance with the proposed theory, increased nutrient availability may have suppressed the production of enzymes that decompose ("mining") the nutrient rich but slowly cycling carbon pool, and the microbes that produce these enzymes (Moorhead and Sinsabaugh, 2006; Craine *et al.*, 2007). Whilst we did not measure microbial community composition, microbial C use efficiency (qCO_2) which has been suggested to be greater in microbial communities dominated by fast-growing copiotrophic taxa (Fierer *et al.*, 2012; Chen *et al.*, 2014) increased in response to high CWM leaf N (see below), indicating consistency with the hypothesised mechanism. Increased nutrient rich plant residue inputs may also have increased the formation of slow cycling C. Greater microbial C use efficiency in the presence of high quality litter may have increased inputs of microbial derived organic matter into the soil matrix (Cotrufo *et al.*, 2013; Cenini *et al.*, 2015). Consistent with findings from other soil types and plant communities (Manning *et al.*, 2015a; Owen *et al.*, 2015) these results provide further evidence that plant community changes can cascade to influence soil C storage.

3.5.2 *Effects on microbial biomass and community function*

Our study revealed that changes to soil pH and plant functional composition can mediate microbial responses to long-term fertilisation, with negative effects of acidification on microbial biomass, C use efficiency and catabolic capacity being particularly strong. Contrary to the findings of previous studies which demonstrate that the application of organic manures

increases the size of the microbial biomass (Witter *et al.*, 1993; Zhong *et al.*, 2010), no such effects were detected here. The lack of direct effects of FYM addition on the microbial biomass suggests that the influence of changes to soil pH were so dominant that the direct effects of FYM were overwhelmed. Soil acidification is associated with increased concentrations of potentially toxic metals such as aluminium and concomitant depletion of base cations, related to physiological stress to microorganisms (Chen *et al.*, 2015a; Chen *et al.*, 2015b). Whilst we cannot be certain of the mechanism responsible here, acidification is associated with increases in the bioavailability of Al^{3+} in this long-term experiment (see Chapter 2). Within the pH range observed here, soil pH can strongly determine microbial community composition (Jones *et al.*, 2009; Lauber *et al.*, 2009; Rousk *et al.*, 2010a), with high acidity selecting for slow-growing oligotrophic taxa (e.g. *Acidobacteria*) typical of resource poor environments. A general shift towards oligotrophs may therefore explain why soil acidification reduced microbial C use efficiency and catabolic capacity.

Evidence of the effects of plant functional change, corroborate the findings of several studies which detected relationships between plant functional composition and microbial dynamics (Orwin *et al.*, 2010; De Vries *et al.*, 2012b; Grigulis *et al.*, 2013). An increase in leaf N, indicative of a shift towards a faster growing and more productive plant community (De Vries *et al.*, 2012b) is likely to result in an increased quantity of high quality (low C:N) inputs to the soil (Grigulis *et al.*, 2013). Enhanced allocation of N rich residues might initiate a shift towards copiotrophic dominance, thus causing the microbial community to operate more efficiently (low qCO_2) (Spohn and Chodak, 2015) and increase the allocation of resources for biosynthesis (microbial growth).

The prevalence of pH-mediated effects of fertilisation in this study are consistent with previous attempts to disentangle the direct and indirect effects of fertilisation on microbial community composition and function in semi-arid Mongolian grasslands (Wei *et al.*, 2013; Chen *et al.*, 2015a; Chen *et al.*, 2015b). Such consistency, given considerable differences in terms of climate, soil type and vegetation, suggests that pH-mediated effects on soil microbes may be general across grassland ecosystems. Whilst we also observed fertilisation effects on microbes via a change in plant functional composition (leaf N), the aforementioned studies detected no such pathway. It is possible that by sampling at a finer resolution (5cm depth) in the topsoil layer where microbial communities have greater access to litter residues and rhizodeposits (Fierer *et al.*, 2003), we had a better opportunity of identifying plant-microbe interactions than the other studies (0-15 cm depth).

In contrast to the patterns described above, a global study demonstrated that changes in plant community composition were of greater importance in shifting microbial community response (increase in copiotrophic:oligotrophic taxa) to nutrient addition than changes in soil pH (Leff *et al.*, 2015). We suggest the lack of consistent drivers between studies is due to differences in the magnitude of fertiliser-induced pH change. In Leff *et al.* (2015), due to the relatively short-term nature of the study (2-4 years), fertiliser-induced pH change was of a different order of magnitude to this study (-0.16 versus ~-2 units) and the Mongolian grassland experiments. This indicates that the relative importance of different mechanisms changes over time and also emphasises the value of using long-term experiments in order to distinguish between long-term and transient effects of fertilisation (Silvertown *et al.*, 2010).

3.5.3 *Effects on aboveground biomass carbon stocks*

The direct effects of fertilisation were stronger than the indirect pathways in driving aboveground biomass C stocks, probably as a result of reduced nutrient limitation enhancing plant growth (Elser *et al.*, 2007; LeBauer and Treseder, 2008). We also found that greater stocks of aboveground biomass C were weakly affected by indirect effects of fertilisation, operating via soil pH and plant functional composition (CWM SLA). Lower stocks of aboveground biomass C associated with increasing acidity are likely due to increased concentrations of Al^{3+} and NH_4^+ (Stevens *et al.*, 2011), and subsequent reductions in soil nutrient status (low rates of nitrification and P solubility), base cation leaching (Bowman *et al.*, 2008), and the formation of a toxic and resource-poor environment not conducive for plant growth. An increase in SLA is typical of a change towards a community dominated by species with high relative growth rates (Garnier *et al.*, 2004). These results emphasise that as well as increasing plant growth, fertilisation can also enhance aboveground biomass C stocks by altering the plant community's capacity to grow rapidly and exploit additional resources (Laliberté and Tylianakis, 2011; Grigulis *et al.*, 2013).

3.5.4 *Lack of plant diversity effects*

Notably, we did not detect any effects of plant species diversity loss, despite considerable variation in the number species between treatment plots (4-21 species per m^2) (Chapter 2). In contrast, biodiversity experiments have demonstrated strong effects of plant diversity on soil microbial communities (Eisenhauer *et al.*, 2013) and C stocks (Fornara and Tilman, 2008; Lange *et al.*, 2015), which can sometimes exceed the effect of global environmental change factors (Thakur *et al.*, 2015). However, in biodiversity experiments the size and composition

of the species pool is typically artificially assembled and maintained, and soils have undergone major management in recent years. Whilst biodiversity loss at Palace Leas is non-random and caused by fertilisation, as in many 'real-world' agricultural systems and soil properties may be closer to equilibrium. In such cases concurrent changes to functional composition may be stronger than diversity effects, particularly for soil functions (Allan *et al.*, 2015). In addition, as this is a very long-term experiment it may be that transient biodiversity effects occurred in the past, but have subsequently become overwhelmed by the effects of other drivers, e.g. acidification.

3.5.5 *Consistency of effects across ecosystems*

This study and others provide consistent evidence that fertilisation alters grassland C and soil function via changes to soil pH- and plant functional composition. However, the strength of indirect pathway effects are likely to be context dependent (e.g. Allan *et al*., 2015) and vary depending on site abiotic (e.g. climate, soil texture, and nutrient status), biotic (plant community composition and traits) and management factors (rates of fertiliser and lime addition). For example, in extensively managed grasslands we may expect that plant-mediated effects (fast traits strategy; increased CWM leaf N and SLA) will dominate in heavier clay soils, due to their inherently higher buffering capacity (Brady and Weil, 2002) slowing the onset of soil acidification. In contrast, a light sandy soil's low buffering capacity may allow rapid soil acidification in response to fertilisation, like that seen in semi-arid grasslands (Chen *et al.*, 2015a; Chen *et al.*, 2015b) resulting in pH-mediated effects that rapidly exceed those of plant community composition change. In order to manage the impacts of fertilisation to maximise the provision of multiple ecosystem services and formulate models which accurately predict ecosystem responses, this context dependence needs to be considered. For instance, in high pH soil (calcareous or limed) the direct effects of fertilisation may be of greater importance (e.g. Ramirez *et al*., 2010). Therefore, it is necessary to perform studies that estimate the importance of these mechanisms in a wider range of grassland ecosystems.

Whilst our results indicate that fertilisation can enhance ecosystem C retention, it is important to emphasise that fertilisation has been shown to influence the ecosystem C balance via effects on other functions not measured here, i.e. greenhouse gas (GHG) emissions (Owen *et al.*, 2015) and the leaching of dissolved organic carbon (DOC) (Liu and Greaver, 2010). We suggest future studies should also consider GHG emissions and DOC loss as they are important contributors to the ecosystem C balance and currently we know little of how they respond to the different pathway effects of fertilisation.

3.6 Conclusions

The findings from our study highlight the importance of soil pH and plant community composition in mediating the effects of very long-term fertilisation on grassland C and soil function. We show that the prominence of pH-mediated effects in determining microbial response was broadly consistent with patterns observed in ecologically dissimilar semi-arid grasslands, thus indicating that strong, acidity-mediated effects may be common across the world's grasslands. In contrast, the plant community effects were weak and better explained by plant functional composition shifts (CWM leaf N and SLA) than biodiversity loss. In synthesis with results from other studies we suggest that the dominance of particular direct and indirect pathways of fertilisation will largely be context dependent (i.e. vary depending on; abiotic, biotic and management factors) and may change temporally. Therefore, agricultural grasslands management practices (e.g. liming, fertilisation and diversification) may require local modification if they are to sustainably produce forage, alongside other ecosystem services. A more general understanding of the pathways described in this study may therefore enable the formulation of effective management strategies and more accurate predictions of grassland responses to future fertilisation.

Chapter 4: The influence of pH on soil carbon dynamics varies within a rotational cropping cycle

Jonathan Kidd, Elizabeth Stockdale, Peter Manning, Christine Watson. To be submitted to *Soil Biology & Biochemistry* as a Short Communication.

Contribution statement: Jonathan Kidd, Elizabeth Stockdale and Christine Watson conceived the study. Soil sampling, laboratory analysis and analysis of the data was carried out by Jonathan Kidd. Jonathan Kidd wrote the paper, all authors contributed to revisions.

4.1 Abstract

Soil pH is a key determinant of belowground carbon (C) dynamics in grassland and arable cropping systems; however, we lack an understanding of how pH effects on soil C are adjusted by other drivers of soil C dynamics. Using a long-term ley-arable crop rotation with an experimentally generated pH gradient we examined the effects of pH on soil organic carbon (SOC) dynamics in three phases of the cropping sequence. We found that increases in soil pH negatively affected SOC stocks, and provide evidence that this relationship is driven by the inhibition of rapid microbial C mineralisation (low molecular weight (LMW) C utilisation) in the ley phase, in more acid soils, when *Trifolium repens* is absent and the supply of N rich rhizodeposits to the microbial community is likely to be reduced. This research demonstrates that pH is key in regulating soil C dynamics in rotational cropping systems and that differences in the short-term functional response of the microbial community across the rotational cycle may dictate long-term soil C stock dynamics.

4.2 Introduction

The soil microbial community plays a critical role in mediating the decomposition of organic matter and the transformation of plant-growth limiting nutrients (Rice, 2002). Due to their role in organic matter mineralisation, the soil microbial community is often linked to the depletion of soil organic carbon (SOC), but there is increasing evidence that they also contribute to its stabilisation (Kindler *et al.*, 2009; Miltner *et al.*, 2012). Soil microbes are also particularly sensitive to changes in soil pH (Chen *et al.*, 2013); across a range of spatial scales increases in soil pH towards neutrality have been shown to alter microbial community composition (Fierer and Jackson, 2006; Lauber *et al.*, 2009), increase bacterial diversity (Fierer and Jackson, 2006; Rousk *et al.*, 2010a) and enhance rates of microbial processing of organic C to decomposition resistant fractions (Fornara *et al.*, 2011).

To date, most research on the impacts of pH on soil C dynamics has been studied in permanent grassland or continuous cropping systems (Pietri and Brookes, 2008; Fornara *et al.*, 2011), and little is known regarding the effects of pH in rotational cropping systems. In contrast to permanent grassland or continuous cropping systems in which we may expect soil C dynamics in response to pH manipulation to reach a relatively steady state over time, rotational cropping involves regular alternation of crops, hence the soil environment is continually perturbed and steady state conditions are inhibited. Therefore, the cumulative impact of pH on soil C in rotational cropping systems is likely to be driven by the interaction of pH with the biological, physical and chemical changes experienced at different stages of the cropping sequence. Previous research has revealed the capacity for specific crops to shape microbial communities (Grayston *et al.*, 1998; Larkin and Honeycutt, 2006; Haichar *et al.*, 2008) via differences in the quantity and quality of resources they supply to the rhizosphere; hence the activities of the microbial community will change temporally between different crops in the rotation. Differences in microbial activities may also occur within crop phase as a result of variation in soil pH, both as a direct response to soil acidity and indirectly via the effects of pH on the availability of C to the microbial community (Kemmitt *et al.*, 2006). However, we currently lack experimental evidence of how pH influences soil C dynamics in crop rotations, or understand how these effects may vary across the cropping sequence.

4.3 Materials and Methods

In a long-term ley-arable rotation in which fertility varies temporally with management actions and soil pH differs across a defined pH gradient (Woodland's Field, Craibstone Estate, Scotland, 57°11'N, 2°12'W, UK National Grid Reference NJ872104), we investigated the effects of pH on soil C dynamics. In short, since 1961 soils, classified as sandy loam (Countesswells Association), have been maintained at pH 4.5-7.5 at 0.5 unit intervals by adding $FeSO_4$ (since 2009, previously $Al_2(SO_4)_3$) and $Ca(OH)_2$, and subject to an eight course crop rotation arranged in a grid pattern (Table 4.1 and Figure 4.1). For a detailed site description see Kemp et al. (1992). In March 2015, composite soil samples (n=3, 0-10 cm) were collected from plots maintained at pH 4.5-6 from three stages of the crop rotation sequence. These broadly represent phases of the fertility build-up and depletion cycle; winter wheat (high fertility, average potentially mineralisable N (PNM) between pH 4.5-6; 43.0 ± 4.0 mg N kg^{-1}), spring barley (medium fertility, PNM; 39.9 ± 2.8 mg N kg^{-1}) and first year pasture (low fertility, PNM; 29.9 ± 2.1 mg N kg^{-1}). Plots were sampled early in the growing season to avoid the immediate effects of fertilisation. An exception was the winter wheat crop which had received a basal application of 65 kg P_2O_5 ha^{-1} and 85 kg K_2O ha^{-1} in December 2014.

Soil pH was measured on air-dried soil (sieved <2 mm) in a 1:2.5 soil-water supernatant. A modified wet sieving method was used to isolate the coarse (2.8 mm-200 µm), fine (200-50 µm) and very fine (50–0.45 µm) soil particle-size fractions (De Deyn *et al.*, 2011). Inorganic C in the individual particle-size fraction and bulk soils was accounted for as described by Fornara *et al.* (2011). Concentrations of organic C in fractions and bulk soil was measured by Vario Macro Cube (Elementar, Hanau, Germany) and expressed as stocks (kg C per m^{-2}) (Manning *et al.*, 2015a). Microbial catabolic profiles were analysed using MicroResp™ (Campbell *et al.*, 2003). Fresh soil (sieved <2 mm) was pre-incubated at 25°C for 5 days (adjusted to 40% WHC) and 0.3 g was added to each well of a deep well plate. 14 dissolved LMW substrates (supplied at 30 mg g^{-1} soil water), including 5 amino acids (L-glutamine, L-asparagine, L-alanine, γ-aminobutyric acid and N-acetyl glucosamine), 5 carbohydrates (D-mannose, D-glucose, L-arabinose, D-fructose and D-galactose) and 4 carboxylic acids (oxalic acid, pantothenic acid, L-malic acid and citric acid) were applied (25 µl) to each soil sample. Due to the low solubility of the amino acids they were applied at 7.5 mg g^{-1} soil water. Gel detection microplates were then measured at 570 nm by a spectrophotometer (SpectraMax Plus, Molecular Devices), inverted on to the deep well plates, sealed and incubated at 25 °C for 6 h then re-measured to determine the respiratory response, according to Campbell *et al.* (2003). Microbial community catabolic response was calculated for each soil sample as the cumulative rate of utilisation (µg CO_2-C g^{-1} h^{-1}) of each of the three chemical groups of substrates. Active microbial biomass was estimated by glucose-induced respiration (Anderson and Domsch, 1978). An estimation of the cover of *Trifolium repens* was made in the 2nd and 3rd year pasture at pH 4.5-6 by laying out three quadrats (50 x 50 cm) at random positions in each plot. Vegetation within each quadrat was photographed, photographs were uploaded on to a computer and cover was determined using a grid-point plate, according to Chen *et al.* (2010). Two-way ANOVA was used to test for the effects of soil pH treatment, rotation stage and their interaction on soil C dynamics, and Tukey's post-hoc test was used to identify differences between treatments.

	Fertiliser			
Crop	N (kg ha^{-1})	P_2O_5 (kg ha^{-1})	K_2O (kg ha^{-1})	Farmyard manure (t ha^{-1})
Pasture 1st year	70	30	50	0
Pasture 2nd year	0	0	0	0
Pasture 3rd year	0	0	0	0
Winter Wheat	100	65	85	0
Potatoes	100	150	120	30
Spring Barley	90	50	70	0
Swede	70	80	100	0
Spring Oats	60	50	70	0

Table 4.1 Rates of fertiliser input to each of the crops in the rotation. Fertiliser N, P and K is applied as ammonium nitrate, triple superphosphate and muriate of potash, respectively.

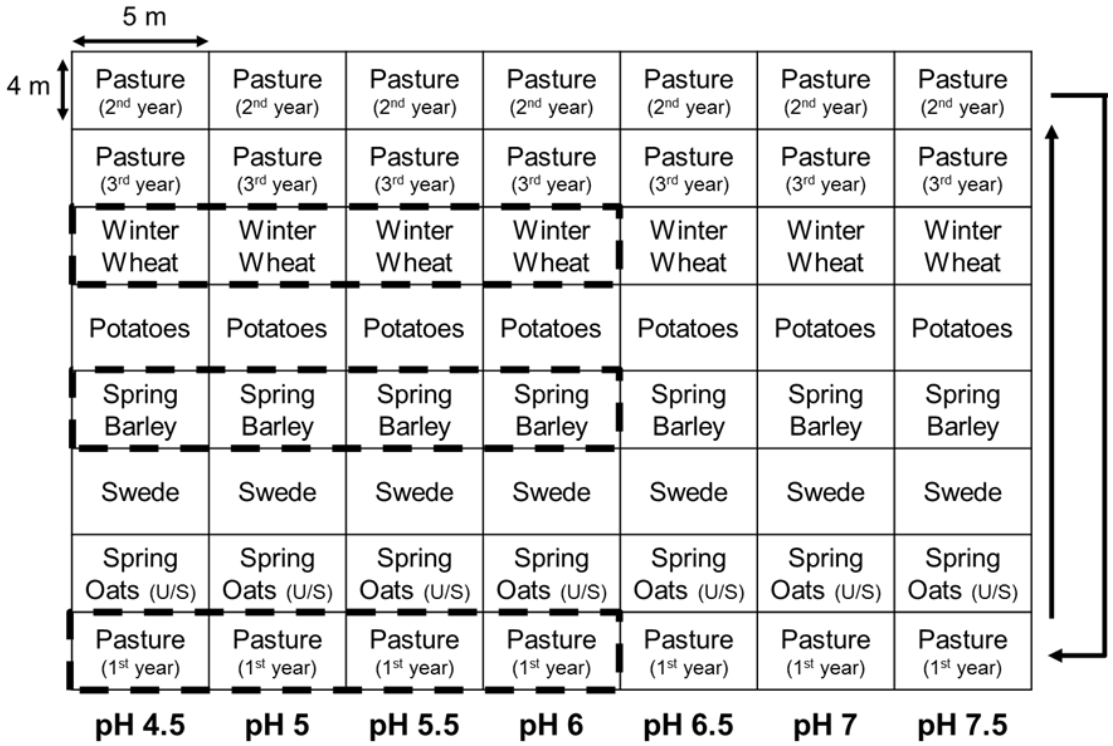

Figure 4.1 Plot layout of the pH gradient rotational cropping experiment for 2015. Treatment plots used in this investigation are highlighted with a dashed line. U/S; undersown with grass-white clover mixture.

4.4 Results and Discussion

SOC in bulk soil and particle-size fractions was greatest in soils maintained at pH 4.5 (P < 0.001) and decreased as soil pH increased (pH 5>5.5>6), with the exception of very fine fraction C stocks, which were not significantly different between pH 5-6 (Figure 4.2 a-d). Lower rates of microbial activity are expected in more acid soils (Pietri and Brookes, 2008) and the greater SOC stocks of acid soils can be explained by changes to substrate utilisation; we observed lower active microbial biomass ($F_{3,32}$ = 14.33, P<0.001; Table 4.2) and lower cumulative rates of LMW-C (carbohydrates; $F_{3,32}$ = 15.41, P<0.001, carboxylic acids; $F_{3,32}$ = 8.24, P<0.001) and -N (amino acid; $F_{3,32}$ = 37.76, P<0.001) utilisation by the microbial community in more acid soils (Figure 4.3 a-c). Lower activity and substrate use of the microbial community is likely to be explained by both reduced plant productivity (Figure 4.4), decreasing plant-derived resource availability, and physiological stress to microorganisms related to increased solubility of toxic metals at low pH e.g. Al^{3+} (Kemmitt *et al.*, 2006; Chen *et al.*, 2015b).

pH treatment	Measured pH	Active microbial biomass (mg kg^{-1})	Cumulative amino acid utilisation/C_{mic} (µg CO_2-C mg^{-1} h^{-1})	Cumulative carbohydrate utilisation/C_{mic} (µg CO_2-C mg^{-1} h^{-1})	Cumulative carboxylic acid utilisation/C_{mic} (µg CO_2-C mg^{-1} h^{-1})
4.5	4.73 (0.03)	79.07 (3.79) b	35.62 (2.22)	46.50 (2.55) ab	25.95 (1.11) ab
5.0	5.05 (0.02)	84.41 (7.76) b	39.53 (3.87)	49.00 (4.06) a	27.50 (2.52) a
5.5	5.41 (0.03)	123.15 (8.83) a	36.67 (2.81)	42.13 (3.49) bc	20.72 (1.52) bc
6.0	5.76 (0.04)	148.60 (12.30) a	34.32 (1.89)	37.67 (1.65) c	18.52 (1.26) c
P		<0.001	ns	<0.05	<0.01

Table 4.2 Measured soil pH, active microbial biomass and cumulative amino acid, carbohydrate and carboxylic acid utilisation rates per unit microbial biomass. Values are means (n=3) and error bars represent the standard error of the mean. Contrasting letters within columns denote significant differences between soil pH treatments, P > 0.05; not significant (ns).

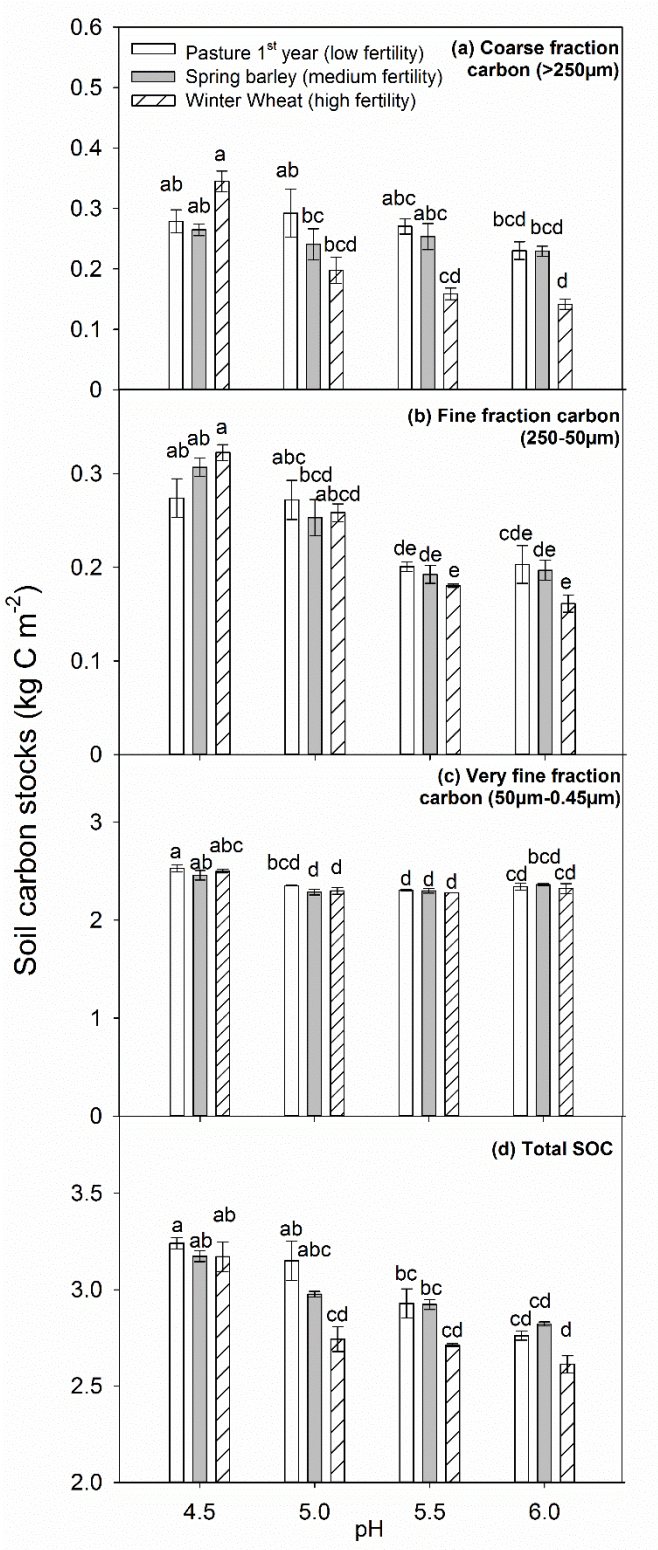

Figure 4.2 The effects of soil pH treatments in the three stages of the crop rotation on particle-size fraction C (a-c) and total SOC stocks (d). Data are means (n=3) and error bars represent ± SEM. Contrasting letters denote significant differences between treatments (two-way ANOVA; $P < 0.05$). The recovery of total SOC from the particle-size fractionation ranged from 85.6-104.7%.

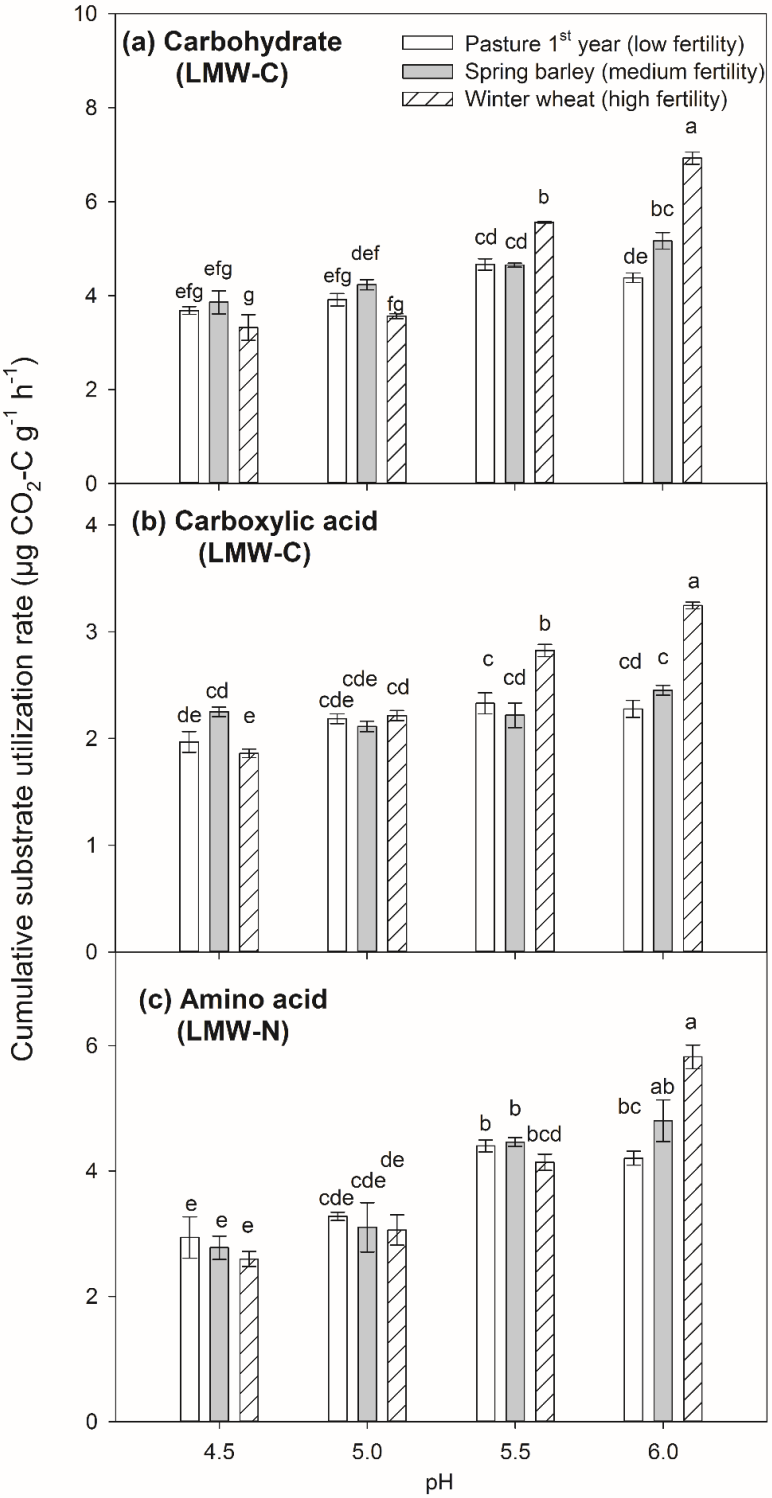

Figure 4.3 The effects of soil pH treatments in the three stages of the crop rotation on the cumulative rates of utilization of carbohydrates (a), carboxylic acids (b) and amino acids (c). Data are means (n=3) and error bars represent ± SEM. Contrasting letters denote significant differences between treatments (two-way ANOVA; $P < 0.05$).

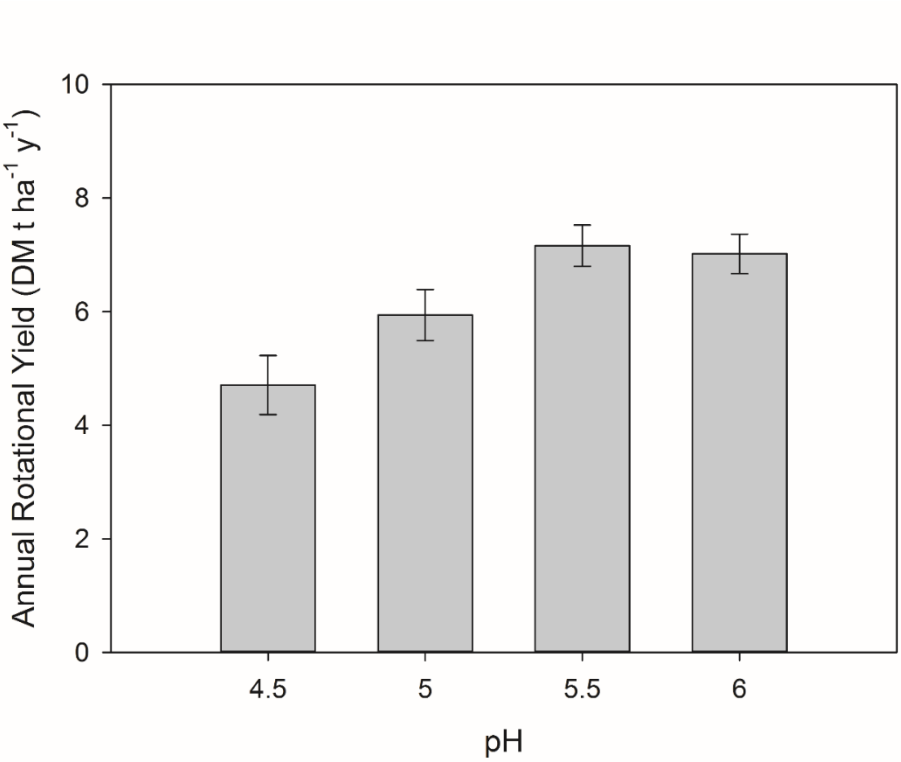

Figure 4.4 Average annual rotational yield for Woodland's Field pH gradient rotational cropping experiment. Estimations based on 100% dry matter offtake from the six annually harvested crops: Pasture (first year), winter wheat, potatoes, spring barley, swede and spring oats from 1 full crop rotation cycle prior to soil sampling (2007-2014). Error bars represent ± SEM.

Despite greater microbial substrate use at higher pH, this did not equate to greater microbial transfer of organic matter to increase the mineral-associated C fractions (i.e. fine and very fine fraction carbon), as observed in other studies (Tonon *et al.*, 2010; Fornara *et al.*, 2011). A combination of the relatively narrow pH range and the sandy textured soil, with a low inherent capacity to physically protect OC (Brady and Weil, 2002; von Lützow *et al.*, 2007) means that the effects of high pH on C stored in stabilised fractions may not have occurred in this study.

LMW-C utilisation appeared not to be governed solely by pH-induced variation in the size of the active microbial biomass. When expressed as per unit active microbial biomass, cumulative rates of LMW-C utilisation were significantly higher at pH 4.5 and 5 than pH 6 (carbohydrates; $F_{3,32} = 2.63$, $P<0.05$, carboxylic acids; $F_{3,32} = 6.28$, $P<0.01$). However, LMW-N utilisation was not affected ($F_{3,32} = 0.63$, $P>0.05$; Table 4.2). Strong shifts in microbial community composition across the pH gradient were previously shown at this site (Bartram *et al.*, 2014) and concomitant variation in microbial physiology may explain the observed variation in LMW-C utilisation.

The long-term effects of pH on soil C dynamics appeared to be regulated by the phase of the cropping cycle. Cumulative rates of LMW-C utilisation were significantly higher in the 'high fertility' winter wheat phase at pH 5.5 and 6 (interaction; carbohydrates; $F_{6,24} = 27.50$, $P<0.001$, carboxylic acids; $F_{6,24} = 20.04$, $P<0.001$), but not at pH 4.5 or 5 (Figure 4.3 a-b). This may be attributable to legacy effects of the legume *Trifolium repens* from the preceding ley phase, which due to inhibitory acidity effects (Wood *et al.*, 1984) was in markedly greater abundance in the ley at pH 5.5 and 6 (Figure 4.5). Greater supply of N to the microbial community via the N-fixing *T. repens*, coupled with cultivation (for winter wheat) may have increased the accessibility of organic matter that accumulates during the 3-year ley, thus accounting for the observed increase in C utilisation at this stage of the rotation. In contrast, comparable rates of LMW-C utilisation between stages of the cropping cycle at low pH (Figure 4.3 a-b) may reflect a lack of biological N fixation in the ley and the further inhibition of microbial activity in highly acidic soil conditions. The continued suppression of microbial community catabolism in the ley phase at low pH can therefore explain why soil carbon stocks were greater in more acid soils.

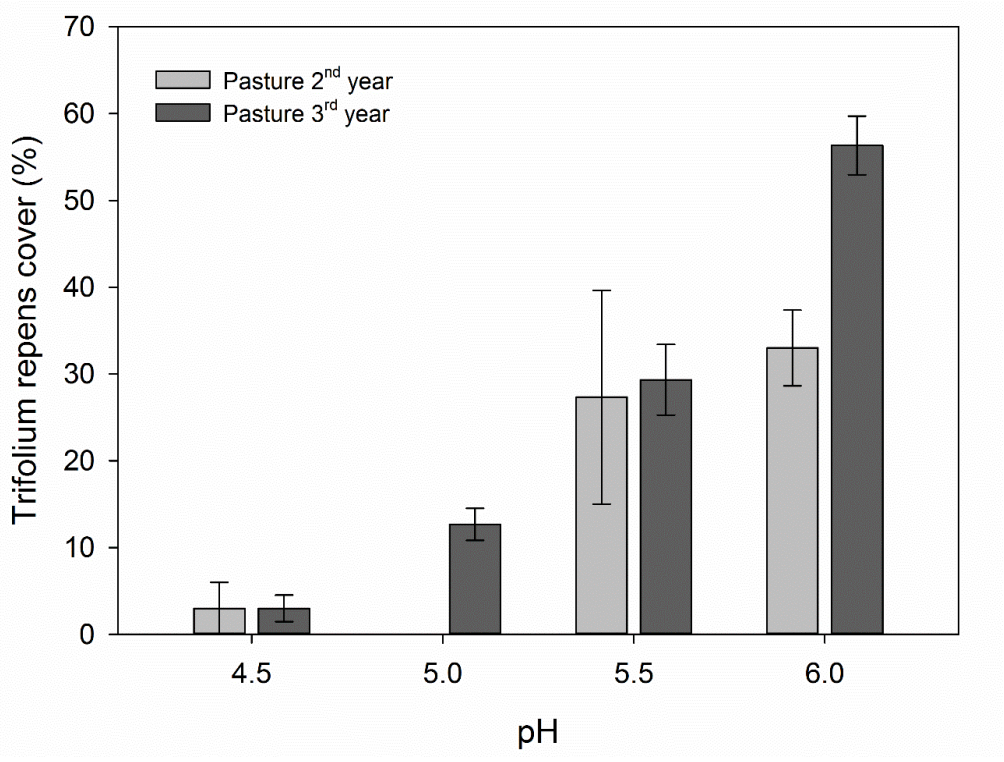

Figure 4.5 *Trifolium repens* cover in the 2nd and 3rd year pasture for Woodland's Field pH gradient rotational cropping experiment. Data are means (n=3) and error bars represent ± SEM.

Contrary to the utilisation of LMW-C, significant differences in cumulative rates of LMW-N utilisation were only detected at pH 6 between the winter wheat ('high fertility') and first year pasture ('low fertility') phases (Figure 4.3 c). The supply of inorganic N from *T. repens* to the rhizosphere may have partially repressed amino acid deamination at pH 6 (Geisseler *et al.*, 2010), thus decoupling microbial utilisation of LMW-C and LMW-N.

Although the physical protection of soil C and the availability of resources to the microbial community may differ in fine-textured soils, the results presented here suggest that pH can be a key regulator of soil C dynamics in ley-arable cropping rotations. More fundamentally these results show that pH effects are not independent of other soil and land management factors, and that these need to be considered together to gain a full understanding of soil C dynamics.

Chapter 5: Grassland microbial communities subject to long-term acidification are resistant to liming-induced pH change

Jonathan Kidd, Peter Manning, Ian Singleton, Alexandria McCallum, Ben Allen, Christine Watson and Elizabeth Stockdale. To be submitted to *Frontiers in Microbiology*.

Contribution statement: Jonathan Kidd, Elizabeth Stockdale and Peter Manning conceived and designed the study. Jonathan Kidd performed the microcosm experiment. Jonathan Kidd measured above- and below-ground plant biomass, soil chemical properties and microbial catabolic profiles. Alexandria McCallum, Ian Singleton and Jonathan Kidd extracted and prepared the genomic DNA for Illumina sequencing. Illumina sequencing was conducted by NU-OMICS at Northumbria University. Ben Allen and Alexandria McCallum processed the raw Illumina sequence data. Jonathan Kidd analysed the data and wrote the paper, all authors contributed to revisions.

5.1 Abstract

Soil pH is widely recognised as a driver of microbial community composition and a mediator of microbially regulated biogeochemical cycles. However, it remains unclear what determines the strength of microbial community response to changes in soil pH and to what extent the impacts of acidification can be remediated by liming. Here we investigated the effect of a short-term (6 month) increase in soil pH (via liming) on microbial community composition and functioning from soils differing greatly in acidity (pH 3.9-5.7) due to 120 years of experimental fertiliser addition. Bacterial community analysis was undertaken using high-throughput Illumina 16S rRNA sequencing, whilst microbial functioning was assessed from the catabolic potential of the communities to utilise multiple low-molecular weight compounds (MicroResp^{TM}). We found that liming only increased microbial functioning in communities from less acidified soils and there was no evidence that these changes were linked to shifts in bacterial community composition. This may be due to the fact that liming was not associated with a clear shift in bacterial community composition towards copiotrophic taxa, i.e. those that typically thrive in less acidic and higher-resource environments, even where liming had increased soil pH. Instead, the change in microbial functioning was potentially related to an increase in the fraction of the microbial biomass induced from a dormant to a physiologically active state or due to the phenotypic plasticity of the standing community. Our results demonstrate that long-term acidification causes changes to bacterial community composition and microbial function that cannot easily be reversed by

liming, particularly where acidification has created highly acid soils. These findings highlight the importance of preventing acidification, as it may cause soil degradation that is not easily rectified.

5.2 Introduction

Soil microbial communities play a critical role in regulating key soil processes including organic matter decomposition, nutrient mineralisation and the formation of a stable soil structure (Schimel and Schaeffer, 2012). It has been frequently documented that pH is one of the most important environmental factors shaping soil microbial communities at both local and global scales (Lauber *et al.*, 2009; De Vries *et al.*, 2012b; Zhalnina *et al.*, 2015). Microbial diversity responds strongly to differences in soil pH, with generally greater taxonomic diversity at neutral pH than in more acid soils (Fierer and Jackson, 2006; Rousk *et al.*, 2010a; Bartram *et al.*, 2014). Soil pH has also been shown to be a key determinant of microbial community composition, differentially affecting major taxonomic clades with contrasting life history characteristics. For example, the relative abundance of *Acidobacteria* has been shown to decline in response to increased soil pH (Jones *et al.*, 2009; Lauber *et al.*, 2009; Rousk *et al.*, 2010a). *Acidobacteria* are considered to have oligotrophic or *K*-selected life history strategies, meaning they tend to exhibit slow growth rates and thrive in resource limited environments (Fierer *et al.*, 2007). In contrast, the *Actinobacteria* and *Bacteroidetes* are categorised as having copiotrophic or *r*-selected life history strategies, meaning they have fast growth rates and prefer habitats with a high resource availability (Fierer *et al.*, 2007). These groups were found to be significantly positively related to higher soil pH in a cross-continent study (Lauber *et al.*, 2009). Conversely, within one arable soil, the abundance of *Actinobacteria* and *Bacteroidetes* did not increase as pH increased. However, the abundance of other copiotrophs (proteobacterial groups) were generally positively related to soil pH (Rousk *et al.*, 2010a). Taken together, results of these previous studies suggest that whilst the strength of the relationship between dominant microbial taxa and pH may vary somewhat between sites, copiotrophic taxa tend to dominate where soils are less acidic, whereas oligotrophic taxa generally increase in abundance at low pH.

Soil pH also exerts a great influence on the way in which microbial communities function, with many studies reporting that soil acidification suppresses microbial activity and microbially mediated processes including nitrification and carbon mineralisation (Kemmitt *et al.*, 2006; Rousk *et al.*, 2009; Yao *et al.*, 2011). However, despite the apparently strong pH-induced effects on both microbial community composition and function, there is a lack of

conclusive evidence to suggest that differences in microbial community composition account for pH driven changes to soil function (Rousk *et al.*, 2009).

Although the relationship between soil pH and soil microbial community properties is well described, there is a paucity of knowledge regarding the dynamics of microbial community changes following a pH change. If intensification of agricultural management practices which are known to influence pH continues (i.e. fertilisation and liming), then rapid changes to microbial community composition and function may become widespread. Studies to date have focused on the impacts of changing pH on bacterial growth, activity and community composition (Pettersson and Bååth, 2003; Pettersson and Bååth, 2004; Pawlett *et al.*, 2009; Fernandez-Calvino and Baath, 2010). However, methodological advances now allow for more detailed and comprehensive insights into pH driven changes to the functional and taxonomic properties of microbial communities.

Two non-exclusive hypotheses may explain how potential pH-induced changes in microbial community composition mediate the microbial community function response. Firstly, the 'mass-ratio hypothesis' (Grime, 1998) postulates that the functional traits of the most dominant species in a community drive ecosystem processes. Accordingly, a management-induced increase in soil pH could shift microbial communities towards dominance by copiotrophic taxa (Pawlett *et al.*, 2009; Fornara *et al.*, 2011) that possess intrinsically higher rates of activity, therefore increasing microbial community activity. Secondly, the 'diversity hypothesis' proposes that diverse communities partition niche space more effectively and use resources more completely, thus leading to higher levels of ecosystem functioning (Tilman, 1997; Hooper *et al.*, 2005). If this mechanism operates then an increase in soil pH may result in higher levels of taxonomic diversity (Fierer and Jackson, 2006; Rousk *et al.*, 2010a), consequently increasing resource use and microbial community function. The capacity for these two hypothetical mechanisms to explain plant community effects on ecosystem processes has been previously assessed (Mokany *et al.*, 2008; Allan *et al.*, 2015; De Vries and Bardgett, 2016). However, their capacity to explain how changes to microbial community composition mediate changes to microbial community function has not. Alternatively, changes in microbial community functioning may not be driven by shifts in community composition, but via changes to microbial physiology and metabolism (Shade *et al.*, 2012; Carey *et al.*, 2015). For example, soil microbes may change from a dormant to an active state or vice versa (Blagodatskaya and Kuzyakov, 2013) or by altering extracellular enzyme production (Mooshammer *et al.*, 2014; Fanin *et al.*, 2016).

Here we set out to investigate how soil microbial communities subject to different degrees of soil acidity respond to a change in pH and explore the relative importance of bacterial community composition effects (mass-ratio vs. diversity) in mediating changes in microbial community function. To do this we used soils from a very long term (120 year) fertilisation experiment, the Palace Leas Hay Meadow. These possess known and marked differences in soil acidity (pH 3.6-5.9 in the 0-10 cm mineral soil layer) and microbial properties (Chapter 3). Upon these we experimentally increased soil pH by liming and determined the effects on microbial community composition and functioning.

We hypothesised that (i) an increase in soil pH would induce a shift in bacterial community composition towards the dominance of copiotrophic over oligotrophic taxa. Further, we tested the hypotheses that (ii) an increase in soil pH would increase microbial functioning, (iii) that these effects could be explained by changes to bacterial community composition and (iv) that composition mediated effects would be better explained by a shift in bacterial functional composition (mass-ratio hypothesis) than by diversity change (diversity hypothesis). We also predicted that (v) an increase in microbial community functional response following an increase in pH would be greater in microbial communities originating from less acidic soils, as those communities have an inherently higher abundance of copiotrophic taxa (Lauber *et al.*, 2009; Rousk *et al.*, 2010a) which are quicker to adapt to disturbance (De Vries and Shade, 2013).

5.3 Materials and methods

5.3.1 *Study site*

The soil used for the study was collected from the Palace Leas Hay Meadow Experiment (55˚13' N, 1˚41' W, UK National Grid Reference NZ 202912), located at Cockle Park Farm, 30 km north of Newcastle upon Tyne, England. The soil is classified as a pelo-stagnogley (Typic Ochraqualf) of the Hallsworth series (Jarvis *et al.*, 1977) with a clay-loam texture (41% sand, 29% silt, 30% clay). Palace Leas is a permanent grassland experiment, consisting of 14 unreplicated plots (the experiment pre-dates replicated and randomised designs) each of which (*c.* 120 m x 15 m) have received an annual application of farmyard manure (FYM) and/or mineral fertiliser since 1896, with the exception of an unfertilised control and treatment plot 14 which was added in 1976 (Chapter 2). FYM is applied in February and mineral fertilisers are applied in late March or early April. Treatment plots are harvested annually in early July to measure hay yield after which livestock freely graze the post-cut aftermath growth. As a result of long-term differences in the fertiliser treatments applied,

vegetation and soil properties differ markedly between the plots (Chapter 2; Shiel and Rimmer, 1984; Hopkins *et al.,* 2011). For full details of the experiment and its characteristics see Chapter 2.

5.3.2 *Experiment design*

The study was conducted using soil collected from four fertiliser treatment plots which were placed in pots, and treated with three rates of lime application, with four replicates for each soil x lime treatment combination resulting in 48 pots in total. We selected four contrasting fertiliser treatments (plot 2 = 20 t FYM ha^{-1}, plot 6 = control (no fertiliser), plot 7 = 35 kg N ha^{-1}, plot 8 = 60 kg P ha^{-1}) to represent a range of soils at different levels of acidity. However long-term fertilisation has also significantly affected a range of other soil and vegetation properties in these plots (Chapter 2). In August 2014 soils were collected from each plot at four replicate positions (24 m, 48 m, 72 m and 96 m from the western edge). At each replicate position, *c.* 2 kg of field moist soil was removed from the upper 10 cm of the mineral soil using 15 soil cores (10 cm depth, 5 cm diam.). Soil taken from the same replicate sampling point was then gently sieved (4 mm) and homogenised, and soil moisture content (dried at 105°C until constant weight) and water holding capacity (WHC; gravimetric water content of saturated soil) was determined. Soil from each replicate sampling point was weighed out into 3 x 500 g subsamples. Subsamples were amended with lime at rates of 0 g in the control treatment, 0.6 g in the intermediate lime treatment (equivalent to 0.55 t ha^{-1}) and 1.2 g in the high lime treatment (equivalent to 1.1 t ha^{-1}), mixed thoroughly and transferred into 1.5 L pots (12 cm depth, 12 cm diam.). To determine the amount of lime required to induce a 1 unit increase in pH in the soils from the four fertiliser treatment plots, a pilot lime requirement study was undertaken. To each pot, *Anthoxanthum odoratum* seeds (0.5 g) were spread evenly across the soil surface and covered with a thin layer of soil. *Anthoxanthum odoratum* was chosen as it grows well across a wide pH range (Davies and Snaydon, 1973) and is present on each of the sampled treatment plots (Table 2.4) . Pots were maintained at *c.*40% of soil WHC via an automatic watering system (Hydrospike®-HS-300) which enabled the uniform distribution of water within the pot, and by manual watering when necessary. Pots were organised in a randomised block design and kept in a controlled glasshouse (light/dark; 16/8 h, 18°C for 16 h followed by 10°C for 8 h) for 180 days. Following the end of the experiment, vegetation was cut to the soil surface and the soils were destructively sampled and transferred to a laboratory within 2 h. Fresh soils were homogenised and passed through a 2 mm sieve. 10 g of soil from each pot was frozen at -80°C for Illumina 16S rRNA sequencing analysis, whilst the remaining soil was divided into two portions, one portion was air-dried at room

temperature until no further mass loss for soil chemical analyses, whilst the other was stored in the dark at 4˚C for microbial catabolic profiling analysis. All soil chemical and biological properties were analysed after the experiment.

5.3.3 *Above- and below-ground biomass*

Aboveground biomass was harvested at four intervals throughout the experiment (days 45, 90, 135 and 180) by clipping the vegetation at 2 cm above the soil surface. At each interval, fresh samples were weighed, oven dried at 65˚C to constant mass and reweighed to determine yield. Yields for each interval were summed to determine the cumulative experiment yield for each pot. After 180 days, before soils were destructively sampled, duplicate soil cores (10 cm depth, 2 cm diam.) were taken from the middle of each pot in order to estimate belowground plant biomass. The contents of the cores were gently rinsed with distilled water through a 1 mm sieve, and the root material was oven dried at 65 ˚C to constant mass and weighed. Belowground plant biomass is expressed as g per pot by extrapolating the volume of the cores to the volume of the pots. Oven dried aboveground and belowground plant samples were milled to a fine powder and a 0.1 subsample was analysed for C and N concentrations using a Vario Macro Cube (Elementar, Hanau, Germany).

5.3.4 *Soil chemical analyses*

Soil pH was measured using 10 g (DW) of soil in a 1:2.5 soil-water suspension. 10g of soil (DW) was ground in a mortar and a 0.1 g subsample was analysed for total C concentrations using a Vario Macro Cube. Inorganic C concentrations were determined by burning 5 g (DW) of ground soil in a muffle furnace for 16 h at 550˚C to remove organic C and a 0.1 g ash sample was then analysed using the aforementioned Vario Macro Cube. The organic C concentration was calculated as the difference between the total and inorganic C.

5.3.5 *Microbial catabolic profiling*

We analysed microbial communities' catabolic profile using the MicroResp^{TM} CO_2 detection system (Campbell *et al.*, 2003). In order to re-establish active microbial communities, soils were pre-incubated at 40% of their WHC at 25°C for 5 days. After pre-incubation, 16 x 0.3 g of each soil sample was dispensed into a 96 deep well plate. These wells contained aqueous solutions of fourteen different low-molecular weight (LMW) substrates and two distilled water controls. The substrates used included a range of amino acids (L-alanine, L-asparagine, L-glutamine, γ-amino-butyric acid, N-acetyl-glucosamine), carbohydrates (D-fructose, D-galactose, D-glucose, D-mannose, L-arabinose), carboxylic acids (citric acid, L-malic acid,

oxalic acid) and a phenolic acid (syringic acid). Substrates were dissolved in water and applied in 25 µl aliquots to provide a concentration of 30 mg g^{-1} soil water. However, due to the low solubility of L-alanine, L-asparagine, L-glutamine, and N-acetyl-glucosamine they were applied at 7.5 mg g^{-1} soil water and syringic acid at 0.3 mg g^{-1} soil water. To minimise the influence of abiotic CO_2, plates were left uncovered at room temperature for 2 h after substrate addition to allow abiotic CO_2 release (García-Palacios *et al.*, 2011; Creamer *et al.*, 2016). Subsequently, a 96 well microtiter colorimetric CO_2 detection plate was inverted and fitted to the top of the 96 deep well plate using a rubber gasket seal and securely clamped (Campbell *et al.*, 2003). Vessels were then incubated at 25°C in the dark for 4 hours. Detection microplates were read at 570 nm on a microplate reader (SpectraMax Plus, Molecular Devices) directly before being fitted to the deep well (0 h) and immediately after the incubation period (4 h). CO_2 evolved during the incubation was corrected for differences in basal respiration (minus the CO_2 response to the control). We created an absorbance versus CO_2 concentration calibration curve by applying different concentrations of CO_2 gas to breakable CombiStrips (Thermo LifeSciences) held in 40ml sealed glass vials which were incubated at 25°C for 4 h, reassembled in a plate carriage and re-read (Campbell *et al.*, 2003). CO_2 evolved was determined as the difference in absorbance at 0 h and 4 h for each well, expressed as µg CO_2-C g^{-1} h^{-1} as described by Campbell *et al.* (2003).

We used principal components analysis (CANOCO 4.5; ter Braak and Smilauer, 2002) to simplify the analysis of microbial catabolic profiling data by combining the measures of microbial respiratory response to 14 LMW substrates. The first principal component (PC1) explained 81.6% of the total variation in the data, and was subsequently used as a single 'microbial catabolic capacity' variable. All 14 microbial catabolic profiling response variables were significantly positively correlated with PC1 (r=0.73-0.98; Appendix C, Appendix D), therefore increases in PC1 scores represent a greater catabolic capacity of the microbial community. By applying a glucose substrate it was also possible to estimate active soil microbial biomass via the substrate-induced respiration method (Anderson and Domsch, 1978).

5.3.6 *Illumina high-throughput sequencing analysis*

Genomic DNA was extracted from 0.5 g of soil sample using a FastDNA Spin Kit (MP Biomedicals, UK) and a FastPrep Ribolyser (MP Biomedicals, France) according to the manufacturer's instructions. The FastPrep machine was set at 6 m s^{-1} for 30 seconds. A procedural blank was carried out using 250 µl of microbiological grade filter sterilised water

(Microzone, UK) in place of soil to confirm that the kits were clear of contaminants. The presence of extracted DNA was checked by agarose gel electrophoresis, DNA extracts were analysed in 0.7 % (w/v) agarose gels, made in 1 x TAE (tris-acetate-EDTA) buffer (40 mM tris, 20 mM acetic acid, 1mM EDTA, pH 8.3) and stained with 1.6 µl of ethidium bromide. For each genomic DNA sample, 5 µl of extract plus 2 µl of 6 x loading buffer (0.25 % bromophenol blue, 0.25 % xylene cyanol FF, and 30 % glycerol in filter sterile water; Sigma, UK) was run on the gel at 100 V constant current for 60 minutes. 2 µl of λ-DNA Hind III Digest marker (Sigma, UK), heated to 60 °C for 3 minutes, was run alongside DNA extracts to assess the relative yields of linear double stranded DNA molecules between 125bp and 23.1 kb. The agarose gels were visualised and photographed using a UV trans-illuminator (UVP iBox, UVP, UK). DNA extracts were stored at -20°C until further use.

DNA extracts from the samples were amplified using 16S bacterial primers (515f and 806r primers; Caporaso *et al.,* 2011) targeting the variable region 4 (V4). All sequencing was conducted by NU-OMICS (Northumbria University, UK) based on the Schloss wet-lab MiSeq standard operation procedure (Kozich *et al.*, 2013). PCR products were sequenced using an Illumina MiSeq Desktop sequencer to produce paired end reads. Sequences were provided demultiplexed and then analysed using the Quantitative Insights into Microbial Ecology pipeline (QIIME, Caporaso *et al.,* 2010a) after checking sequence quality using FastQC software (Andrews, 2014). Briefly, for bacterial sequences, the UCLUST algorithm (Edgar, 2010) was employed for picking operational taxonomic units (OTU). OTUs were picked at a 97% similarity level (species-equivalent) and singletons were excluded from the dataset. PyNAST (Caporaso *et al.*, 2010b) was used for sequence alignment, chimeric sequences were identified with ChimeraSlayer (Haas *et al.*, 2011) and the Greengenes reference database version 12_10 was used for bacterial taxonomy assignment (McDonald *et al.*, 2012).

To avoid heterogeneity in sequencing depth, samples were rarefied to 350 sequences/sample. Due to insufficient sequence coverage (<350 sequences/sample) five samples were discarded from further analysis, although each treatment combination still had at least three replicates. A script in QIIME was used to compute three alpha diversity indices using the rarefied OTU data; Shannon's diversity, Simpson's diversity and OTU richness. Overall, 76,525 quality sequences and 4917 bacterial OTUs were identified. Of the total number of sequences, 99% were classified to the phylum level, 23% to the genus level and 0.5% to the species level.

5.3.7 *Statistical analysis*

The effects of lime amendment, original fertiliser treatment and their interaction on soil, vegetation and microbial response variables were analysed using generalised linear models (GLM) and likelihood ratio F tests (LRTs). Minimum adequate models were produced by initially fitting maximal models that contained the main effects (lime amendment and fertiliser treatment) and their interaction. Maximal models were then simplified using a manual stepwise procedure; non-significant terms were sequentially deleted from the maximal models and compared to the previous more complex model using LRTs and ANOVA until only significant terms remained ($P<0.05$) (Crawley, 2007). Where main effects and the interaction between factors were significant, differences between factor levels were identified using Tukey's HSD post-hoc test in the multcomp package (Hothorn *et al.*, 2008). To assess if the impacts of liming on a change in microbial catabolic capacity was dependent on the pH of the soil in the original fertiliser treatment plot, a filled contour plot was created using the function visgred2d for the visreg package (Breheny and Burchett, 2012).

Mediation model analysis (a form of structural equation modelling) was used to simultaneously analyse hypothesised direct and indirect effects of a change in soil pH on a change in microbial catabolic capacity (change in limed treatments relative to the control), using the lavaan package in R (Rosseel, 2012). Based on *a priori* knowledge, we created a simple hypothetical model as the structure for the analysis (Figure 5.1). We included an indirect pathway (via bacterial community composition) in order to assess if the effects of a change in bacterial community composition on microbial catabolic capacity were better explained by the 'mass-ratio hypothesis' or the 'diversity hypothesis'. To represent the 'mass-ratio hypothesis' we used the relative abundances of two higher-level bacterial clades with contrasting functional traits (according to Fierer *et al.*, 2007); *Acidobacteria* (oligotrophic taxa) and *Betaproteobacteria* (copiotrophic taxa) and an integrating Copiotrophic:Oligotrophic ratio. We calculated the Copiotrophic:Oligotrophic ratio as the sum of the relative abundances of phyla considered to be copiotrophic (*Actinobacteria, Alphaproteobacteria, Bacteroidetes, Betaproteobacteria, Firmicutes* and *Gammaproteobacteria*) compared to the sum of the relative abundances of bacterial clades considered to be oligotrophic (*Acidobacteria, Chloroflexi, Deltaproteobacteria, Planctomycetes* and *Verrucomicrobia*). To represent the 'diversity hypothesis' we used three bacterial diversity indices; Shannon's diversity, Simpson's diversity and OTU richness. The best fitting bacterial community composition measure was selected as the model that produced the lowest Akaike's Information Criterion (AIC) and a significant *P* value leading to

and from the mediating variable. A direct pathway from a change in soil pH to a change in microbial catabolic capacity was also included to account for all mechanisms not operating via a shift in bacterial community composition. For example, a change in abiotic and biotic conditions following liming may induce a physiological change within the already-established ("resident" hereafter) community (De Nobili *et al.*, 2001; Shade *et al.*, 2012). Standardised path coefficients (SPC) were calculated to enable comparison between the hypothesised pathways. Bollen-Stine bootstrapping was employed to deal with non-normal data, nullifying potentially adverse effects on the standard errors of parameter estimates (Grace, 2006). All statistical tests were performed using R version 3.2.5 (R Core Team, 2015).

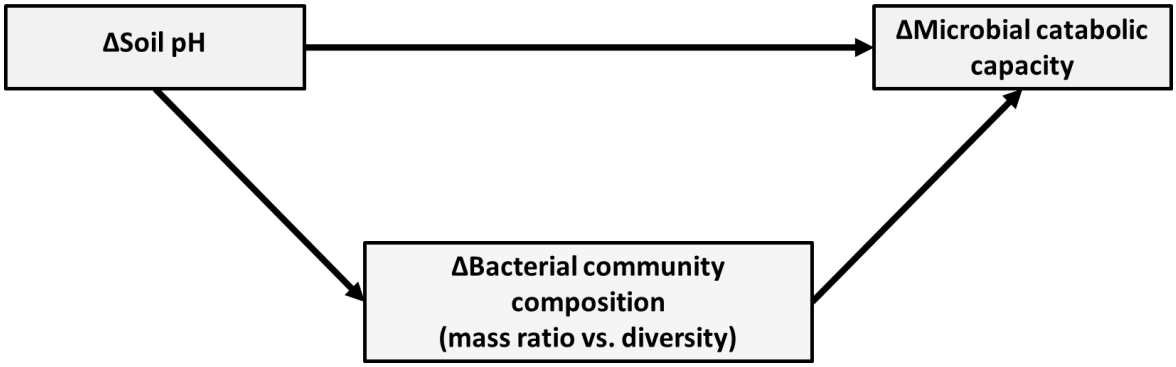

Figure 5.1 Hypothetical diagram of potential causal relationships between the direct and indirect (diversity and mass ratio indices) effects of soil pH on microbial catabolic capacity.

5.4 Results

5.4.1 *Effect of lime amendment and fertiliser treatment on soil and plant properties*

Lime amendment treatment significantly increased soil pH ($F_{2,36}$ = 150.71, P<0.001; Table 5.1). Lime amendment increased soil pH by ~0.45 units between the control and the intermediate liming treatment and by ~0.95 units between the control and the high liming treatment (Figure 5.2 a). The original fertiliser treatment also significantly affected soil pH ($F_{3,36}$ = 342.72, P<0.001; Table 5.1). The FYM fertilised plot 2 had significantly higher pH than all other fertiliser treatments, whilst the N fertilised plot 7 had the lowest (Figure 5.2 a). SOC content depended on the original fertiliser treatment ($F_{3,36}$ = 27.48, P<0.001; Table 5.1) but not lime amendment. SOC content was higher in plot 2 and lower in plot 7 (Figure 5.2 b). Active microbial biomass was significantly increased by lime amendment and also affected by the original fertiliser treatment ($F_{2,36}$ = 5.04, P = 0.012, $F_{3,36}$ = 147.46, P<0.001, respectively; Table 5.1). Similarly to patterns observed for soil pH and SOC, active microbial

biomass was highest in FYM treated plot 2 and lowest in the low pH N fertilised plot 7 (Figure 5.2 c).

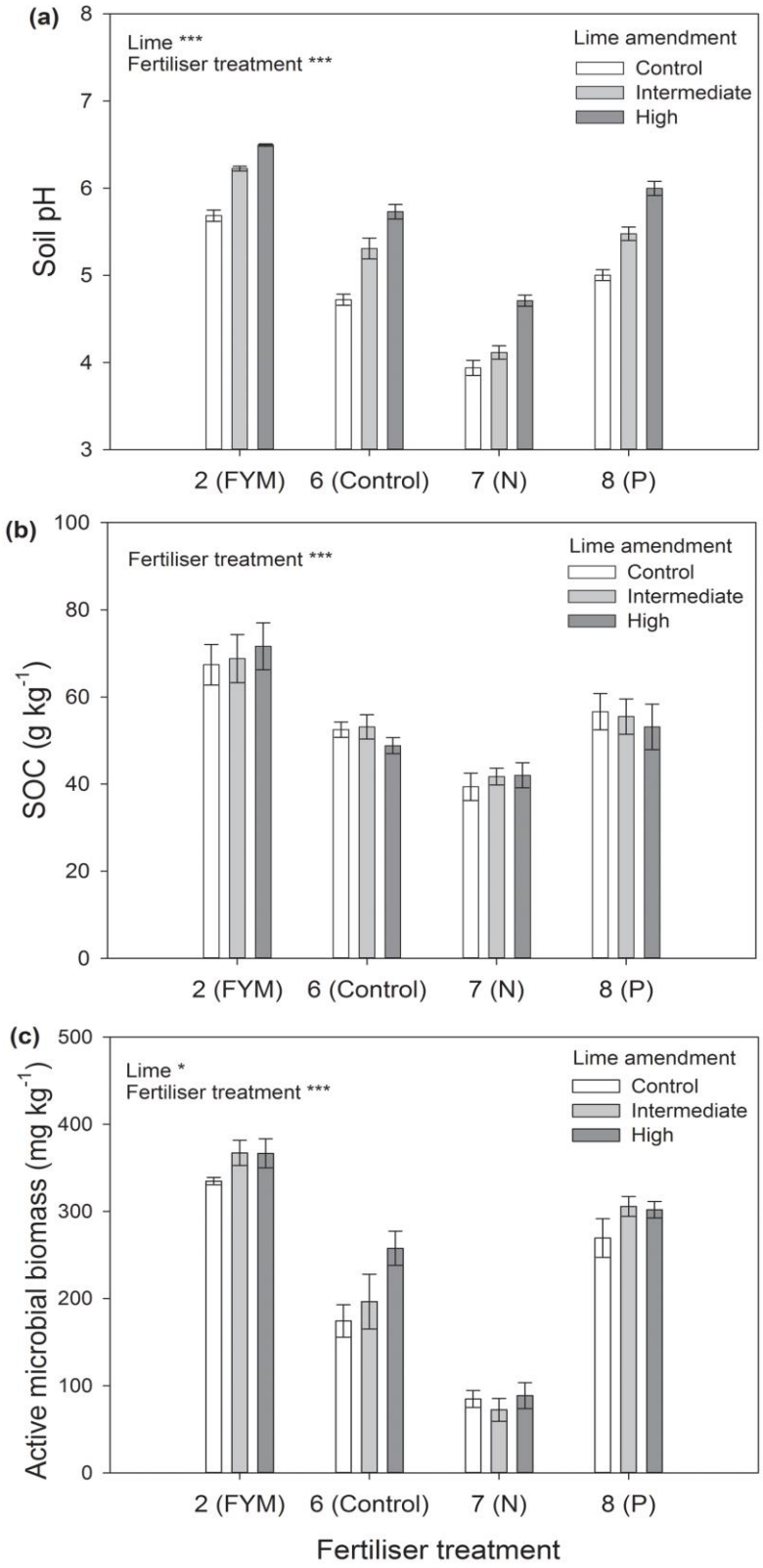

Figure 5.2 . Soil pH (a), SOC (b) and active microbial biomass (c) as affected by lime amendment and fertiliser treatment. Significance at P<0.001;***, P<0.01;**, P<0.05;*. Data are means (n=4) and error bars represent ± SEM.

	Lime amendment		Fertiliser treatment		Lime amendment x Fertiliser treatment	
	F	*P*	F	*P*	F	*P*
Soil						
pH	**150.71**	**<0.001**	**342.72**	**<0.001**	2.15	0.072
SOC	0.06	0.940	**27.48**	**<0.001**	0.32	0.921
Active microbial biomass	**5.04**	**0.012**	**147.46**	**<0.001**	1.45	0.222
Plant						
Aboveground:belowground biomass ratio	**2.99**	**0.039**	**4.61**	**0.011**	0.41	0.867
Aboveground biomass C:N	**4.78**	**0.014**	**5.44**	**0.003**	0.53	0.779
Belowground biomass C:N	**3.96**	**0.028**	**7.24**	**<0.001**	**4.39**	**0.002**

Table 5.1 The effects of lime amendment and fertiliser treatment and their interaction on soil and plant properties. Significance level $P < 0.05$ are in bold.

The ratio of plant biomass aboveground to belowground was significantly affected by lime amendment and original fertiliser treatment ($F_{2,36} = 2.99$, $P = 0.039$, $F_{3,36} = 4.61$, $P = 0.011$, respectively; Table 5.1). Aboveground:belowground plant biomass ratio was significantly higher in the high lime amendment treatment than where no lime had been applied and significantly higher in plot 2 than plots 6 and 7 (Figure 5.3 a). Above- and below-ground plant biomass C:N depended on both lime amendment ($F_{2,36} = 4.78$, $P = 0.014$ and $F_{2,36} = 3.96$, $P = 0.028$, respectively) and original fertiliser treatment ($F_{3,36} = 5.44$, $P = 0.003$ and $F_{3,36} = 7.24$, $P<0.001$, respectively; Table 5.1). Above- and below-ground plant biomass C:N was significantly higher where no lime had been applied than the high rate of lime (Figure 5.3 b and c). Aboveground plant biomass C:N was significantly higher in the FYM treated plot 2 than the unfertilised plot 6 and the N amended plot 7, whilst belowground plant biomass C:N was greater in unfertilised plot 6 than the fertilised plots 2 and 7. There was also a significant interaction between the two treatments ($F_{6,31} = 4.39$, $P = 0.002$; Table 5.1), reflecting the fact that lime amendment effects were more pronounced on belowground plant biomass C:N in the P treated plot 8.

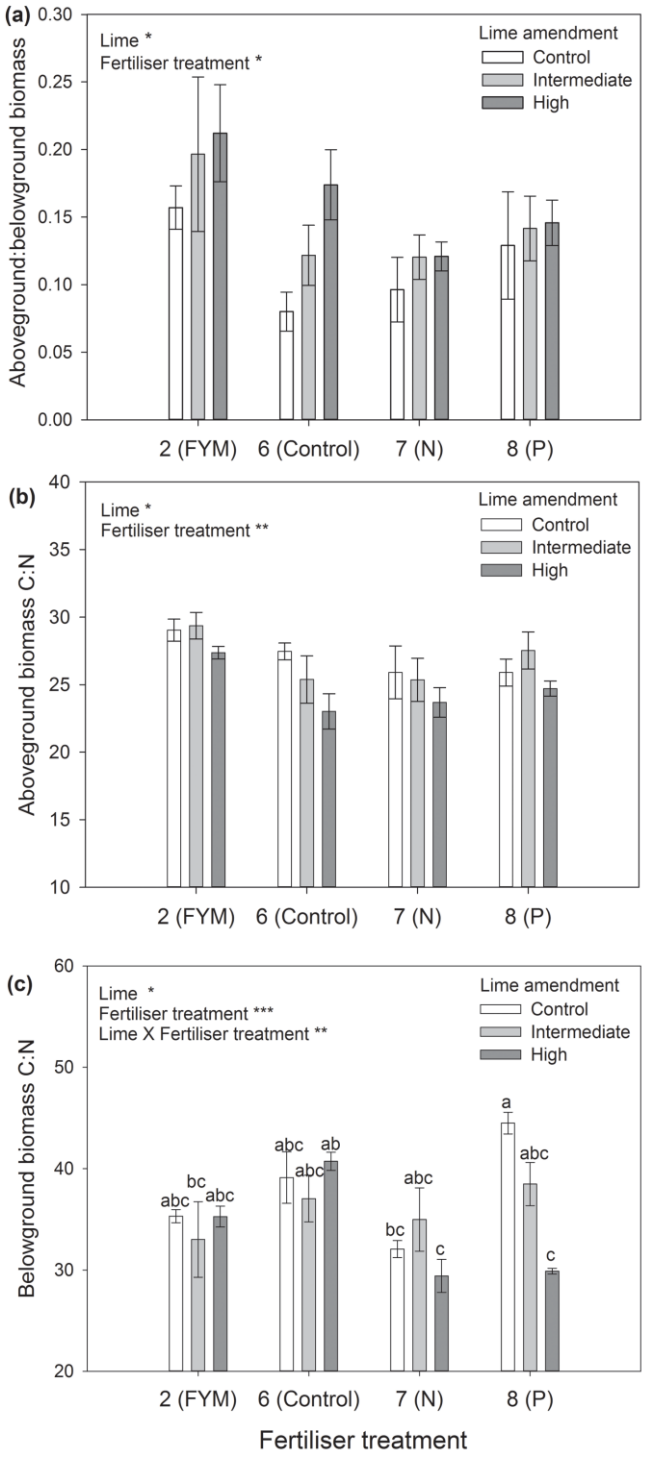

Figure 5.3 Aboveground:belowground plant biomass ratio (a), aboveground plant biomass C:N (b), belowground plant biomass (c) and belowground plant biomass C:N (d) as affected by lime amendment and fertiliser treatment. Significance at P<0.001;***, P<0.01;**, P<0.05;*. Contrasting letters denote significant differences between treatments (P < 0.05) and are shown for the variable where the interaction was significant only. Data are means (n=4) and error bars represent ± SEM.

5.4.2 *Effect of lime amendment and fertiliser treatment on microbial function*

Microbial catabolic capacity (PC1) was significantly increased by lime amendment ($F_{2,36}$ = 12.44, P<0.001), was dependent upon original fertiliser treatment ($F_{3,36}$ = 281.34, P<0.001) and there was also an interaction between factors ($F_{6,36}$ = 2.70, P = 0.029; Table 5.2). Microbial catabolic capacity was highest in plots originally of intermediate pH (plots 2 and 8). Interaction effects were only observed in the unfertilised plot 6, with significantly greater effects in the high lime amendment treatment than the treatment receiving no lime (Figure 5.4). The interaction effect was visualised using a filled contour plot (Figure 5.5), which shows that the observed relationship between lime amendment and the change in microbial catabolic capacity was dependent on the initial soil pH. The greatest increase in microbial catabolic capacity (+150-200%) occurred in response to high lime amendment in soils with an original pH of 4.7-5.2. Similarly, in soils originally of pH 5.2-5.7 microbial catabolic capacity increased in response to the intermediate lime amendment (+100%). However, there was no further change at the high amendment rate. Below pH 4.2 lime amendment had only minor effects (Figure 5.5), thus indicating that catabolic capacity of microbial communities in very acid soils is relatively insensitive to pH increases.

	Lime amendment		Fertiliser treatment		Lime amendment x Fertiliser treatment	
Phylum	F	*P*	F	*P*	F	*P*
Acidobacteria	0.28	0.757	**21.95**	**<0.001**	0.48	0.812
Actinobacteria	0.43	0.656	**10.62**	**<0.001**	0.89	0.513
*Alphaproteobacteria**	0.21	0.814	**5.45**	**0.004**	1.90	0.111
Bacteroidetes	2.42	0.105	**6.25**	**0.002**	**3.29**	**0.013**
*Betaproteobacteria**	0.14	0.869	**37.61**	**<0.001**	1.47	0.220
Chloroflexi	0.98	0.386	**7.56**	**<0.001**	1.28	0.293
*Deltaproteobacteria**	0.29	0.753	**3.83**	**0.019**	0.93	0.490
Firmicutes	0.22	0.801	**5.30**	**0.005**	1.76	0.142
*Gammaproteobacteria**	0.17	0.839	**3.02**	**0.044**	1.01	0.432
Planctomycetes	1.70	0.198	**4.92**	**0.007**	2.15	0.076
Verrucomicrobia	0.52	0.599	**21.94**	**<0.001**	0.76	0.605
Diversity						
OTU richness	0.19	0.830	**10.25**	**<0.001**	1.02	0.431
Shannon's diversity	0.33	0.724	**17.21**	**<0.001**	0.32	0.921
Simpson's diversity	0.25	0.779	**9.37**	**<0.001**	0.47	0.824
Function						
Microbial Catabolic Capacity	**12.44**	**<0.001**	**281.34**	**<0.001**	**2.70**	**0.029**

Table 5.2 The effects of lime amendment and fertiliser treatment and their interaction on the relative abundance of higher-level bacterial taxa (with a mean >1% across treatments) and bacterial diversity indices. Significance level $P < 0.05$ are in bold. * denotes subphylum of *Proteobacteria*.

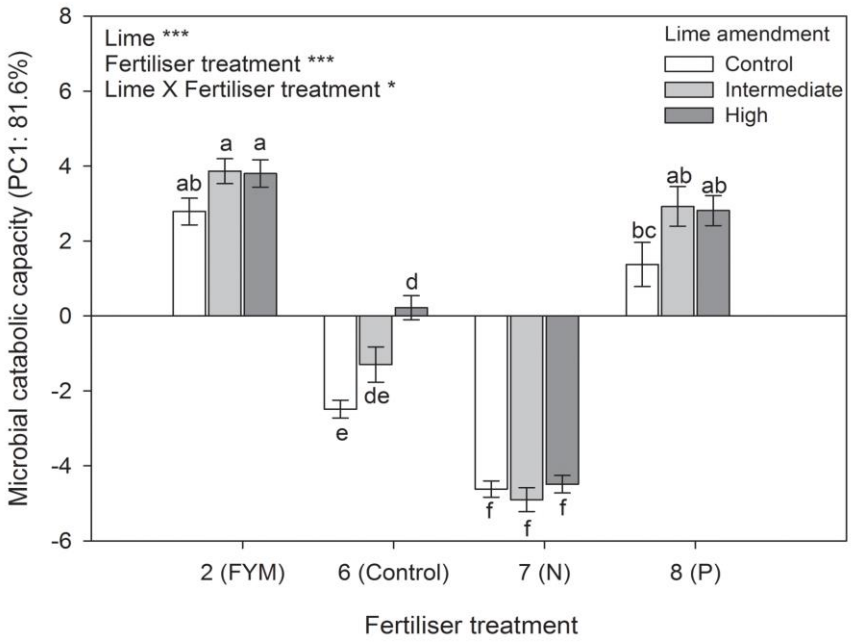

Figure 5.4 Microbial catabolic capacity (PC1) as affected by lime amendment and fertiliser treatment. Significance at P<0.001;***, P<0.01;**, P<0.05;*. Contrasting letters denote significant differences between treatments (P < 0.05). Data are means (n=4) and error bars represent ± SEM.

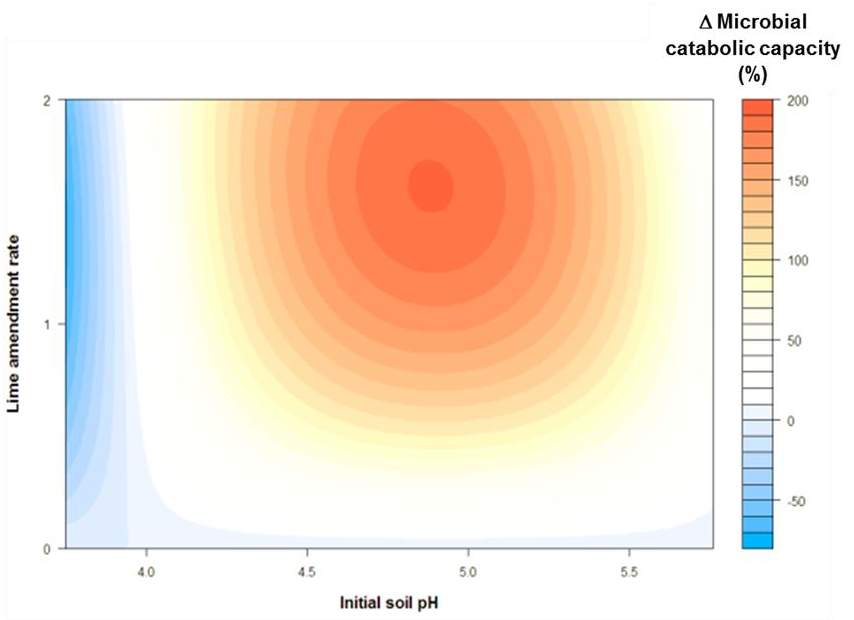

Figure 5.5 Fitted multiple quadratic regression relationship of a change in microbial catabolic capacity as a function of initial soil pH of the fertiliser treatment and lime amendment rate. Lime amendment: 0 = 0 t ha^{-1}, 1 = 0.55 t ha^{-1}, 2 = 1.1 t ha^{-1}. The change in microbial catabolic capacity at the intermediate and high lime amendment rate for each data point was calculated as the difference in PC1 scores from the corresponding soil receiving no lime (control).

5.4.3 *Effect of lime amendment and fertiliser treatment on microbial community composition and diversity*

Averaged across all samples, the bacterial community was dominated by the following higher-level bacterial taxa; *Acidobacteria* (24%), *Actinobacteria* (11%), *Alphaproteobacteria* (11%), *Gammaproteobacteria* (5%), *Planctomycetes* (10%) and *Verrucomicrobia* (12%) (Figure 5.6). The relative abundances of the most dominant bacteria at phylum and subphylum levels (>5%) were not significantly affected by lime amendment, but the abundance of all phyla and subphyla were significantly different between the original fertiliser treatments (Table 5.2, Figure 5.6). Only the relative abundance of *Bacteroidetes* was significantly affected by the lime amendment x fertiliser treatment interaction ($F_{6,31} = 3.29$, $P = 0.013$); this group were ~6.0% less abundant in the limed treatments of least acidic plot 2 (Table 5.2, Figure 5.6). This response of *Bacteroidetes* appeared to be determined by the order *Bacteroidales*, which in plot 2 were significantly less abundant in limed treatments than in the control ($F_{6,31} = 4.03$, $P = 0.004$; Table 5.3). Similarly to the patterns observed at the phylum level, the relative abundance of bacteria in the most dominant classes and OTUs were generally significantly affected by the original fertiliser treatment but not by lime amendment (Table 5.3 and Appendix E), thus demonstrating that lime amendment did not affect bacterial community composition at either coarse nor fine levels of taxonomic resolution. At the individual OTU level, lime amendment and its interaction with fertilisation history had significant effects on only 4 of the 40 most abundant OTUs (Appendix E). The relative abundance of an OTU belonging to the *Planctomycetes* phylum (family; *Gemmataceae*) was significantly reduced (-1.54%) by high lime amendment ($F_{2,36} = 7.13$, $P = 0.002$) and even lower in the intermediate lime amendment treatment (-3.71%) in the soils of the unfertilised plot 6 ($F_{6,31} = 2.92$, $P = 0.022$). In contrast, an OTU from the *Firmicutes* phylum (genus; *Bacillus*) significantly increased in abundance (~+0.5%) in response to liming ($F_{2,36} = 5.64$, $P = 0.008$). The relative abundance of an OTU from the *Bacteroidetes* phylum (order; *Bacteroidales*) was significantly reduced by liming (-2.93%) in plot 2 ($F_{6,31} = 4.76$, $P = 0.002$), whilst the relative abundance of an OTU in the *Alphaproteobacteria* subphylum (family; *Hyphomicrobiaceae*) was significantly higher in the intermediate (+1.21%) than the high lime amendment treatment in plot 6 ($F_{6,31} = 2.85$, $P = 0.025$; Appendix E). Despite changes in the abundance of a very low number of OTUs, those OTUs that were significantly affected amounted to a minor proportion of the whole bacterial community (0.75-3.3%).

Due to the general resistance of bacterial community composition to liming, bacterial diversity as measured by Shannon's diversity, Simpson's diversity and OTU richness was not

statistically significantly different between lime amendment treatments, but it was significantly affected by the original fertiliser treatment ($F_{3,31}$ = 17.21, P<0.001, $F_{3,31}$ = 9.37, P<0.001 and $F_{3,31}$ = 10.36, P<0.001, respectively; Table 5.2). Bacterial diversity was greater in less acid fertiliser treatments and was significantly higher in plot 2 than the highly acidic plot 7 (Figure 5.7 a-c). Interactions between lime amendment and original fertiliser treatment were not significant for any of the diversity indices.

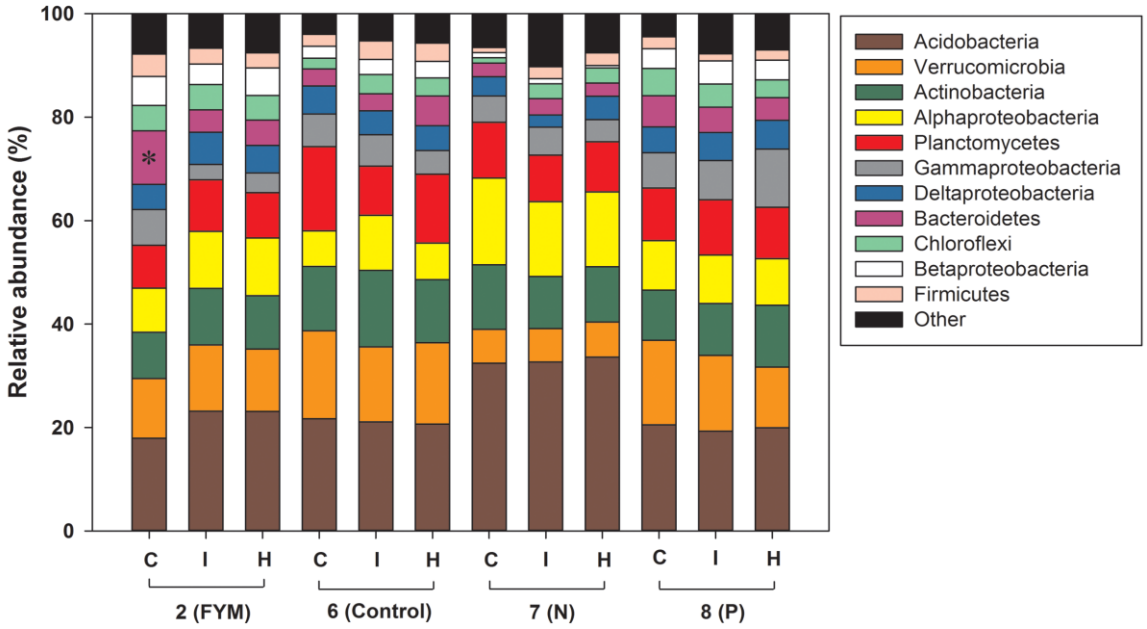

Figure 5.6 Relative abundance of major bacterial taxa as affected by lime amendment and fertiliser treatment. C = Control (no lime), I = Intermediate lime amendment and H = High lime amendment. Only major bacterial taxa which account for >1% of total OTU abundance are shown while those <1% are grouped as other. *denotes significant interactions (lime amendment x fertiliser treatment; P<0.05).

Phylum/subphylum	**Class**	Lime amendment		Fertiliser treatment		Lime amendment x Fertiliser treatment	
		F	*P*	F	*P*	F	*P*
Acidobacteria	*Acidobacteriia*	0.13	0.875	**79.65**	**<0.001**	0.22	0.967
	Acidobacteria-Gp6	0.08	0.915	**24.13**	**<0.001**	0.34	0.901
	Solibacteres	0.68	0.516	**20.29**	**<0.001**	0.53	0.800
Actinobacteria	*Actinobacteria*	0.35	0.908	**24.73**	**<0.001**	0.34	0.908
	Thermoleophilia	1.17	0.324	**19.51**	**<0.001**	1.32	0.277
Alphaproteobacteria	*Rhizobiales**	0.29	0.749	**7.65**	**<0.001**	1.48	0.216
	*Rhodospirillales**	1.65	0.209	**12.77**	**<0.001**	0.97	0.458
Bacteroidetes	*Saprospirae*	0.48	0.620	**11.91**	**<0.001**	0.77	0.597
	Bacteroidia	1.71	0.184	2.92	0.068	**4.03**	**0.004**
Chloroflexi	*Ellin6529*	1.36	0.273	**74.51**	**<0.001**	1.67	0.162
Deltaproteobacteria	*Myxococcales**	0.24	0.785	**3.31**	**0.032**	1.17	0.344
	*Desulfuromonadales**	0.90	0.509	0.95	0.428	0.27	0.762
Firmicutes	*Bacilli*	0.46	0.831	**3.63**	**0.023**	1.27	0.292
Gammaproteobacteria	*Xanthomonadales**	0.82	0.450	2.670	0.064	1.22	0.324
	*Enterobacteriales**	1.38	0.265	**7.14**	**<0.001**	2.37	0.053
	*Pseudomonadales**	0.67	0.514	2.60	0.070	1.18	0.340
Planctomycetes	*Planctomycetia*	**5.62**	**0.008**	**17.43**	**<0.001**	2.31	0.058
	Phycisphaerae	0.45	0.644	**3.28**	**0.034**	0.99	0.448
Verrucomicrobia	*Pedosphaerae*	1.35	0.273	**4.41**	**0.011**	1.26	0.303
	Spartobacteria	0.68	0.513	**40.22**	**<0.001**	1.20	0.331

Table 5.3 The effects of lime amendment and fertiliser treatment and their interaction on the relative abundance of the 20 most dominant bacterial taxonomic classes. Significance level P < 0.05 are in bold. * denotes order of the *Proteobacteria* subphylum.

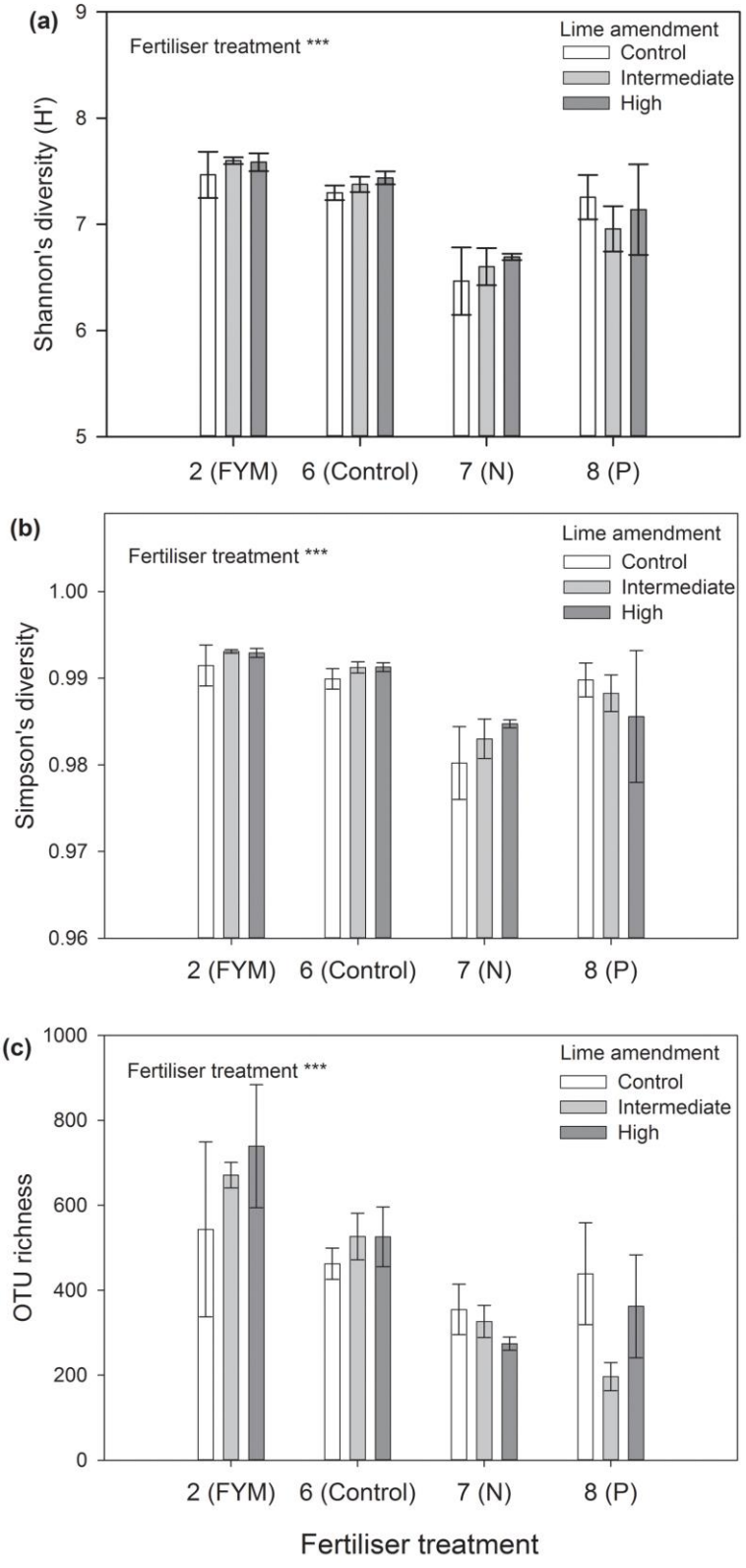

Figure 5.7 Bacterial diversity indices as affected by lime amendment and fertiliser treatment. Significance at P<0.001;***, P<0.01;**, P<0.05;*. Contrasting letters denote significant differences between treatments (P < 0.05). Data are means (n=4) and error bars represent ± SEM.

5.4.4 *Relationship between soil pH, bacterial community composition and microbial catabolic capacity*

In the mediation models the direct effects of a change in soil pH (limed – control) on a change in microbial catabolic capacity (limed – control) were significant; a change in soil pH was positively related to a change in microbial catabolic capacity (SPC = 0.34; Figure 5.8). However, the effects of a change in soil pH explained a relatively low proportion of the total variance of a change in microbial catabolic capacity (R^2 = 0.12). There was little evidence that changes in microbial community composition (limed – control) regulated the response of microbial catabolic capacity as neither measures representing the diversity hypothesis nor the mass-ratio hypothesis were significant (Table 5.4).

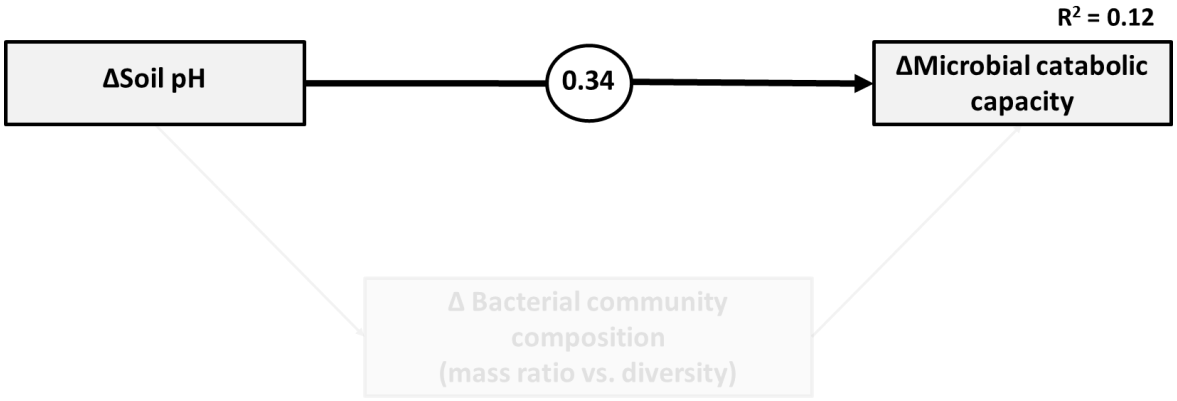

Figure 5.8 Mediation analysis model for a change in microbial catabolic capacity as a function of the direct and indirect effects (via bacterial community composition) of a change in soil pH.

Bacterial community composition mediated effect	AIC	ΔAIC	Path	Estimate	Standard Error	Path P value	Model P value	R^2
ΔShannon's diversity	362	0	ΔpH → ΔMCC	0.40	0.16	0.01	0.59	0.19
			ΔpH → ΔShannon's diversity	0.20	0.13	0.11		0.04
			ΔShannon's diversity → ΔMCC	-0.28	0.14	0.06		
ΔSimpson's diversity	366	4	ΔpH → ΔMCC	0.35	0.15	0.02	0.17	0.13
			ΔpH → ΔSimpson's diversity	0.11	0.16	0.49		0.01
			ΔSimpson's diversity → ΔMCC	-0.13	0.15	0.39		
ΔOTU richness	363	1	ΔpH → ΔMCC	0.38	0.15	0.01	0.58	0.20
			ΔpH → ΔOTU richness	0.14	0.12	0.21		0.02
			ΔOTU richness → ΔMCC	-0.29	0.14	0.06		
Δ*Acidobacteria*	368	6	ΔpH → ΔMCC	0.34	0.14	0.02	0.20	0.12
			ΔpH → Δ*Acidobacteria*	-0.01	0.12	0.96		0.00
			Δ*Acidobacteria* → ΔMCC	-0.07	0.14	0.61		
Δ*Betaproteobacteria*	367	5	ΔpH → ΔMCC	0.35	0.14	0.01	0.00	0.12
			ΔpH → Δ*Betaproteobacteria*	0.16	0.12	0.18		0.03
			Δ*Betaproteobacteria* → ΔMCC	-0.04	0.15	0.80		
ΔCopiotrophic:ΔOligotrophic ratio	367	5	ΔpH → ΔMCC	0.34	0.14	0.02	0.23	0.13
			ΔpH → ΔCopiotrophic:ΔOligotrophic	-0.04	0.12	0.75		0.00
			ΔCopiotrophic: ΔOligotrophic → ΔMCC	0.13	0.17	0.45		

Table 5.4 Mediation analysis for microbial catabolic capacity (ΔMCC) as a function of hypothesised direct (ΔpH →ΔMCC) and indirect effects (ΔpH → Δbacterial community composition → ΔMCC) of soil pH. Bacterial community composition mediated effects could be explained via diversity (ΔShannon's diversity, ΔSimpson's diversity and ΔOTU richness) and mass ratio indices (Δrelative abundance of *Acidobacteria*, *Betaproteobacteria* and a ΔCopiotrophic:ΔOligotrophic ratio). Models were compared using AIC to determine the most parsimonious plant pathway, the pathway judged to be the most appropriate had the lowest AIC. Values shown for each model are AIC, ΔAIC (difference in AIC between a model and the model with the lowest AIC score), the P value associated with the χ^2 and the R^2 for mediating variable and MCC.

5.5 Discussion

In this study, we hypothesised that a liming-induced increase in soil pH would be associated with a shift in bacterial community composition towards dominance of taxa characterised as having copiotrophic (or *r*-selected) life history strategies over those with oligotrophic (or *K*-selected) life history strategies. According to the copiotrophic-oligotrophic concept (Fierer *et al.*, 2007); copiotrophic taxa are adept at assimilating LMW compounds, possess fast turnover rates and thrive in areas of enhanced resource availability (Fierer *et al.*, 2007). It has been reported in the past that towards neutral pH the abundance of copiotrophic taxa including *Actinobacteria* and *Bacteroidetes* increase (Lauber *et al.*, 2009; Rousk *et al.*, 2010a), whilst *Acidobacteria*, considered to exhibit oligotrophic traits prefer acidic environments (Jones *et al.*, 2009; Griffiths *et al.*, 2011). Despite an increase in resource availability in limed soils, indicated by a greater allocation of biomass to aboveground plant organs and lower plant C:N ratios (Craine, 2006; Di Palo and Fornara, 2015) the bacterial community was resistant as evidenced by the general lack of significant effects on the abundance and diversity of bacterial taxa. Our first hypothesis was therefore not supported. At the highest taxonomic rank (phylum), only the relative abundance of *Bacteroidetes* was affected by liming and these changes were exclusive to soils originally fertilised by FYM (plot 2). This indicates that the effects of increasing soil pH on bacterial community composition are not uniform, but rather that they are contingent on the legacy effects of fertilisation. Lower abundance of OTUs belonging to the order *Bacteroidales* following liming was responsible for the observed effects on *Bacteroidetes*. There is little previous evidence to explain a relationship between *Bacteroidales* abundance and soil properties. However, at the phylum level *Bacteroidetes* are considered copiotrophs (Fierer *et al.*, 2007), meaning a decline in their abundance following an increase in soil pH is contrary to their suggested life strategy.

Negligible effects of a change in soil pH on bacterial community composition were unexpected given that bacteria have short generation times, meaning that they can turnover rapidly in response to changing environmental conditions (Schmidt *et al.*, 2007). Moreover, clear shifts in bacterial community composition in short-term microcosm studies have previously been reported. For example, in a 6 week experiment the abundance of copiotrophic phyla including *Actinobacteria* and *Gammaproteobacteria* was shown to respond positively to NH_4NO_3 amendment, whilst oligotrophs such as *Acidobacteria*, *Planctomycetes* and *Verrucomicrobia* responded negatively (Männistö *et al.*, 2016). Similarly, a separate study observed significant increases in copiotroph abundance just 24 hours after the addition of labile C substrates (glucose, glycine and citric acid) (Eilers *et al.*, 2010). Although our

findings relating to plant biomass properties suggest that resource availability increased in response to liming, such a change may not have been of great enough magnitude to override the long-term management effects to which the communities have adapted. This is supported by Carey *et al.* (2015), they reported that 5-6 years after the establishment of a field experiment subject to all combinations of exotic plant invasion, vegetation clipping and N fertilisation, bacterial community composition was largely unchanged. This was attributed to strong temporal variation in soil conditions; therefore the treatments may not have elucidated an effect great enough to require compositional shift away from the types already present. The lack of community change may also be partially related to the use of closed microcosms, preventing the migration of microorganisms (Hopkins *et al.*, 1990). It is conceivable that under field conditions more evident shifts in bacterial community composition may have occurred as taxa which are physiologically adapted to the experimentally altered conditions may have colonised and competed with members of the resident community.

Categorising clades of bacteria into copiotrophic and oligotrophic groups is undoubtedly a coarse approach (Fierer *et al.*, 2007) as it has been previously shown that taxa belonging to the same phyla are diverse and may respond in a dissimilar manner (Rousk *et al.*, 2010a; Zeng *et al.*, 2016). Indeed, Bartram *et al.* (2014) observed distinct differences in bacterial community composition across a pH gradient (pH 4.5-7.5) but only within phyla, indicating that changes in community structure following a change in soil pH may be more apparent at finer levels of taxonomic resolution. However, it would appear that in this study this was not the case because even at finer levels of taxonomic resolution (class and OTU) no major shifts in bacterial abundance were observed.

In contrast to the negligible shifts in bacterial community composition, but consistent with our second hypothesis we observed a significant influence of a change in soil pH on microbial catabolic capacity. It was also hypothesised that a liming-induced increase in microbial functioning would be mediated by shifts in bacterial community composition towards copiotrophic dominance, associated with higher C mineralisation rates (Fierer *et al.*, 2007), than by diversity change. Consequently, we rejected our third and fourth hypothesis as microbial catabolic capacity appeared to increase independently of changes in bacterial community composition. Although some studies suggest clear links between shifts in bacterial community composition and changes to microbial function (Cleveland *et al.*, 2007; Eilers *et al.*, 2010; Ramirez *et al.*, 2012), there is also mounting evidence that change in microbial community composition do not always underpin changes in soil and ecosystem function (Rousk and Frey, 2015; Rousk *et al.*, 2015; Wood *et al.*, 2015; Fanin *et al.*, 2016; Fry *et al.*,

2016). In the absence of a community composition change, such shifts in microbial community function may be explained by physiological adjustments in the resident microbial community. Of the total microbial biomass, it is thought that only 0.1-5% is in an active state (Blagodatskaya and Kuzyakov, 2013) with the rest being 'potentially active' or 'dormant', but may become active should conditions change, e.g. an increase in resource availability (Chen *et al.*, 2014). Greater resource availability following an increase in soil pH may have removed microbial C or nutrient limitation and therefore induced a greater portion of the soil microbes into an active state (De Nobili *et al.*, 2001). Consistent with this, active microbial biomass was significantly increased at the high lime amendment rate. Moreover, in order to address potential microbial stoichiometric imbalance (C:N:P ratio) caused by the addition of labile substrates, microorganisms may increase their allocation of extracellular enzymes (EEs) to degrade soil organic matter, liberating a larger reservoir of LMW organic matter (Kuzyakov and Blagodatskaya, 2015). Enhanced availability of organic substrates via the mining of soil organic matter, e.g. for N rich compounds, could also explain the overall higher levels of microbial functioning in limed soils (Manning *et al.*, 2008).

Although liming generally increased microbial functioning, the magnitude of change depended on the fertiliser treatment from which the soils were collected (fifth hypothesis). Microbial catabolic capacity increased following liming in the less acidic fertiliser treatments (plots 2, 6 and 8), whilst no change was observed in the very acidic fertiliser treatment (plot 7). These results therefore support the idea that an increase in microbial function in less acidic soils reflects a general shift in the microbial biomass towards an active state (De Nobili *et al.*, 2001). The persistence of low levels of active microbial biomass and functioning following the addition of lime to N amended plot 7 soils may be attributable to long-term exposure to higher levels of acidity (pH 3.9), shifting the resident microbial community to a more oligotrophic low-diversity state. Consistent with the characteristics of a low-diversity state, bacterial diversity was low and there was an increase in oligotrophic groups (*Acidobacteria*) compared to the less acidified fertiliser treatments. Low-diversity states have previously been described in plant communities following perturbation (Silvertown *et al.*, 2006; Isbell *et al.*, 2013b), and recent evidence has shown comparable responses in bacterial communities (Calderon *et al.*, 2016). There is evidence that the response of oligotrophic communities to disturbance is often slower than in those dominated by more copiotrophic microorganisms, due to their higher inherent resistance to environmental change and slow growth rate (De Vries and Shade, 2013). Similarly, Storkey *et al.* (2015) reported that following the cessation of long-term fertilisation there was a lack of recovery in grassland plant communities where

fertiliser-induced acidification had created highly acid soils. Together, these findings confirm that long-term acidification is likely to have severe lasting legacy effects on both above- and below-ground communities. Results from two studies which sampled a similarly highly acidified soil from the Park Grass Experiment at Rothamsted (pH 3.6) revealed that long-term liming (started in 1903) has caused partial recovery of microbial diversity (Zhalnina *et al.*, 2015) and increased levels of functioning as more soil C has been microbially processed and transferred to the organo-mineral fraction (Fornara *et al.*, 2011). Whilst these findings confirm that the adverse effects of intense acidification on the microbial community may be reversible via liming, a lack of time series data means it is unclear how long the community persisted in the acidic state before recovery occurred. A potential strategy to accelerate microbial community recovery may involve the introduction of a new microbial community (Pettersson and Bååth, 2004). Calderon *et al.* (2016) successfully increased microbial diversity following the inoculation of microbial communities into degraded soils. However, the effects of degradation on microbial diversity were not remediated in all communities and no significant change in function (N cycling) was observed. Clearly, the full restoration of microbial community composition and functioning following degradation is a lengthy and complex process (Calderon *et al.*, 2016), underlining the importance of carefully managing soil pH (i.e. corrective liming) to avert the constraints of acidification.

5.6 Conclusion

The composition of bacterial communities subject to long-term soil acidification is highly resistant to relatively short-term but significant changes in soil pH. Our results showed that an increase in soil pH did not favour copiotrophic over oligotrophic taxa and indicated that the magnitude of the effects associated with a change in soil pH were not strong enough to override the legacy of management to which the resident community had acclimated. Substantial changes in microbial functioning following liming appeared to be independent of the small shifts in bacterial community composition that did occur, suggesting that the microbial functional response was associated with physiological adjustments in the resident microbial community, e.g. via the breaking of dormancy and increased enzyme production. Changes in soil pH following liming were positively related to a change in microbial function, but only in communities subject to less intense long-term acidification. This inconsistency may be explained by the fact that following liming, microbial communities historically exposed to less intense acidification were able to physiologically adjust whilst those subject to highly acidic conditions were not. Thus, our results suggest that intense acidification may pose a serious threat to microbial recovery at very low pH, consistent with the response

observed for grassland plant communities (Storkey *et al.*, 2015). Such degradation following intense acidification could potentially prevent the recovery of important biogeochemical cycles regulated by these communities, despite efforts to improve soil conditions. As such, soil pH needs to be managed vigilantly, e.g. by minimising the use of acidifying fertilisers, if soils are to be sustainably farmed.

Chapter 6. General Discussion

6.1 Objective 1: To characterise the responses of a wide range of ecosystem properties to long-term management and compare ecosystem responses with those observed at other long-term fertilised sites and with results from short-term studies.

Whilst the addition of fertilisers is of critical importance in increasing the availability of plant-growth limiting nutrients to enhance agricultural yield (Gough *et al.*, 2000; Lee *et al.*, 2010), studies have also demonstrated the dramatic consequences nutrient addition can unintentionally have for numerous ecosystem properties and services. To date, much of our understanding of the effects of fertilisation has arisen from short-term studies, the findings of which are undoubtedly relevant in areas of the world where fertilisers are only now starting to be applied or where management is changing. However, these responses may not be representative in areas that have received fertiliser applications for prolonged periods, hence inferring a long-term effect from the findings of short-term studies may be misleading. To address this issue a comprehensive assessment of the effects of long-term fertilisation on ecosystem properties at the Palace Leas Hay Meadow Experiment (est. 1896) was made and where possible responses were compared with those reported from other long-term fertilisation experiments and short-term studies (Chapter 2). In addition, to further examine the effects of management on SOC stocks, two long-term experiments receiving fertiliser amendment were also used at Woodland's Field, Craibstone Estate, Aberdeen. However, only the results from one of the experiments were discussed in papers currently submitted for publication and presented earlier in this thesis (pH gradient experiment; Chapter 4). Findings from a scoping study at the Old Rotation experiment are briefly presented below to add to the argument but were not included in the thesis chapters as there was no evidence that total and fraction SOC stocks differed between fertilised plots (Table 6.1).

At Palace Leas, long-term addition of FYM was shown to be beneficial for agricultural productivity by increasing nutrient availability (P and K), buffering against soil acidification (>pH 5) and enhancing hay yield, whilst also increasing mineral-associated SOC stocks. However, where ammonium-containing fertiliser had been applied, nutrient availability and hay yield was low and although SOC accumulated at the soil surface in these plots, much of the C was stored in the coarse and fine carbon fractions, rather than being protected against microbial decomposition as mineral-associated carbon. This was thought in part to be due to the effects of ammonium-induced acidification (Guo *et al.*, 2010). In the past, increased soil acidity has been associated with a reduction in the availability of plant-growth limiting

nutrients, constraining primary productivity (Haynes and Naidu, 1998; Haynes and Mokolobate, 2001), and lower levels of microbial activity which reduces SOC turnover (Pietri and Brookes, 2008; Fornara *et al.*, 2011) (Chapter 2).

In contrast to the findings at Palace Leas, the long-term addition (90 years) of ammonium-containing fertiliser at the Old Rotation experiment, Woodland's Field (Figure 6.1) affected neither total SOC nor particle-size fraction C stocks (Table 6.1). The discrepancy in the SOC stocks between the two contrasting systems can be attributed to the regular addition of lime to the Old Rotation, which was not applied at Palace Leas. By maintaining the soil pH at a comparable level across fertiliser treatments, it is likely that the indirect pH-mediated effects of fertilisation on SOC stocks observed at Palace Leas were nullified in the ley-arable rotation (Table 6.1). Support for this hypothesised mechanism was provided by the study of Chapter 4 using a separate long-term ley-arable rotation experiment which is also found at Woodland's Field. In this field trial, the long-term ley-arable experiment is subject to an experimentally generated pH gradient (pH 4.5-6 used). In addition, specific fertiliser treatments are applied to each of the eight crops in the rotation, however over an entire rotational cropping cycle all treatment plots receive the same cumulative rate of fertiliser input (Table 4.1). If pH was not a key control over SOC stocks then it would have been expected that total and particle-size fraction SOC stocks would change between the courses of the cropping cycle (short-term nutrient addition effect) or alternatively be consistent across all treatment plots (long-term nutrient addition effect) rather than being predominantly controlled by pH. However, consistent with the findings at Palace Leas; total, coarse and fine SOC fractions were markedly higher in soils maintained at lower pH. This showed that long-term acidity-induced effects of management (e.g. via fertilisation) strongly influence SOC stocks and that these effects are less likely to be observed where lime is applied alongside fertilisers to buffer soil pH.

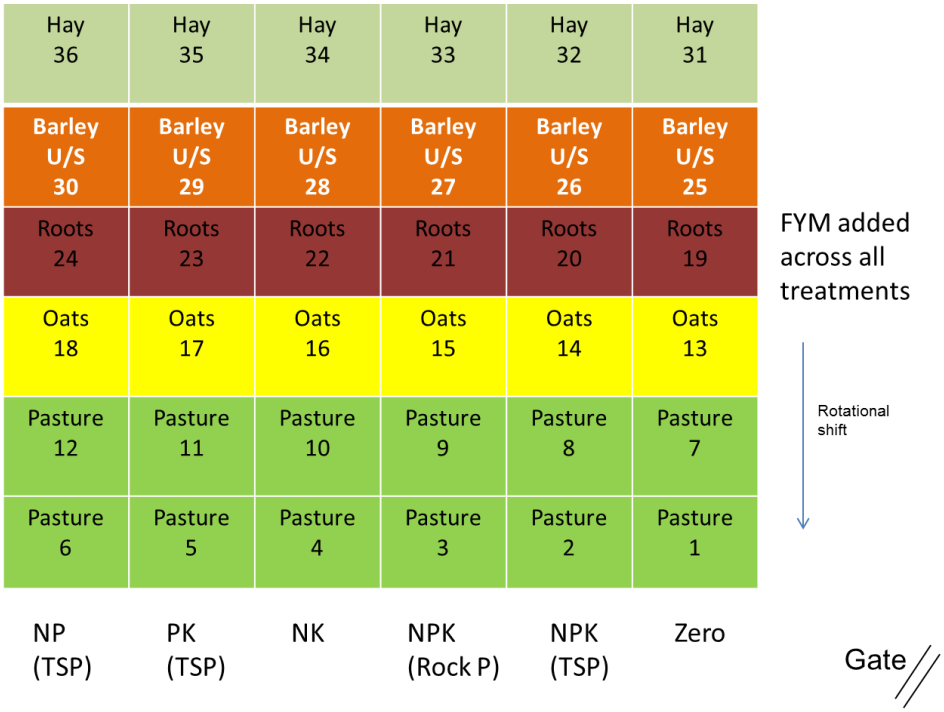

Figure 6.1 Plot layout of Woodland's Field Old Rotation experiment for 2012.

Treatment	pH	Total SOC (kg C m^{-2})	Coarse fraction carbon stocks (kg C m^{-2})	Fine fraction carbon stocks (kg C m^{-2})	Very fine fraction carbon stocks (kg C m^{-2})
Control	5.64(0.04)	2.69(0.07) b	0.44(0.02)	0.54(0.02)	1.98(0.02) b
NP^1K	5.73(0.04)	2.80(0.08) ab	0.46(0.01)	0.64(0.02)	2.04(0.03) ab
NP^2K	5.67(0.05)	2.93(0.03) a	0.43(0.02)	0.60(0.02)	2.01(0.01) ab
NK	5.68(0.05)	2.78(0.01) ab	0.42(0.01)	0.54(0.02)	2.15(0.07) a
KP	5.67(0.03)	2.97(0.02) a	0.40(0.01)	0.53(0.04)	2.12(0.04) ab
NP	5.69(0.04)	2.94(0.09) a	0.42(0.01)	0.62(0.03)	2.18(0.03) a
P value	0.150	0.002	0.135	0.055	0.004

Table 6.1 The effects of fertiliser treatment on pH, total SOC, coarse fraction carbon stocks, fine fraction carbon stocks, very fine fraction carbon stocks (0-10 cm) in the Old Rotation experiment, Woodland's Field. [1]triple superphosphate, [2]ground mineral phosphate, N; ammonium nitrate, K; muriate of potash. Contrasting letters denote significant differences between treatments (ANOVA; $P < 0.05$).

In Chapter 2 it was demonstrated that the direction and magnitude of the long-term effects of fertilisation on a number of the measured ecosystem properties at Palace Leas were consistent with responses observed in other long-term fertilised grassland sites. This can be attributed to the fact that the duration of long-term experiments makes it possible to observe both the direct

and indirect longer-term effects of management (Knapp *et al.*, 2012). For example, the extent of fertiliser-induced acidification were very similar in the N only and NK treated plots at Palace Leas and their equivalent at Park Grass (plot 1d and 18d; ~2 unit change) (Crawley *et al.*, 2005). Also, at both sites fertiliser-induced acidification has resulted in the formation of an O horizon above the mineral soil due to impedance of organic matter decomposition (Hopkins *et al.*, 2009). Plant responses at Palace Leas were also consistent with those at both Park Grass and the Steinach Grassland experiment. In all three studies, plant species richness was negatively affected not only by increased biomass production in response to increased nutrient supply, but also by loss of species unable to tolerate highly acidic conditions (Crawley *et al.*, 2005; Hejcman *et al.*, 2014). In contrast, when the findings at Palace Leas were compared with those from studies of shorter duration it was revealed that whilst the trajectory of the short- and long-term effects of fertilisation on plant and soil properties often concurred, the magnitude of the changes were markedly different (Knapp *et al.*, 2012). Differences between the magnitude of the short- and long-term effects appeared to be greatest where the indirect pH-mediated effects of long-term fertilisation were clearly influencing ecosystem response. For example, a comparison of the short-term (1897-1901) versus the long-term (1976-2015) hay yield response between plots amended with FYM and plots amended with $(NH_4)_2SO_4$ revealed that the difference between treatments in the short-term was less apparent than the long-term response. The initial growth response early in the experiment was likely to have been driven by nutrient input, but over time the repeated addition of $(NH_4)_2SO_4$ progressively acidified the soil resulting in a reduction in nutrient availability and constraining plant growth (Haynes and Naidu, 1998; Haynes and Mokolobate, 2001). Short-term studies often benefit from more modern experimental designs and greater replication (Richter *et al.*, 2007) and are valuable when the objective is to realise the initial system response to disturbance (Knapp *et al.*, 2012). However, such experiments lack the timeframe necessary for indirect impacts or complex interactions to occur and to determine not just the trajectory but also the full magnitude of the impact (Richter *et al.*, 2007; Knapp *et al.*, 2012). Furthermore, there is a high likelihood that at the point of measurement in short-term studies, ecosystem responses are transient or influenced by artefacts either derived from previous management or by disturbances caused by establishing the experiment (Knapp *et al.*, 2012). Therefore, a short-term response is unlikely to represent the true cumulative impact (Silvertown *et al.*, 2010) and it may be misleading to extrapolate the long-term effect from a short-term experiment (Richter *et al.*, 2007).

6.2 Objective 2: To investigate the relative importance of direct nutrient, soil pH-, and plant community-mediated effects of fertilisation upon agroecosystem C and soil function.

As discussed above, there is evidently a multivariate relationship between fertilisation and ecosystem properties. Recent studies have shown that the effects of fertilisation on agroecosystem C dynamics are mediated via at least three major pathways. These include the direct effects of nutrient input (Ramirez *et al.*, 2010b) and the indirect effects of a change in soil pH (Chen *et al.*, 2015b) and a shift in the plant community (Leff *et al.*, 2015). In 'real-world' agroecosystems these mechanisms operate simultaneously, therefore disentangling such effects is complex and until now there has been few attempts to decompose these relationships. It is important to distinguish the relative importance of these pathways in order to inform management, to mitigate potential impacts of fertilisation and improve the reliability of predictive ecosystem models. Doing so also increases our fundamental knowledge of how ecosystems function.

In Chapter 3, a structural equation modelling (SEM) framework was applied to an extended dataset from Chapter 2 (Palace Leas Experiment) to examine the relative importance of the three classes of fertiliser mechanism on measures of grassland C and soil function. A long-term ley-arable rotation was then used in Chapter 4 to validate the consistency of the drivers in a contrasting agroecosystem. The findings from the SEM of the Palace Leas dataset (Chapter 3) demonstrates the importance of soil pH and plant community composition as well as direct nutrient addition in mediating the effects of long-term fertilisation on grassland C and soil function. Direct nutrient addition increased total SOC stocks and their component size fractions and the magnitude of the effects were generally greater than the indirect effects of fertilisation. Nevertheless, C stocks in the coarse and fine fractions were promoted by the weaker indirect effects of soil acidification, whilst very fine fraction C stocks increased with an increase in CWM leaf N, indicative of a shift in plant community composition towards dominance by fast-growing species with exploitative growth strategies. In contrast to the findings in the ley-arable rotation (Chapter 4), at Palace Leas the effects of pH on very fine fraction C stocks were not significant, rather the stocks were positively affected by FYM addition and the abovementioned plant functional composition change. The discrepancy between the findings of these two experiments may simply be due to differences in the treatments applied. At the pH gradient experiment it can be expected that soils are equally affected by FYM addition as all plots receive the same rate of FYM (30 t ha^{-1}) during the potato phase of the rotation. In contrast, at Palace Leas only a subset of the 14 treatment plots

receive FYM and in those that do it is applied at a considerably higher rate than in the pH experiment (pH experiment = 3.75 t ha^{-1} y^{-1}, Palace Leas = 10 - 20 t ha^{-1} y^{-1}). It is conceivable that the relatively strong positive direct effects of fertiliser addition may have overwhelmed the pH-mediated effects and explain why pH was not significantly associated with C stored in the very fine fraction at Palace Leas.

Results from the SEM also established that soil acidification was dominant in explaining the microbial response to long-term fertilisation; it reduced active microbial biomass and microbial functioning (catabolic capacity), whilst direct nutrient addition was not significant. Whilst it is not definite, the greater abundance of oligotrophic *Acidobacteria* (Chapter 5), which are considered to possess lower rates of activity (Fierer *et al.*, 2007), where acidity was greatest suggests a shift in microbial community composition that may partially explain the long-term acidity-induced effects of fertilisation on microbial functioning. Although, further research is necessary to determine if this relationship is causal. The prominence of pH-mediated effects of fertilisation corroborate the findings of recent experimental studies in semi-arid Mongolian grasslands which have similarly tried to unravel the drivers of fertilisation on soil C dynamics (Wei *et al.*, 2013; Chen *et al.*, 2015a; Chen *et al.*, 2015b). That the significance of pH-mediated effects of fertilisation on microbial properties are in line with those observed in ecologically dissimilar semi-arid grasslands suggests acidification may be an important driver of fertilisation impacts across the world's grassland ecosystems. Similarly to the observed patterns in grasslands, in the ley-arable rotation experiment in Chapter 4, active microbial biomass and microbial functioning (utilisation rate of LMW-C and –N substrates) was also supressed in the more acidified soils. Therefore, it can be concluded that the effects of management-induced acidification is a key mechanism through which soil C dynamics may also be affected in arable agroecosystems.

At both Palace Leas and the ley-arable rotation there was also evidence of plant-community mediated effects on microbial dynamics. Notably, in contrast to the reported findings of a number of grassland biodiversity experiments (Eisenhauer *et al.*, 2010; Strecker *et al.*, 2015; Thakur *et al.*, 2015), at Palace Leas the plant-mediated effects were not explained by a change in diversity, rather they were associated with shifts in plant functional composition (CWM leaf N). An increase in CWM leaf N, indicative of a shift in plant community composition towards dominance by fast-growing species with exploitative growth strategies (De Vries *et al.*, 2012b; Reich, 2014) was associated with an increase in active microbial biomass and microbial functioning (Chapter 3). In the ley-arable rotation it was suggested that microbial

functioning was markedly increased at higher pH in the winter wheat phase of the rotation due to the legacy effects of the N rich legume *Trifolium repens* from the ley phase (Chapter 4).

Although some studies have shown that the effects of fertilisation on microbial C dynamics are largely driven by the direct effects of nutrient input (Ramirez *et al.*, 2010a; Ramirez *et al.*, 2010b; Ramirez *et al.*, 2012) or changes in plant community composition (Leff *et al.*, 2015), there was no indication that these mechanisms were a dominant control over the soil microbial properties measured in the experiments described in Chapters 3 and 4. Studies in the past have demonstrated that the effects of acidification on microbial biomass (Geisseler and Scow, 2014), microbial community composition (Fierer and Jackson, 2006) and respiration (Pietri and Brookes, 2008) are greatest below pH 5. Unlike the experiments in Chapters 3 and 4, pH did not fall below 5 in a laboratory study of fertilised soils by Ramirez *et al.* (2010a) or in long-term fertiliser experiments described by Ramirez *et al.* (2010b), whilst in national-scale (Ramirez *et al.*, 2012) and global-scale (Leff *et al.*, 2015) studies the relatively short timescale of the experiments (1 and 2-4 years, respectively) meant that the pH change was minor and such effects were likely to have been overwhelmed by direct nutrient input and plant community composition change. Taking the findings of this thesis and the results reported in the literature together, I suggest that the relative importance of particular direct and indirect pathway effects of fertilisation on soil C dynamics are unlikely to be consistent across agroecosystems and may vary temporally. In soils with a high buffering capacity (high clay or organic matter content), those that develop from limestone and shale or where lime is regularly applied, changes in soil pH may be minimal or at least the onset of acidification may be slowed. In such cases, we may expect to see relatively greater direct nutrient or plant-mediated effects, whilst in soils of low pH (<pH 5) the effects of soil acidification may exert greater control, especially on the microbial community. However, further study will be required to investigate the validity of this hypothesis (see *scope for future research*). Such context dependence of the drivers of fertilisation will need to be considered in order to manage the impacts and improve accuracy of predictive ecosystem models in the future.

6.3 Objective 3: To test the effects of an increase in soil pH on the recovery of microbial communities subject to different degrees of fertiliser-induced acidification.

It has been established that soil acidification is a dominant pathway through which fertilisation influences the microbial community (Chapter 3; Chen *et al.*, 2015a; Chen *et al.*,

2015b). Numerous studies have shown soil pH to be a key determinant of microbial community composition that differentially affects major taxonomic clades with contrasting life-history characteristics (Jones *et al.*, 2009; Lauber *et al.*, 2009; Rousk *et al.*, 2010a). pH also exerts an influence on the processes which microorganisms mediate, including nitrification and carbon mineralisation (Chapter 4; Kemmitt *et al.*, 2006; Rousk *et al.*, 2009; Yao *et al.*, 2011). Whilst the relationship between soil pH and soil microbial community properties is reasonably well described, it remains unclear how rapidly and dynamically microbial communities respond following a soil pH change. If intensification of agricultural management practices which are known to influence pH continues (i.e. fertilisation and liming), then rapid changes to microbial community composition and function may become widespread.

In Chapter 5, a microcosm experiment was carried out to examine the influence of a liming-induced pH change on the community composition and functioning of microbial communities subject to long-term management-induced acidification. Soils collected from Palace Leas were treated with three rates of lime to increase soil pH, transferred to pots and seeded with *Anthoxanthum odoratum*. After 180 days, bacterial community composition and microbial community functioning was measured. It was hypothesised that an increase in soil pH would induce a shift in bacterial community composition towards the dominance of copiotrophic taxa and increase microbial community functioning. In addition, it was hypothesised that an increase in microbial community functioning would be greater in communities originating from less acidic soils. However it was shown that the composition of bacterial communities was resistant to an increase in soil pH following liming, as evidenced by a lack of change in bacterial abundance and diversity. The lack of a community change was surprising given that copiotrophic taxa tend to dominate in less acidic soils and can shift rapidly in response to short-term changes in soil conditions (Eilers *et al.*, 2010; Männistö *et al.*, 2016). Despite only minor changes in bacterial community composition, in partial support of the hypothesis, microbial community functioning increased significantly in response to liming, but only in communities subject to less intense fertiliser-induced acidification. That microbial functioning changed independently of a shift community composition suggests that changes in the microbial functional response were associated with physiological adjustments in the resident microbial community (De Nobili *et al.*, 2001; Shade *et al.*, 2012). Consistent with this, there was an increase in active microbial biomass following liming in the less acidic fertiliser treatments but no change in the highly acidic N fertilised treatment. The effects of historic long-term intense acidification appeared to have caused the bacterial community in the highly

acidic plot to become more oligotrophic and less diverse; such communities tend to express slow growth rates and greater resistance to a change in environmental conditions (De Vries and Shade, 2013) which may lead to the lack of microbial recovery despite an improvement in soil conditions. This corresponds to the patterns observed for grassland plant communities where similarly long-term and intense fertiliser-induced acidification constrained plant community recovery once fertilisation ceased (Storkey *et al.*, 2015). Therefore, the findings demonstrate that not only do management-induced effects of acidification markedly affect microbial communities (Chapters 3 and 4) but where acidification is most intense the effects cannot easily and quickly be rectified by liming. Recent work has suggested that the restoration of degraded microbial communities is a complex and lengthy process (Calderon *et al.*, 2016), as such the soil's pH will need to be managed vigilantly e.g. by corrective liming and by minimising the use of acidifying fertilisers to avert the serious threat to soil microorganisms and the biogeochemical cycles which they regulate.

6.4 Scope for future research and conclusions

As a result of the work carried out during this thesis the following key areas were identified as priorities for future research:

- It was an objective of this thesis to go a step further than previous observational studies by applying a SEM framework to investigate the relative importance of three of the major fertiliser-mediated pathways affecting soil C dynamics. Whilst SEM allows the partitioning of potentially causal relationships (Grace, 2006), the underlying mechanisms responsible are not as well identified as they would be using manipulative experiments. Findings from this thesis and the work of others have established the importance of the pH-induced effects of land management in explaining the response of the microbial community. Disentangling the effects is challenging in because pH simultaneously influences multiple biotic and abiotic factors (e.g. soil moisture, nutrient availability, C availability and base cation availability) (Lauber *et al.*, 2009; Rousk *et al.*, 2009) which drive changes in soil C dynamics. To enhance understanding of the underlying mechanistic effects of soil acidification more controlled manipulative experiments in a wide range of ecosystems are now required.
- The findings from this thesis and results reported in the literature indicate that the negative acidity-induced effects of fertilisation may exert greater control over the response of soil microorganisms in soils <pH 5 (Geisseler and Scow, 2014). To

statistically assess if the hypothesised relationship between fertilisation and microbial properties is pH dependent future studies could carry out multigroup SEM analyses (see Grace, 2006), which enables the evaluation of the same model framework for more than one group of samples (e.g. above and below pH 5). If the association between parameters differs between groups then this would support the case that the modelled relationship is pH dependent. It was not possible to test this in Chapter 3 as sample size was too small to run multigroup SEMs. This approach should be applied to a larger dataset that encompasses data from across a range agroecosystems to allow researchers; 1) to examine the generality of the pH dependent relationship across ecosystems and 2) determine if the pH threshold (hypothesised to be ~pH 5) varies depending on abiotic, biotic and management factors. Establishing such a pH threshold could help to provide guidelines for farmers and land owners regarding the pH above which soil must be maintained in order to avert the most negative impacts of acidification.

- The analytical methods used in this thesis to measure soil microbial properties (16S rRNA gene sequencing and MicroResp^{TM}) tend to focus on the bacterial component of the community rather than fungi. However, fungal communities also play an important role in soil carbon cycling including the decomposition of chemically recalcitrant organic materials such as lignocellulolytic C and in increasing the content of physically protected C in soil macroaggregates (Rillig, 2004; Six *et al.*, 2006; Strickland and Rousk, 2010). The fungal community has previously been shown to be sensitive to the direct (Fog, 1988) and indirect plant- (Bardgett and McAlister, 1999; Bardgett *et al.*, 1999) and pH-mediated (Rousk *et al.*, 2011a) effects of fertilisation and are likely to be affected differently compared to bacteria (Rousk *et al.*, 2010b; Strickland and Rousk, 2010), yet little has been done to disentangle these pathway effects and estimate their relative importance. Therefore, the effects of long-term fertilisation on fungal communities warrants further investigation. The MicroResp^{TM} assay has also now been adapted so it is possible to obtain fungal catabolic profiles (Sassi *et al.*, 2012), which in combination with the sequencing of 18S rRNA genes could provide a more detailed understanding of the effects of fertilisation on fungal community composition and functioning.
- In the microcosm experiment in Chapter 5 it was shown that bacterial communities subject to long-term fertiliser-induced acidification are relatively resistant to a short-term but significant increase in soil pH. While short-term studies such as this are valuable to realise the initial trajectories of change (Knapp *et al.*, 2012), a logical next

step would be to study the response of microbial communities for a longer duration (e.g. decades) and in more complex field systems. In the past, researchers have examined the recovery of grassland plant communities following the cessation of fertilisation (Isbell *et al.*, 2013b; Storkey *et al.*, 2015). However, microbial community recovery has not been studied in this way. It may be possible to establish this experiment at the Palace Leas Hay Meadow (i.e. by dividing plots and applying lime) whilst also maintaining the existing fertiliser treatments, albeit over a smaller area. This would enable researchers to monitor the rate of community composition and ecosystem function change through time and in a system which may more accurately represent the natural environment. Further work could also focus on the metabolically active community by using RNA extraction rather than the DNA-based analysis (whole bacterial/fungal community) employed in Chapter 5. Targeting RNA will enable researchers to characterise the active members of microbial taxa responsible for the measured change in community function (Blagodatskaya and Kuzyakov, 2013; Freedman *et al.*, 2015).

- The focus of this thesis was on the effects of fertilisation on soil C dynamics at the soil's surface as this is the most biologically active layer and where large concentrations of SOC occur (Jobbagy and Jackson, 2000; Griffiths *et al.*, 2003). While the impacts of management are often greatest in the topsoil, there is growing evidence to suggest that the dynamics of SOC deep in the soil profile, where significant stocks of SOC are known to be stored, are also very sensitive to management (Fontaine *et al.*, 2007; Gregory *et al.*, 2014; Ward *et al.*, 2016). Greater consideration for the effects of fertilisation on soil C dynamics at depth could aid the formulation of more robust global carbon models capable of predicting future soil C stocks across the entire soil profile rather than just the topsoil and help to inform land management practice on how best to enhance the storage of deep soil carbon to mitigate climate change.
- In conclusion, long-term fertilisation has significant consequences for many key ecosystem processes and services beyond simply increasing primary productivity. In 'real-world' agroecosystems the mechanisms that mediate the effects of fertilisation are both direct and indirect, which operate simultaneously, therefore disentangling such effects is difficult. Indirect effects of fertilisation on soil C dynamics, particularly pH-mediated effects, may be as great, or greater, than direct nutrient addition impacts. However, the relative importance of particular direct and indirect pathway effects of fertilisation may vary temporally and are unlikely to be universal across

agroecosystems, therefore extrapolating the long-term effect from a short-term single site experiment should be avoided. Microbial communities are degraded by intense fertiliser-induced acidification and the process of restoration may be complex and long-term, as such the use of acidifying fertiliser should be minimised and soil pH corrected by liming. Whilst this thesis provides an important insight into the effects of long-term fertilisation on agroecosystem properties, further work is required across a range of climatic and environmental gradients to improve our understanding of fertilisation effects in contrasting ecosystems. It is also essential that new long-term experiments are created that take into consideration current and projected future trends in fertiliser management.

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Appendix A

Plot number	Treatment	Depth (cm)	pH	SOC (kg C m^{-2})	Total nitrogen content (kg N m^{-2})	Soil C/N	Coarse fraction carbon stocks (kg C m^{-2})	Fine fraction carbon stocks (kg C m^{-2})	Very fine fraction carbon stocks (kg C m^{-2})	Olsen-P (mg P kg^{-1})	Exchangeable K (mg K kg^{-1})	Exchangeable Al (mmol kg^{-1})
1	FYM and NPK	0-5	5.45 (0.05)	3.22 (0.11)	0.28 (0.01)	11.45 (0.20)	0.17 (0.04)	1.66 (0.25)	1.21 (0.07)	85.98 (7.20)	347.56 (72.34)	0.00 (0.00)
		5-10	5.23 (0.04)	2.66 (0.04)	0.23 (0.02)	11.53 (0.80)	0.08 (0.02)	0.74 (0.11)	1.54 (0.24)	63.22 (4.75)	153.76 (27.70)	0.25 (0.25)
2	FYM	0-5	5.72 (0.08)	3.09 (0.15)	0.28 (0.01)	11.00 (0.08)	0.22 (0.02)	1.58 (0.26)	1.55 (0.12)	96.95 (9.42)	400.95 (52.50)	0.00 (0.00)
		5-10	5.24 (0.05)	2.49 (0.06)	0.21 (0.01)	13.03 (1.66)	0.07 (0.01)	0.76 (0.11)	1.62 (0.15)	51.29 (3.06)	291.06 (48.17)	0.00 (0.00)
3	FYM and NPK	0-5	5.47 (0.07)	2.89 (0.23)	0.23 (0.02)	12.32 (0.30)	0.20 (0.04)	1.08 (0.22)	1.34 (0.09)	43.64 (7.42)	257.52 (75.48)	0.74 (0.43)
		5-10	5.18 (0.07)	2.27 (0.06)	0.19 (0.01)	12.91 (0.93)	0.07 (0.01)	0.41 (0.05)	1.42 (0.06)	39.33 (7.57)	214.79 (71.99)	1.12 (1.12)
4	FYM	0-5	5.41 (0.05)	2.70 (0.20)	0.22 (0.01)	12.05 (0.34)	0.22 (0.05)	1.08 (0.22)	1.37 (0.11)	32.22 (2.60)	202.59 (13.03)	0.74 (0.43)
		5-10	4.94 (0.07)	2.33 (0.07)	0.21 (0.01)	13.86 (0.97)	0.06 (0.01)	0.53 (0.04)	1.51 (0.08)	17.61 (1.25)	109.49 (10.14)	0.87 (0.51)
5	FYM and NPK	0-5	5.22 (0.01)	2.81 (0.10)	0.23 (0.01)	12.10 (0.49)	0.20 (0.04)	1.61 (0.22)	1.30 (0.05)	51.34 (5.54)	257.51 (45.11)	0.12 (0.12)
		5-10	4.95 (0.03)	2.39 (0.05)	0.21 (0.01)	11.73 (0.27)	0.08 (0.02)	0.53 (0.02)	1.51 (0.08)	31.40 (8.26)	94.23 (8.28)	0.50 (0.29)
6	Control	0-5	4.81 (0.04)	2.35 (0.19)	0.17 (0.01)	13.49 (0.27)	0.15 (0.02)	1.25 (0.24)	0.92 (0.09)	1.49 (0.44)	178.13 (25.71)	4.12 (0.31)
		5-10	4.36 (0.07)	2.07 (0.02)	0.17 (0.01)	13.26 (0.38)	0.09 (0.02)	0.44 (0.11)	1.34 (0.09)	3.15 (0.65)	100.49 (5.46)	12.71 (0.93)
7	N	O horizon	3.26 (0.03)	3.69 (0.15)	0.24 (0.03)	14.45 (0.67)	1.17 (0.16)	1.59 (0.17)	0.64 (0.15)	n/a	n/a	37.64 (0.98)
		0-5	4.20 (0.04)	2.34 (0.07)	0.15 (0.01)	15.38 (0.81)	0.18 (0.08)	0.75 (0.06)	1.62 (0.15)	3.44 (0.45)	47.62 (3.55)	47.83 (1.07)
		5-10	4.26 (0.06)	1.68 (0.20)	0.11 (0.01)	15.04 (0.51)	0.11 (0.04)	0.22 (0.02)	1.08 (0.08)	4.17 (0.27)	32.26 (2.89)	33.97 (3.30)
8	P	0-5	4.87 (0.10)	2.24 (0.09)	0.18 (0.01)	12.74 (0.42)	0.24 (0.10)	1.16 (0.11)	1.07 (0.08)	27.89 (1.51)	109.65 (10.15)	4.62 (0.24)
		5-10	4.93 (0.05)	1.86 (0.09)	0.16 (0.01)	12.80 (1.11)	0.06 (0.02)	0.48 (0.05)	1.41 (0.08)	27.82 (4.19)	84.14 (9.36)	3.11 (0.71)
9	K	0-5	4.57 (0.07)	2.50 (0.06)	0.17 (0.01)	14.62 (0.39)	0.35 (0.07)	1.31 (0.13)	0.85 (0.08)	5.74 (2.43)	515.47 (47.15)	12.70 (1.85)
		5-10	4.63 (0.03)	2.35 (0.04)	0.16 (0.01)	14.87 (0.52)	0.06 (0.01)	0.60 (0.15)	1.41 (0.16)	9.14 (2.35)	332.38 (20.10)	16.79 (2.81)
10	NP	0-5	4.43 (0.07)	2.29 (0.09)	0.17 (0.01)	13.38 (0.15)	0.30 (0.13)	1.09 (0.12)	1.00 (0.03)	35.74 (3.45)	117.70 (7.45)	18.68 (0.48)
		5-10	4.60 (0.05)	2.15 (0.14)	0.16 (0.01)	15.23 (1.76)	0.06 (0.01)	0.44 (0.07)	1.48 (0.05)	30.45 (4.30)	75.75 (7.01)	11.90 (1.25)
11	NK	O horizon	3.17 (0.06)	3.59 (0.13)	0.31 (0.05)	14.42 (0.68)	0.50 (0.11)	2.50 (0.23)	1.12 (0.14)	n/a	n/a	37.78 (1.00)
		0-5	3.89 (0.09)	2.22 (0.30)	0.13 (0.01)	16.23 (0.55)	0.16 (0.04)	0.78 (0.06)	1.44 (0.15)	3.72 (0.82)	81.85 (5.57)	42.03 (4.42)
		5-10	4.29 (0.04)	1.77 (0.15)	0.11 (0.01)	14.97 (0.28)	0.14 (0.04)	0.20 (0.01)	1.13 (0.07)	7.29 (0.13)	90.25 (11.78)	31.60 (1.44)
12	PK	0-5	4.88 (0.07)	2.27 (0.20)	0.17 (0.02)	13.54 (0.51)	0.26 (0.13)	0.96 (0.22)	1.13 (0.04)	28.18 (2.55)	78.80 (4.53)	3.99 (0.79)
		5-10	4.81 (0.03)	2.32 (0.02)	0.16 (0.01)	15.73 (1.53)	0.05 (0.01)	0.47 (0.11)	1.23 (0.03)	22.75 (1.89)	103.66 (9.44)	5.72 (1.16)
13	NPK	0-5	4.07 (0.06)	2.21 (0.13)	0.16 (0.01)	13.96 (0.60)	0.28 (0.06)	1.06 (0.06)	0.90 (0.04)	37.50 (4.71)	191.89 (11.42)	19.84 (1.79)
		5-10	4.44 (0.08)	1.97 (0.10)	0.15 (0.01)	14.61 (1.00)	0.07 (0.01)	0.50 (0.11)	1.11 (0.17)	27.45 (1.47)	71.17 (20.10	14.66 (1.89)
14	NPK	0-5	3.72 (0.07)	2.61 (0.10)	0.21 (0.01)	13.91 (0.52)	0.68 (0.10)	1.50 (0.23)	0.88 (0.09)	49.09 (8.81)	121.25 (1.06)	31.11 (2.01)
		5-10	4.34 (0.07)	2.00 (0.04)	0.14 (0.01)	15.29 (1.84)	0.07 (0.01)	0.49 (0.08)	1.30 (0.03)	12.43 (2.76)	62.31 (5.25)	31.67 (3.99)

Appendix A Soil pH, organic carbon stocks (SOC), total nitrogen content, soil carbon to nitrogen ratio (C/N), coarse fraction carbon stocks, fine fraction carbon stocks, very fine fraction carbon stocks, Olsen-P, exchangeable K and exchangeable Al under the different fertilizer treatments at 0-5cm, 5-10 cm and the O horizon. Values at 0-5 cm and 5-10 cm are means (±1 SE). The O horizon in plots 7 and 11 was not analysed for Olsen-P or exchangeable K, n/a indicates not applicable.

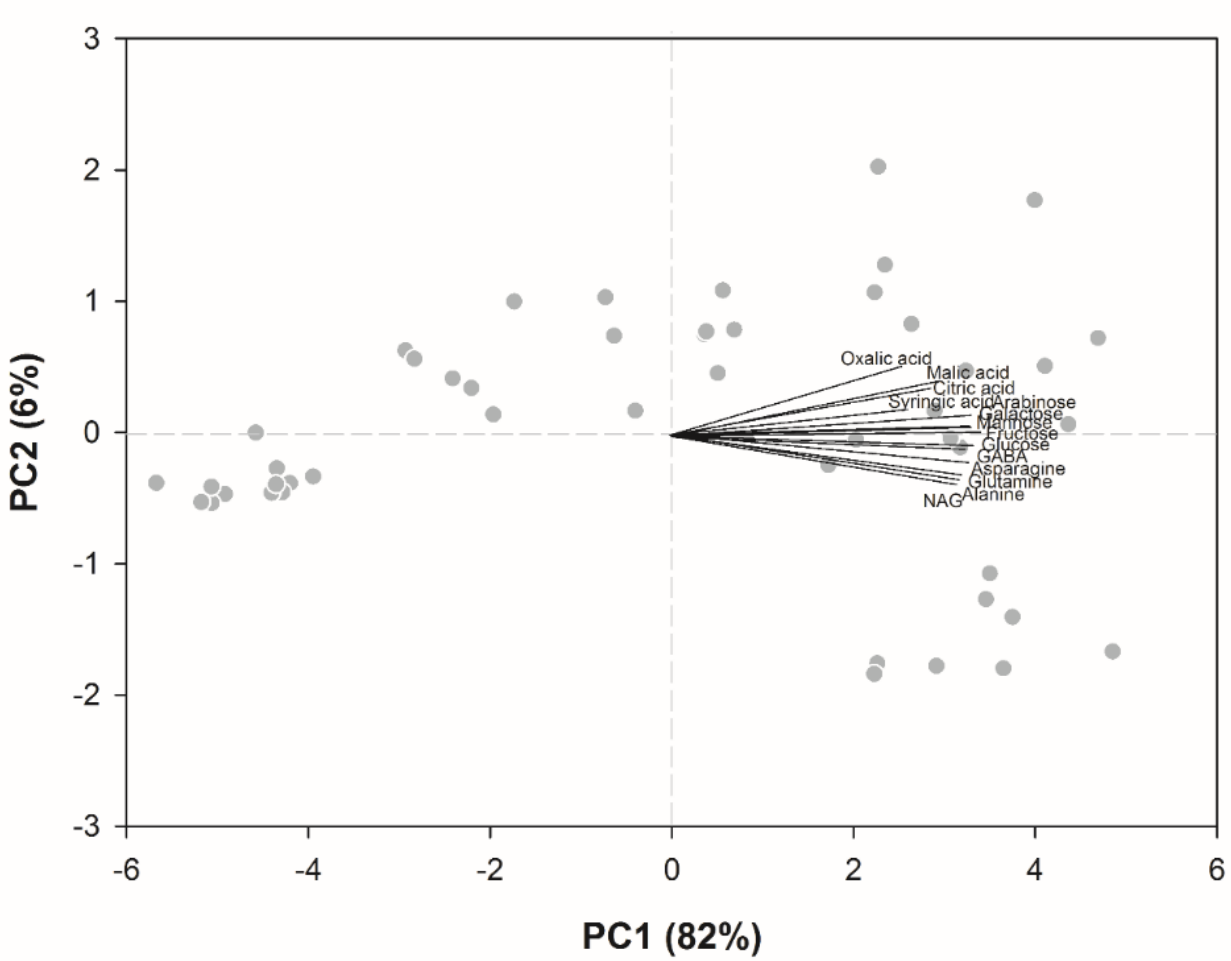
Appendix B

Microbial catabolic capacity threshold (%)	Depth (cm)	Path	Estimate	Standard Error	Path P value	χ^2	Model P value	R^2
30	0-5	FYM ↔ Inorganic P	-0.31	0.10	0.00	8.236	0.13	0.70
		FYM ↔ Inorganic N	-0.30	0.10	0.00			
		Inorganic P ↔ Inorganic N	0.42	0.16	0.01			
		pH → CWM leaf N	0.42	0.15	0.01			0.55
		Inorganic P → CWM leaf N	0.37	0.10	0.00			
		FYM → CWM leaf N	0.39	0.16	0.01			
		Inorganic N → pH	-0.60	0.08	0.00			0.86
		Inorganic P → pH	0.27	0.06	0.00			
		FYM → pH	0.66	0.06	0.00			
		CWM leaf N→ MCC 30	0.17	0.10	0.04			
		pH → MCC 30	0.72	0.08	0.00			
	5-10	FYM ↔ Inorganic P	-0.31	0.11	0.00	4.579	0.31	0.43
		FYM ↔ Inorganic N	-0.30	0.09	0.00			
		Inorganic P ↔ Inorganic N	0.42	0.16	0.01			
		Inorganic N → pH	-0.45	0.07	0.00			0.83
		Inorganic P → pH	0.39	0.06	0.00			
		FYM → pH	0.78	0.06	0.00			
		pH→ MCC 30	0.66	0.08	0.00			
50	0-5	FYM ↔ Inorganic P	-0.31	0.10	0.00	4.394	0.35	0.64
		FYM ↔ Inorganic N	-0.30	0.10	0.00			
		Inorganic P ↔ Inorganic N	0.42	0.16	0.01			
		pH → CWM leaf N	0.42	0.15	0.01			0.55
		Inorganic P → CWM leaf N	0.37	0.10	0.00			
		FYM → CWM leaf N	0.39	0.17	0.02			
		Inorganic N → pH	-0.60	0.08	0.00			0.86
		Inorganic P → pH	0.27	0.06	0.00			
		FYM → pH	0.66	0.06	0.00			

		CWM leaf N→ MCC 50	0.28	0.10	0.00			
		pH → MCC 50	0.59	0.08	0.00			
	5-10	FYM ↔ Inorganic P	-0.31	0.11	0.00	3.216	0.38	0.39
		FYM ↔ Inorganic N	-0.30	0.09	0.00			
		Inorganic P ↔ Inorganic N	0.42	0.16	0.01			
		Inorganic N → pH	-0.45	0.06	0.00			0.83
		Inorganic P → pH	0.39	0.06	0.00			
		FYM → pH	0.78	0.06	0.00			
		pH→ MCC 50	0.62	0.09	0.00			
70	0-5	FYM ↔ Inorganic P	-0.31	0.10	0.00	2.371	0.63	0.44
		FYM ↔ Inorganic N	-0.30	0.10	0.00			
		Inorganic P ↔ Inorganic N	0.42	0.17	0.01			
		pH → CWM leaf N	0.42	0.15	0.00			0.55
		Inorganic P → CWM leaf N	0.37	0.10	0.00			
		FYM → CWM leaf N	0.39	0.16	0.02			
		Inorganic N → pH	-0.60	0.08	0.00			0.86
		Inorganic P → pH	0.27	0.06	0.00			
		FYM → pH	0.66	0.06	0.00			
		CWM leaf N→ MCC 70	0.19	0.11	0.08			
		pH → MCC 70	0.53	0.10	0.00			
	5-10	FYM ↔ Inorganic P	-0.31	0.11	0.01	6.153	0.16	0.18
		FYM ↔ Inorganic N	0.30	0.09	0.00			
		Inorganic P ↔ Inorganic N	0.42	0.16	0.01			
		Inorganic N → pH	-0.45	0.07	0.00			0.83
		Inorganic P → pH	0.39	0.06	0.00			
		FYM → pH	0.78	0.06	0.00			
		pH→ MCC 70	0.43	0.12	0.00			

Appendix B Sensitivity analysis of the thresholds used to calculate microbial catabolic capacity. Two extra thresholds (30% and 70%) are presented, the 50% threshold of the maximum is shown for comparison.

Appendix C



Appendix C Principal components analysis for the microbial community respiratory response (μg CO_2-C g^{-1} h^{-1}) to 14 LMW substrates.

Appendix D

Carbon source	Correlation coefficient (r)
D-glucose	0.979
D-mannose	0.958
D-fructose	0.983
D-galactose	0.944
L-arabinose	0.945
Oxalic acid	0.765
Citric acid	0.841
L-malic acid	0.862
N-acetyl-Glucosamine	0.903
L-asparagine	0.951
L-glutamine	0.937
γ-amino butyric acid	0.907
L-Alanine	0.895
Syringic acid	0.734

Appendix D The correlation between the first principal component (PC1) and substrate-induced respiration response to the 14 LMW substrates.

Appendix E

						LA		FT		LA x FT	
OTU	Phylum	Class	Order	Family	Genus	F	*P*	F	*P*	F	*P*
1	*Verrucomicrobia*	*Spartobacteria*	*Chthoniobacterales*	*Chthoniobacteraceae*	*DA101*	0.24	0.791	**33.93**	**<0.001**	1.60	0.182
2	*Actinobacteria*	*Actinobacteria*	*Actinomycetales*	Unclassified	Unclassified	0.12	0.880	**33.66**	**<0.001**	0.34	0.909
3	*Acidobacteria*	*Acidobacteria-Gp6*	*iii1-15*	Unclassified	Unclassified	0.08	0.921	**27.98**	**<0.001**	0.45	0.837
4	*Acidobacteria*	*Acidobacteriia*	*Acidobacteriales*	*Acidobacteriaceae*	Unclassified	0.48	0.622	**76.20**	**<0.001**	0.66	0.682
5	*Acidobacteria*	*Acidobacteriia*	*Acidobacteriales*	*Koribacteraceae*	Unclassified	0.74	0.479	**65.03**	**<0.001**	1.09	0.386
6	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	Unclassified	0.01	0.987	**3.85**	**0.019**	0.64	0.700
7	*Planctomycetes*	*Planctomycetia*	*Gemmatales*	*Gemmataceae*	Unclassified	**7.13**	**0.002**	**18.75**	**<0.001**	**2.92**	**0.022**
8	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Hyphomicrobiaceae*	*Rhodoplanes*	2.16	0.132	2.34	0.092	0.59	0.735
9	*Bacteroidetes*	*Saprospirae*	*Saprospirales*	*Chitinophagaceae*	Unclassified	0.33	0.719	**9.85**	**<0.001**	0.86	0.536
10	*Planctomycetes*	*Phycisphaerae*	*WD2101*	Unclassified	Unclassified	0.53	0.592	**11.97**	**<0.001**	1.41	0.243
11	*Proteobacteria*	*Gammaproteobacteria*	*Xanthomonadales*	*Sinobacteraceae*	Unclassified	1.98	0.155	**10.63**	**<0.001**	2.01	0.094
12	*Proteobacteria*	*Deltaproteobacteria*	*Myxococcales*	Unclassified	Unclassified	0.07	0.926	**3.59**	**0.025**	1.43	0.234
13	*Acidobacteria*	*Solibacteres*	*Solibacterales*	*Solibacteraceae*	*Candidatus Solibacter*	0.09	0.914	**8.70**	**<0.001**	0.54	0.775
14	*Proteobacteria*	*Alphaproteobacteria*	*Rhodospirillales*	*Acetobacteraceae*	Unclassified	1.66	0.206	**23.91**	**<0.001**	0.44	0.839
15	*Verrucomicrobia*	*Pedosphaerae*	*Pedosphaerales*	*Auto67_4W*	Unclassified	1.09	0.348	**10.54**	**<0.001**	1.74	0.144
16	*Actinobacteria*	*Thermoleophilia*	*Gaiellales*	*Gaiellaceae*	Unclassified	1.80	0.183	**15.02**	**<0.001**	0.90	0.506
17	*Acidobacteria*	*Solibacteres*	*Solibacterales*	Unclassified	Unclassified	0.55	0.578	**23.58**	**<0.001**	0.44	0.846
18	*Acidobacteria*	*Acidobacteria-Gp6*	*iii1-15*	*RB40*	Unclassified	0.90	0.415	**13.06**	**<0.001**	0.36	0.896
19	*Proteobacteria*	*Alphaproteobacteria*	*Rhodospirillales*	*Rhodospirillaceae*	Unclassified	0.45	0.642	**5.48**	**0.004**	2.02	0.074
20	*Verrucomicrobia*	*Spartobacteria*	*Chthoniobacterales*	*Chthoniobacteraceae*	*Candidatus Xiphinematobacter*	0.87	0.428	**19.15**	**<0.001**	1.70	0.154
21	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Bradyrhizobiaceae*	Unclassified	0.94	0.402	**6.02**	**0.002**	0.81	0.567
22	*Planctomycetes*	*Planctomycetia*	*Gemmatales*	*Isosphaeraceae*	Unclassified	0.01	0.998	**12.88**	**<0.001**	1.94	0.106
23	*Chloroflexi*	*Ellin6529*	Unclassified	Unclassified	Unclassified	1.39	0.263	**74.49**	**<0.001**	1.67	0.162
24	*Acidobacteria*	*Acidobacteriia*	*Acidobacteriales*	*Koribacteraceae*	*Candidatus Koribacter*	0.72	0.493	**6.83**	**0.001**	1.11	0.378
25	*Proteobacteria*	*Gammaproteobacteria*	*Xanthomonadales*	*Xanthomonadaceae*	Unclassified	1.02	0.372	**13.35**	**<0.001**	0.88	0.518
26	*Acidobacteria*	*DA052*	*Ellin6513*	Unclassified	Unclassified	0.12	0.883	**11.84**	**<0.001**	0.23	0.963
27	*Acidobacteria*	*iii1-8*	*32-20*	Unclassified	Unclassified	0.83	0.446	**14.30**	**<0.001**	1.20	0.331
28	*WPS-2*	Unclassified	Unclassified	Unclassified	Unclassified	1.89	0.169	**26.28**	**<0.001**	1.81	0.129
29	*Firmicutes*	*Bacilli*	*Bacillales*	*Bacillaceae*	*Bacillus*	**5.64**	**0.008**	2.01	0.132	1.47	0.220
30	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	Unclassified	Unclassified	2.81	0.077	2.79	0.057	**4.76**	**0.002**
31	*Planctomycetes*	*Planctomycetia*	*Planctomycetales*	*Planctomycetaceae*	*Planctomyces*	2.82	0.075	**3.09**	**0.041**	0.45	0.839

32	*Verrucomicrobia*	*Pedosphaerae*	*Pedosphaerales*	Unclassified	Unclassified	0.67	0.517	1.73	0.181	0.64	0.694
33	*Proteobacteria*	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*	0.33	0.719	**3.83**	**0.019**	0.75	0.617
34	*Verrucomicrobia*	*Pedosphaerae*	*Pedosphaerales*	*Ellin515*	Unclassified	0.72	0.494	1.61	0.205	1.09	0.390
35	*Proteobacteria*	*Deltaproteobacteria*	*Desulfuromonadales*	*Geobacteraceae*	*Geobacter*	0.36	0.702	0.63	0.602	0.60	0.728
36	*Proteobacteria*	*Betaproteobacteria*	*SC-I-84*	Unclassified	Unclassified	1.49	0.240	**15.02**	**<0.001**	0.99	0.449
37	*Proteobacteria*	*Alphaproteobacteria*	*Rhizobiales*	*Hyphomicrobiaceae*	Unclassified	1.68	0.203	**7.71**	**<0.001**	**2.85**	**0.025**
38	*Planctomycetes*	*Planctomycetia*	*Pirellulales*	*Pirellulaceae*	Unclassified	0.01	0.987	**5.17**	**0.005**	0.52	0.787
39	*Actinobacteria*	*Thermoleophilia*	*Solirubrobacterales*	Unclassified	Unclassified	1.23	0.305	**9.73**	**<0.001**	0.59	0.712
40	*Proteobacteria*	*Deltaproteobacteria*	*Syntrophobacterales*	*Syntrophobacteraceae*	Unclassified	0.23	0.791	**8.05**	**<0.001**	1.18	0.343

Appendix E Mediation analysis for microbial catabolic capacity (ΔMCC) as a function of hypothesised direct (ΔpH →ΔMCC) and indirect effects (ΔpH → Δbacterial community composition → ΔMCC) of soil pH. Bacterial community composition mediated effects could be explained via diversity (ΔShannon's diversity, ΔSimpson's diversity and ΔOTU richness) and mass ratio indices (Δrelative abundance of *Acidobacteria*, *Betaproteobacteria* and a ΔCopiotrophic:ΔOligotrophic ratio). Models were compared using AIC to determine the most parsimonious plant pathway, the pathway judged to be the most appropriate had the lowest AIC. Values shown for each model are AIC, ΔAIC (difference in AIC between a model and the model with the lowest AIC score), the P value associated with the χ^2 and the R^2 for mediating variable and MCC.