Soil carbon sequestration by grasslands to mitigate climate change

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Declaration

I herewith declare that this thesis has been written by myself and that it embodies the results of my own research. No content herein has previously been submitted to obtain a degree in any form. Where appropriate, the nature and extent of work carried out in collaboration with others is acknowledged.

Radim Šarlej,

Lancaster University, March 2020.

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Abstract

Grasslands over a quarter of land free area and due to large carbon (C) stock they represent a key global C pool. Grassland soil C sequestration is an alternative method for reduction of atmospheric carbon dioxide (CO_2) which can also deliver other benefits including greater ecosystem stability and increase of biodiversity resulting from improved land management in agroecosystems or through restoration of degraded land. Available options for increasing C stock include increasing biomass yield and related delivery of more plant organic matter into the soil, decreasing losses of organic C from the soil and increasing mean residence time of C in the soil.

Surveying regenerating grasslands with different time period of extensive land management (land management history) and performing field experiments, this thesis sought to increase understanding of soil organic C storage in grasslands in order to improve knowledge for greater soil C sequestration. This involved detailed measurements of responses of soil aggregate fractions (coarse and fine silt, clay and POM (POM)) and associated organic carbon (OC) concentration in soils with different land management histories together with microbial community composition. Secondly, grassland field experiment was established where soil pH was modified and potential drivers of ecosystem C cycle were monitored together with ecosystem process rates including soil microbial community composition, soil and plant nutrient pools, plant growth and community composition, soil potential extracellular enzyme activities and ecosystem C fluxes. Further, fungicide was applied to part of the grassland field experiment in order to determine response of the system to a disturbance. Thirdly, field mesocosms experiment was established testing response of community C and nitrogen (N) cycles to interactions of plant species differing in their nutrient acquisition strategies as well as response of soil microbial community composition to these plant interactions.

The findings indicated that coarse silt and fine silt fractions responded differently to land management history and greater C accumulation in coarse fraction which increased with time without significant intensive agricultural management was suggested to be related to POM presence and organic C associated with clay fraction. Soil pH was found to be a strong driver of soil processes and microbial and plant communities when increasing soil pH by lime application promoted substrate availability in the soil, which resulted in changes in microbial composition and a greater microbial activity resulting in increased mineral N availability, which further affected plant growth and composition and ecosystem C respiration. Changes in soil C were not observed in this relatively short term study. Application of fungicides affected N availability and ecosystem C fluxes after the application but these largely returned to control levels later in the season. Comparing system responses on different pH levels plots showed different impact of applied fungicides depending on soil pH level. Studying plant species interactions demonstrated different impact of plant species with opposite life strategies on soil N cycle and further suggested higher N use efficiency with plant species mixtures than for monocultures of these species. These findings are especially interesting because all the species were selected from family *Poacea*. Overall assessment of microbial community composition showed responses of bacterial and fungal communities to land management history and soil pH initiated nutrient availability differences.

The thesis demonstrated that soil organic carbon (SOC) pool consists of different sub-pools with different dynamics as determined based on soil aggregate fractionation. Traits and life strategies approach for characterizing functional differences of different species, as demonstrated on plant species interactions, can be further used for understanding of microbial community diversity and its impact on soil processes and soil C storage, especially at smaller spatial scales such as individual aggregate scale.

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Abbreviations

ASV	amplicon sequence variant
C	carbon
DTP SP	Defence Training Estate Salisbury Plain
DOC	dissolved organic carbon
DON	dissolved organic nitrogen
EEA	extracellular enzyme assay
GLM	generalised linear model
GHG	greenhouse gas
M-GLM	multivariate generalised linear model
N	nitrogen
NPP	net primary productivity
MWD	mean weight diameter
OC	organic carbon
OTU	operational taxonomic unit
POM	particulate organuc matter
SOC	soil organic carbon
TOC	total organic carbon

Specific aggregate fractions

(NA)	non-aggregated matter
(m)	free microaggregates
(intra-M/iM)	intra macroaggregate matter
(iM-m)	microaggregates within macroaggregates

Chapter 1: **General Introduction**

1.1 Modern climate change, impacts and mitigation

1.1.1 Modern climate change

Composition of the atmosphere has an important role in Earth's climate regulation, and as such, it also plays a key role in the modern climate change. Atmospheric greenhouse gasses (GHGs) act as a blanket over the Earth's surface by absorbing heat energy radiated from the surface or atmosphere and emitting a portion of absorbed energy downwards towards the Earth's surface (Stocker et al., 2005). The natural greenhouse effect increases Earth's surface temperature by 30 °C resulting in a global average surface temperature of 15 °C, supporting the life on Earth as we know it. Water vapour is the strongest GHG while other natural GHGs are C dioxide, methane and nitrous oxide. In addition to natural cycles of GHGs, human activities gradually increased concentration of anthropogenic GHGs during the period since industrial revolution (≈ year 1750). Consequently, this increase of atmospheric GHG concentration has affected energetic balance of the Earth resulting in global warming. Average global Earth surface temperature is over 1 °C higher than an estimate of preindustrial global mean temperature (years 1880 – 1920) and it grows by 0.18 °C per decade (Hansen et al., 2017).

Humans have been releasing several different GHGs into the atmosphere, however anthropogenic CO_2 has caused most of the human-induced change in energetic balance of climate system within the period from 1750 to 2011 (Stocker et al., 2005). C dioxide was almost solely responsible for increase of greenhouse effect in the period of 2005 – 2011 (Stocker et al., 2005). Overall, CO_2 concentration increased from preindustrial era level of 278 ppm (year 1750) to recent level of over 400 ppm (2015) (Betts et al., 2016). Land use change activities were primary driver of this increase in the period before 1920, while CO_2 released from burning fossil fuels become the major contributor to its atmospheric pool since then and reached 86 % share of total anthropogenic CO_2 emissions (2008 – 2017) (Le Quéré et al., 2018).

Climate change is predicted to affect weather patterns, and specifically extremes in temperature and precipitation (Karl and Trenberth, 2003), when drought events are expected to start earlier in the year and be more intense under the climate change (Trenberth et al., 2014). Indeed, it has been already shown that patterns or increased number of recent heatwaves and precipitation extremes can be linked to the climate change (Coumou and Rahmstorf, 2012). Moreover, seasonal and perennial snow and ice extent is expected to decrease, resulting in sea level rise (Karl and Trenberth, 2003). Vermeer and Rahmstorf (2009) connected sea level to global temperature and predicted sea level rise by 0.7 – 1.95 m in 2100. Climate change has also a potential to affect crop yield, since climate is an important variable for agricultural production (Kukal and Irmak, 2018). Weather disasters reported during 1964 – 2007, such as droughts and high temperatures, reduced national wheat productions in countries across the globe by 9 – 10 %, and more recent events showed greater losses (Lesk et al., 2016). Reduction of global yield of four major crops including maize, rice, wheat and soy beans due to temperature increase have been predicted (without accounting for CO_2 fertilization effect) (Zhao et al., 2017). Widespread crop yield decrease, such as potential synchronized yield loss in the major maize-exporting countries, could lower global supply and affect price stability with consequences for society (Tigchelaar et al., 2018). Furthermore, climate change has triggered species distribution range shift (Parmesan and Yohe, 2003) and it can lead to biodiversity loss (Thomas et al., 2004) which can accelerate with future global temperatures (Urban, 2015).

1.1.2 Soil C stocks

Exchange of C between terrestrial and atmospheric pools is the largest C flux in the global C cycle (Figure 1.1). Soils represent the greatest C stock within the terrestrial pools and it is more than double the atmospheric C stock (soil C pool determined within 3 m depth). Soil-atmosphere C exchange is a prominent control of atmospheric C concentration and the climate (Crowther et al., 2016). Due to the size of soil C stock and a relatively fast turnover of some of its pools, soil has a capacity to become a significant C source or sink (e.g. Post and Kwon, 2000; Amundson, 2001; Guo and Gifford, 2002; Lal, 2004; Minasny et al., 2017; Smith et al., 2008). Hence, soils are predicted to play an important role in climate change mitigation (Minasny et al., 2017; Lal, 2018).

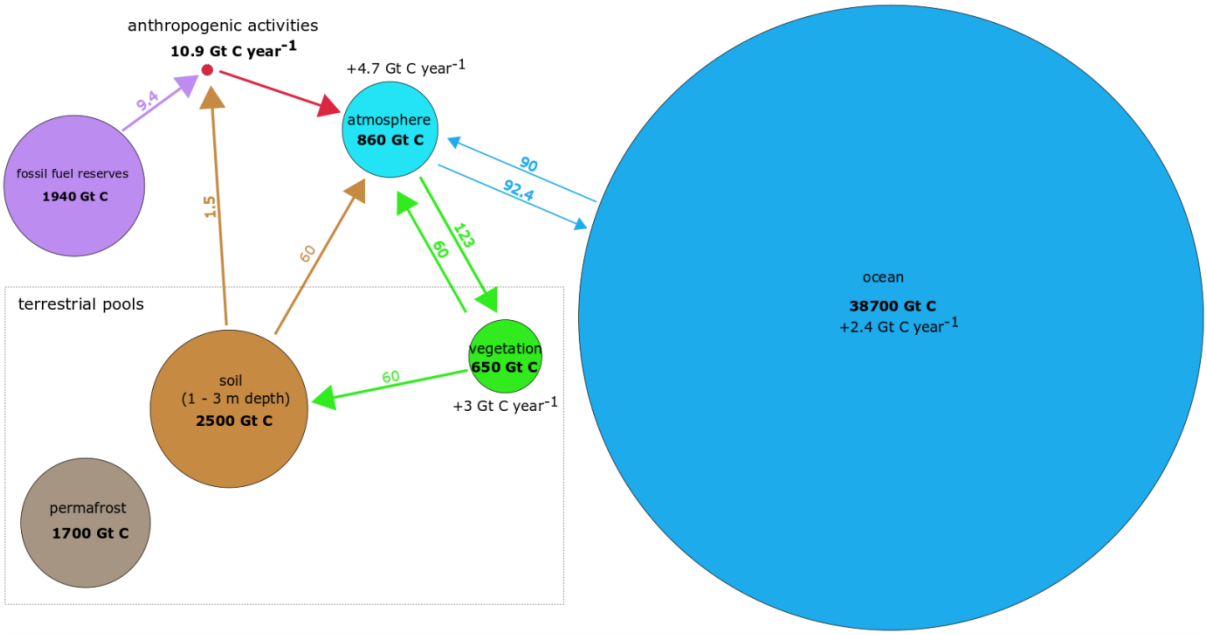


Figure 1.1 Global C cycle. Size of circles representing pools scaled according to pool size. Arrows represent fluxes (in Gt C year^{-1}) between pools. Fossil fuel reserves calculated as sum of max proven gas, oil and coal reserves. Other pools such as coast or ocean surface sediment not considered. Data sources from Lal (2018) and (Le Quéré et al., 2018).

Management of the land was shown to have a strong effect on total soil C stock (Stockmann et al., 2013; Wiesmeier et al., 2019). Land use change towards agriculture was shown to result in C stock decrease (Guo and Gifford, 2002). Indeed, agriculture has been found responsible for a soil C debt of 133 Gt (within 2 m depth) (Sanderman et al., 2018). Changing land use away from agricultural practise by introducing perennial vegetation can increase soil C stock (Post and Kwon, 2000). Similarly, change in practise within agricultural land management can improve soil C balance (Kallenbach et al., 2019; Lal, 2018; Paustian et al., 2016) including measures such as sustaining C inputs to the soil in between crops (Malik et al., 2018) or reduced tillage and improved crop rotation (Paustian et al., 2016).

Climate is usually regarded as the most important driver of soil C stocks globally (Jobbágy and Jackson, 2000; Wiesmeier et al., 2019), however other factors may be equally important such as biogeochemistry (Doetterl et al., 2015). The response of SOC decomposition process rates to predicted future changes in climate is unclear, as well as indirect effects of climate change on environmental constraints of decomposition (Davidson and Janssens, 2006). Hence, better understanding of soil mechanisms driving terrestrial-atmospheric C exchanges and the process of C stabilization in different soils can improve predictions of soil C stock response to future climate change (Bardgett et al., 2008; Bradford et al., 2016; Cotrufo et al., 2013; Jackson et al., 2017).

Outputs of soil biogeochemistry models are sensitive to assumptions about SOC decomposition dynamics (Schmidt et al., 2011) and as such, climate change feedback prediction will be improved by explicit representation of microbial mechanisms controlling SOC dynamics (Wieder et al., 2013). Better knowledge of SOC storage can help to improve land management practise for enhanced soil C sequestration in agroecosystems (Lal, 2003; Paustian et al., 2016).

Overall, climate change represents enormous challenge to humanity, and it has been recognized globally resulting in international agreements to take action against. Response of natural and managed ecosystem to future climate change is of immense importance because these ecosystems hold relatively large C stocks, stored in the soil and vegetation, when compared to current atmospheric stock (Figure 1.1). Soil, storing over four times more C than vegetation, can play an important role in increased C sink by terrestrial ecosystem but we need to better understand soil C cycle in order to improve our land management practices as well as to better predict soil-climate change feedbacks.

1.2. Soil organic C cycle and storage

1.2.1 Soil organic C pools

Soil organic C cycling is essentially a function of (1) inputs of OC compounds into the soil from plant net primary production and (2) outputs resulting from decomposition processes in the soil (Batjes et al., 1996; Davidsons and Janssens, 2006). Overall SOC pools consist of living components including microbial biomass typically accounting for 1 – 3 % of SOC (Fierer et al., 2009) and soil organisms, which do not exceed 1 % of SOC (Lützow et al., 2006), and remaining non-living OC.

Overall, SOC consists of a variety of diverse sub-pools, which can be categorized according to their origin, chemistry, association with soil matrix, size or age among others. Plant primary production can enter soil in the form of aboveground or belowground litter or as root exudates (Figure 1.2). Recent evidence shows that root derived C entering soil accounts for 5 – 33 % of net primary productivity (NPP) (Jones et al., 2009) and represents the greatest part of SOC (Jackson et al. 2017). The degrading tissues form particulate organic matter (POM) which is differentiated from soil matrix by its size or density. The origin of SOC also determines its chemical composition (Paul, 2016) and overall nutrient content when POM originating from plant litter is characterized by a wider range of C:N ratio which is also typically higher than material originating from microbial biomass whose C:N ration is more constrained. Furthermore, SOC can interact with soil matrix as it can form organo-mineral complexes bonding to surfaces of soil mineral matrix or it can be occluded within soil aggregates in the form of POM or organo-mineral complexes (Figure 1.2). Alternatively, it can be part of non-aggregated SOC or dissolved in soil solution (dissolved organic C (DOC)). Organo-mineral SOC is quantitatively the greatest part of SOC stock (Wiesmeier et al., 2019) and includes C of different ages (Kögel-Knabner et al., 2008).

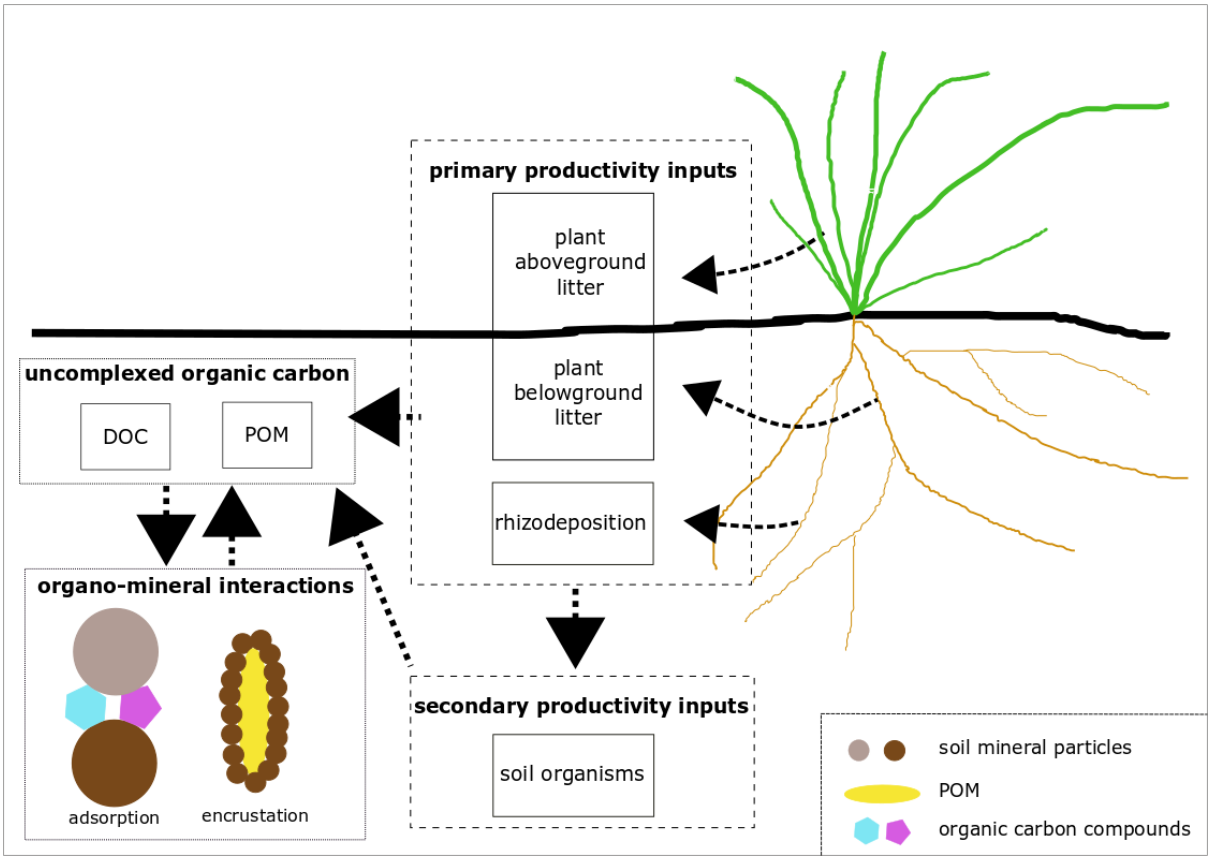


Figure 1.2 Simplified representation of soil organic matter inputs to the soil and their transition into soil organic C pools. Arrows represent movement of plant biomass or organic C compounds. DOC = dissolved organic C, POM = particulate organic matter.

1.2.2 Long-term persistence of organic C in the soil

Soil organic C chemical characterization, its composition and decomposability (or recalcitrance), and its dynamics have been widely debated recently resulting in the shift of old paradigms describing its long-term persistence (Lützow et al., 2006; Kleber, 2010; Jangid et al., 2011; Gleixner et al. 2013). Concepts of mechanisms of SOC storage are based on theories of humification, selective preservation and progressive decomposition (Lehmann and Kleber, 2015). It is becoming accepted that concepts of humification and selective preservation do not play a significant role for long term SOC persistence (e.g. Kleber, 2010; Schmidt et al., 2011). This is because *de novo* synthesis of complex molecules in the soil (humification process) has never been confirmed (Lehman and Kleber, 2015) and simple molecules are preserved during the decomposition instead (Schmidt et al., 2011), such as proteins and peptides (Gleixner et al., 1999). Moreover, the microbial community has the capacity to degrade even material previously presumed resistant (e.g. lignin) more quickly than had been anticipated (Klotzbücher et al., 2011). Lignin was considered to be the main part of SOC but its proved decomposability does not suggest its preferential preservation in the soil (Thevenot et al., 2010). Lehman and Kleber (2015) proposed viewing the SOC pool as a mixture of various compounds at different stages of decomposition which are stabilized within soil matrix and being processed by microbial community as they become available. They further argued that as the original tissues become progressively decomposed into smaller molecules, the importance of matrix interactions for their persistence increases. Their view is in line with recent evidence stating that the SOC pool consists of both plant and microbial origin when microbial compounds form a large part of it (Lutzow et al., 2006). It is also supported by evidence showing that the age of organic molecules determines the period

when C was taken up from atmosphere but not age of organic molecules themselves (Gleixner, 2013) and decomposability of an organic compounds in the soil is not related to its chemical composition (Kleber, 2010).

It has been widely argued recently, that C interactions with matrix are important to its long-term persistence in the soil (e.g. Kogel-Knabner et al., 2008b). SOC consists of simple organic compounds (Schmidt et al., 2011) that are usually easily degradable but still the bulk of C related to mineral phase of the soil can be thousands of years old (Trumbore, 2009) which suggests that something is preventing its decomposition. SOC decomposition can be constrained when decomposers are in spatial isolation from substrates or when soil structure retards movement of enzymes or available substrates as well as through unfavourable conditions for decomposition (Dungait et al., 2012; Ekschmitt et al., 2008). Long-term persistence of soil organic C becomes an environmental property instead of related to its own chemistry (Schmidt et al., 2011).

1.3 Soil microbial community

1.3.1 Microbial community role in soil C cycling and organic C storage

It is widely agreed that soil microbes play a key role in biogeochemical cycling of C (and also of other nutrients) (Schimel and Weintraub, 2003; Bardgett et al., 2008; Allison et al., 2010; Schimel and Schaeffer, 2012; Litchman et al., 2015) which is due to both their catabolic and anabolic activities (Liang et al., 2017). Microbial catabolism includes degradation of complex organic compounds by extracellular enzymes (Burns et al., 2013; Sinsabaugh, 1994) as well as mineralization of substrates inside of cells. Alternatively, substrates brought into the cell can be utilized for production of various compounds (anabolism). Relative contribution of each of these processes to the overall cell metabolism is determined by demand for substrates and energy (Geyer et al., 2016). The importance of microbial metabolism for soil SOC stock relies on the production of biomass, thus stabilizing available SOC compounds, and the production of compounds that are relatively more stable or can be stabilized within soil (Liang et al., 2017). This points towards stabilization of microbial assimilated C compounds as an important channel for soil organic C stock (Simpson et al., 2007; Liang and Balser, 2012). It implies thus that microbial control over the fate of C within soil processes is directly related to long term persistence of SOC.

Microbes differ in their metabolism (Allison, 2005 ; Fierer et al., 2007; Litchman et al., 2015; Roller and Schmidt, 2015). At the same time, soil microbial communities typically contain a large number of different microbial taxa (Schimel and Schaeffer, 2012) and they differ in the abundance of different taxa at small as well as large spatial scales (e.g. Fierer et al., 2012; Zhalnina et al., 2014). As a consequence of variation of microbial community composition, it may be expected that communities differ in their overall metabolism and effect on biogeochemical cycles. Schimel and Schaefer (2012) argued that for microbial community composition to have an effect on ecological processes it must consist of taxa differing in their functional traits as well as the organism functioning must be controlling the nature of the processes including either rate of or the fate of the process.

Overall, the emerging view of soil C dynamics and long term persistence of SOC (Lehmann and Kleber, 2015) agrees with importance of soil structural characteristics for the substrate availability for soil decomposers (Schmidt et al., 2011; Dungait et al., 2012) as well as control of microbial community on transformation of organic compounds in the soil (Bardgett et al., 2008; Schimel and Schaefer, 2012) which can be then stabilized in the soil (Liang et al., 2017). Nevertheless, we still lack an understanding of the relationship of community composition with its functioning (Martiny et al., 2015; Fierer, 2017).

1.3.2 Linking microbial community to soil C cycling

Using microbial traits and life strategies instead of species is critical to link microbial community with its biogeography, function and effect on biogeochemistry, and such an approach is gaining popularity recently (Krause et al., 2014; Nemergut et al., 2014; Litchman et al., 2015; Martiny et al., 2015; Ho et al., 2017; Fierer, 2017). It can be further used to predict response of microbial communities and function to a change in environmental conditions (Berlemont et al., 2014). The concept is borrowed from plant and animal science (Martiny et al., 2015) but can be adjusted to unique characteristics of microbial communities (Malik et al., 2020). Community composition can be linked to its effect on ecosystem if traits determining response to environmental filters are linked to traits that contribute to ecosystem processes (Lavorel and Garnier, 2002) and they further argued that these linkages will occur due to trait correlations and trade-offs within the organisms. Trait trade-offs can occur for example when organisms allocate finite resources into investment in one function but at the expense of another function (Treseder and Lennon, 2015) such as when investment of microbial cell into biomass growth trade-offs with investment into resource acquisition or stress alleviation (Malik et al., 2020). These trade-offs representing similarities in important physiological traits selected by biotic and abiotic environmental pressures can be generalized to form life history strategies (Ho et al., 2017).

Microbial investments, represented by processing and utilization of acquired resources (Geyer et al., 2016) as a result of selection of dominant microbial life strategy, with inherent trade-offs in microbial traits, determine the role of microbial processes within ecosystem C cycling. Microbial life strategies frameworks include the copiotroph-oligotroph dichotomy (Fierer et al., 2007; Thomson et al., 2013) and a three-way variation of Grime's (1977) C-S-R framework (Wood et al., 2018; Malik et al., 2019). However, it is an open question whether and how these strategies are linked to microbial taxonomic groups and at what resolution. For example, if these life strategies can be linked to microbial community at higher taxonomic resolution, then amplicon sequencing at this resolution may be sufficient to characterize such community and infer its potential response to changes in environmental parameters in relation to effect on ecosystem C cycle. This inference from taxonomy may not be possible if the traits are conserved at a shallow taxonomic resolution such as individual species.

1.4 Control of soil organic C stocks

Soil organic C stock can be understood as a long term expression of cycling of OC within an ecosystem at a particular location. It is a result of a hierarchy of biotic and abiotic factors controlling processes of inputs of OC into the soil and decomposition of OC in the soil (Stockman et al., 2013; Wiesmeier et al., 2019) (Figure 1.3).

The input of OC into the soil is a portion of NPP which enters the soil in the form of litter or exuded from the roots (Figure 1.2). In principle, it is driven by ability of primary producers to capture resources including CO_2, nutrients, water as well as energy to build their biomass. Because CO_2 and energy from the sun are generally in ample supplies, the primary production is controlled by availability of nutrients and water in the soil. N represents the fourth most important element of plant biomass and as such it can control NPP (Vitousek and Howarth, 1991; LeBauer and Treseder, 2008) as well as plant tissue chemistry (C:N ratio) such as of roots (Fornara and Tilman, 2012). Other factors can also affect plant biomass yield and tissue chemistry such as plant life strategy whereby nutrient acquisitive species are characterized by higher leaf C:N ratio as oppose to nutrient conservative species (Wright et al., 2004).

Decomposition of OC in the soil is related to (1) abiotic factors controlling substrate availability for soil heterotrophs (section 1.2) and (2) biotic factors controlling substrate transformation (section 1.3). In brief, ability of soil to create conditions where OC is locked in the soil structure is a prerequisite for its long-term persistence (Schmidt et al., 2011; Dungait et al., 2012). Soil C can be protected by aggregation or interactions with soil matrix (Lutzow et al., 2006). The release of this locked C can be achieved by destabilization shifting OC from a protected to an available state by different processes including release from physical occlusion through mechanical disturbance (e.g. tillage, dry-wet cycle), C desorption from soil matrix (e.g. after pH increase) and C metabolism increase (Bailey et al., 2019).

Processing of available substrates is under control of the microbial community and is essentially determined by a need for energy and resources by individual cells within the community (section 1.3) (Figure 1.3). Microbes have limited ability to control nutrient stoichiometry in the biomass, as suggested by a relatively constant global microbial biomass nutrient stoichiometry (Cleveland and Liptzin, 2007; Xu et al., 2013). The most probable way that a microbial community responds to potential imbalances of nutrients in available substrates is by alteration of its metabolism through release of the nutrient in excess (Mooshammer et al., 2014a; Mooshammer et al., 2014b). Such adjustment of microbial metabolism will lead to losses of soil C due to overflow respiration (Manzoni et al., 2012). Chemistry of primary inputs to the soil can thus control its degradation and incorporation into stable SOC pool through efficiency of its microbial processing (Cotrufo et al., 2013). Similarly, disturbance induced stress can affect microbial substrate processing which is related to preference of microbial investment between growth and maintenance with implication to soil C budget (Schimel et al., 2007; Malik et al., 2017).

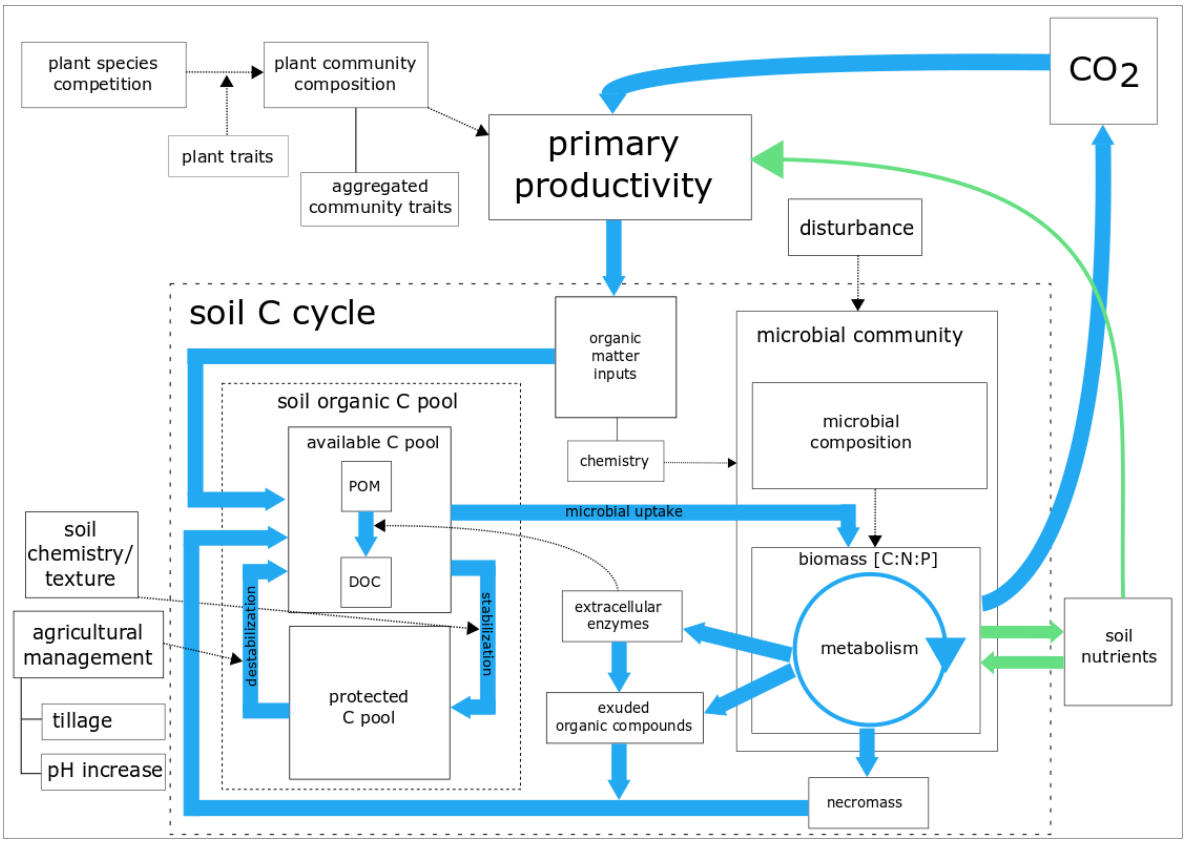


Figure 1.3 Simplified ecosystem C cycle. Blue and green arrows represent C and N flow respectively. Black dashed arrows represent direct effects.

1.5 Thesis outline – aims, objectives and research hypotheses

Grasslands represent an important ecosystem globally (with coverage estimated at 26 % of the global ice-free land area (Lorenz and Lal, 2018)). Their native rage includes sites preventing forest development due to low rainfall and fire (Bond et al., 2005). They are also maintained at sites where aboveground vegetation is removed by grazing and moving but with sufficient rainfall (e.g. north-western and central Europe) and these are more productive (Soussana et al., 2010). They represent an important global reservoir of C with most of it stored belowground (Jones and Donnelly, 2004) estimated at 343 Pg of soil C in 1 m (Lorenz and Lal, 2018) (estimated at 20 % – 30 % of global terrestrial C stock (Conant et al., 2001)). They provide essential services such as biomass production and soil C storage. Studies of management impact on grassland soil C stock show mixed results with losses as well as increases (Smith, 2014). Poor or inappropriate management can result in reduction of SOC in grasslands (Conant et al., 2017). Sequestration of C in the soil can decrease its depletion level and help mitigating climate change (Lal, 2004). Strategies enhancing SOC sequestration include increasing input of biomass C, decreasing losses of SOC by erosion and decomposition and increasing stabilization of the sequestered SOC in order to enhance its residence time (Lal, 2018). This thesis thus aimed at increasing understanding of factors important for SOC storage in grasslands in an effort to promote soil C sequestration. Chapter 2 specify common methods used throughout the research work.

Land use change and intensive land management have been shown to strongly negatively affect soil C stock (Six et al., 1998; Guo and Gifford, 2002). An important insight into controls of SOC storage can be obtained by following recovery of SOC during ecosystem regeneration from intensive land use (O'Brien and Jastrow, 2013). This idea was utilized in Chapter 3 which studied a chronosequence of extensively managed grasslands (differing in time since the last intensive management) and arable sites. In this case, studying the distribution of SOC in specific soil fractions as affected by age since the last intensive land use would highlight the importance of specific SOC fractions for soil C sequestration and increase understanding of OC dynamics in the soil. Specifically, soil fractionation procedure was employed (Six et al., 2000) in order to determine differences of specific SOC pools during the ecosystem regeneration. Utilization of age of developing grassland ecosystem after a disturbance to the soil processes by intensive land management was utilized in the past (e.g. Baer et al., 2002; Hernandez et al., 2013; Barber et al., 2017) as well as when determining dynamics in different SOC pools (Jastrow, 1987; Jastrow, 1996; O'Brien and Jastrow, 2013; Scott et al., 2017). It remains to be determined, whether dynamics of sub-pools of the specific SOC pools such as for instance coarse and fine silt which altogether represent an important soil C pool (Virto et al., 2008, 2010) differ in their dynamincs of OC acquisition with time (O'Brien and Jastrow, 2013) and thus such a detailed fractionation have been employed in the present research. At the same time, the microbial community (bacteria and fungi) was characterized at the different stages of ecosystem regeneration in order to understand its successional development after land use change (Barber et al., 2017).

Soil pH is typically determined as a strong driver of soil microbial community composition (e.g. Lauber et al., 2009; Rousk et al., 2010; Griffiths et al., 2011). This is due to the effect of soil pH on microbial metabolism (Malik et al., 2015) as well as effect on availability of substrates in the soil such as dissolved SOM (Bailey et al., 2019) or through an effect on aggregation (i.e. effect of Ca) (von Lutzow et al., 2006). Availability of substrates can affect microbial community composition (Fierer et al., 2007; Fierer, 2017). Microbial community is responsible for processing of available substrates in the soil (Schimmel and Schaefer, 2012) and thus changes in soil microbial community composition initiated by soil pH can affect soil processes and overal availability of nutrients in the soil (Soong et al., 2020). . Due to the positive effect of soil pH increase on soil nutrient availability (Kemmit et al., 2006), soil pH manipulation represents a frequent agricultural measure for yield

improvement. Understanding the role of soil pH in soil C and nutrient cycling can be important for improvement in delivery of multiple ecosystem services in agroecosystems such as C storage apart from biomass yield (e.g. Malik et al., 2018). Response of various soil and overall ecosystem characteristics was studied after soil pH manipulation of extensively managed grassland (Chapter 4).

At the same time, changes of soil biotic characteristics due to soil pH might be important for response of an ecosystem to a disturbance (Allison and Martiny, 2008; Shade et al., 2012; DeVries and Shade, 2013). Differences in soil nutrient availability are expected to result in different soil microbial communities at different soil pH levels whereby higher nutrient availability at non-acidic soil pH is expected to promote microbes with copiotrophic life styles and bacteria over the fungi as oppose to acidic soils (Fierer et al., 2007; Bárcenas-Moreno et al., 2016). These pH-related differences in microbial communities will be important for response of soil abiotic and biotic characteristics to a disturbance by application of a biocide (mixture of two fungicides). This is because of the specificity of fungicide effect on microbial community, prefferentialy tagetting soil fungi, and the role of soil fungi in plant litter and SOM decomposition (Štursová & Baldrian, 2011; Voriskova & Baldrian, 2013) as well as hypothesized differences in the response of microbial communities consisting of different life strategies to a disturbance (DeVries and Shade, 2013). Fungicides are commonly used in agriculture and have been shown to affect soil biotic and abiotic characteristics (e.g. Bending et al., 2007; Monkiedje et al., 2002; Muñoz-Leoz et al., 2011; Sukul, 2006). Understanding their effect on soil is might be important in determining their role in soil nutrient cycles and thus feedback to the climate change. Morevover, such studies have been typically peformed in laboratory conditions and field studies including plant-soil interactions are needed (Chapter 5).

Plants can affect soil microbial community and soil processes for instance by differences in quality and quantity of primary productivity entering the soil (De Deyn et al., 2008; Orwin et al., 2010b). These differences in the plant primary productivity can be expressed through plant life strategies in the continuum from nutrient acquisitive to conservative strategy whereby leaf traits such as C:N ratio are informative in distinguishing between plant life strategies (Wright et al., 2004). Plant communities differing in their cumulative traits can result in differences in important soil cycles (e.g. N cycle) (Grigulis et al., 2013). Moreover, competition between plants of different life strategies might determine the resulting plant composition for instance through plant soil feedbacks (Van der Putten et al., 2013; Baxendale et al., 2014) and it might also determine cumulative plant community traits affecting soil and ecosystem processes (de Vries et al., 2012; Lavorel et al., 2011). This idea was followed in Chapter 6 when plant species from family *Poaceae* differing in their life strategies were studied in monocultures and species mixtures for their effects on soil microbial community and soil N cycle and ecosystem respiration.

Overall, the PhD research work aimed to increase understanding of specific controls on C cycle and SOC stock in grasslands by setting the following objectives:

1. Study changes within SOC sub-pools in grasslands with different time periods of extensive management in order to determine relative importance of these sub-pools for overall SOC stock.
2. Study soil C cycle through manipulation of microbial community by
    a. changes in soil abiotic characteristics such as soil pH
    b. a direct effect on microbial community through a biocide effect.
3. Study plant species interactive effect on soil C cycle.

These objectives are individualy discussed within the corresponding chapters together with specific research hypotheses (listed below) and the thesis is completed by a discussion (Chapter 7)

addressing grassland management improvement measures targetting increase of SOC stock with inclusion of relevant insights obtained from the research chapters.

1.5.1 Specific Research hypotheses

Chapter 3

(1) Land use will affect soil aggregation and bulk SOC concentration whereby intensive arable crop production will show lower soil aggregation as well as SOC concentration than extensively managed grasslands.
(2) Grasslands with the most recent history of intensive agricultural practise will show lower bulk SOC concentration than sites with the longest regenerating status.
(3) Silt-size fraction will be the most important fraction in relation to OC storage and it will consist of two sub-fractions of different sizes which can be characterized as two distinct fractions based on aggregation and/or OC concentration.
(4) Microbial communities of both bacteria and fungi will differ between arable and Recent regenerating grasslands as well as between Recent regenerating grasslands and Old regenerating grasslands.
(5) Copiotrophic bacterial species will show a trend of decreasing their relative abundance while oligotrophic bacterial species will show opposite trend from arable and Recent regenerating grasslands towards Old regenerating grassland.

Chapter 4

(1) Liming application, increasing soil pH to near neutral level, will change bacterial community structure and promote microbial taxa known as copiotrophic while lowering of soil pH would promote opposite changes.
(2) Liming related increase of soil N availability and N mineralization will be detected by an increase of activity of extracellular enzymes involved in N cycle.
(3) The changes in soil N availability as expected on high pH plots will affect plant growth when plants on limed plots will increase investment into aboveground growth resulting in greater biomass yield while decrease investment into root growth in comparison to acidic soils of the other treatments.
(4) Soil respiration will increase on limed plots.

Chapter 5

(1) Effect on fungal community will be more pronounced than effect on bacterial community because the biocide application primarily targets fungal community. At the same time, an interactive effect of soil pH treatment and biocide application on soil bacterial and fungal communities will be observed whereby the two acidic soils (low pH and control pH treatment plots) communities will respond in a similar way and different to the communities in high pH treatment soils.
(2) Different effects on fungal diversity across the pH gradient will be apparent, due to altered physiological constraints and compositions. Fungal community diversity in low pH and control pH treatment soils will decrease while fungal community on high pH plots will increase its diversity.

Chapter 6

(1) Plant species with different growth rates and trait characteristics will be functionally differentiated along the resource acquisition-conservation spectrum, allowing prediction of their effect on soil processes in the monocultures. Specifically, resource acquisitive plant species characterized by higher biomass N content and quality as well as higher growth

rate will be associated with faster C and N cycling in the ecosystem and higher proportion of copiotrophic members of soil microbial community than resource conservative plant species.

(2) Plant species interaction in species mixed communities will affect growth of individual plant species and will affect rate of soil processes at the ecosystem level.

Explanation of frequently used terms

Soil organic C and organic matter are used interchangeably in this report and both indicate OC compounds of different composition and origin. POM is related specifically to undecomposed organic debris of plant and microbial origin.

Chapter 2: **General methods**

2.1 Soil abiotic characteristics

Gravimetric moisture content

Aluminium dish was weighed, between 5 and 10 g of fresh soil was weighed into the aluminium dish, dried at 105 °C for 24 h and weighed again. Gravimetric moisture was calculated.

Soil pH

pH of soil slurry was measured. The slurry was prepared using 6 g of fresh soil suspended in 15 ml of MiliQ H_2O by shaking for 30 minutes on an orbital shaker followed by settling for 1 hour.

Dissolved mineral N and net N mineralization

Dissolved mineral N ions (NO_3–N and NH_4–N) were estimated using 1 M KCl extracts, when 5 g of fresh soil was suspended in 25 ml of the extractant by shaking for 1 hour on an orbital shaker and filtered through Whatman Number 1 filter paper. Extracted mineral fractions were determined using standard protocol on AA3 segmented flow analyser (SEAL Analytical Inc., Mequon, USA).

Net N mineralization was determined as release of mineral N ions after laboratory incubation of 5 g of soil at 70 % water holding capacity for 21 days at 25 ˚C. After incubation, dissolved mineral N ions were extracted and determined as above.

Dissolved organic C and dissolved organic N

Dissolved organic C (DOC) and nitrogen (DON) compounds were measured in single water extract when 3.5 g of dry weight equivalent of fresh soil was suspended in 35 ml of MiliQ H_2O by shaking on an orbital shaker for 10 min, centrifuging at 3500 rpm for 3 min and filtering through Whatman no. 42 filter paper. DOC was determined analysing the water extract on total OC (TOC) analyser (Shimadzu, Milton Keynes, UK). DON was determined as the difference between total N and mineral N in the water extracts; both measured as mineral N ions by procedure described above. Preparation of samples for total N determination included oxidation of DON by potassium persulphate method: 5 ml of 0.148 M potassium persulphate, 0.25 ml of 3 M NaOH and 15 ml of the extract were combined, autoclaved at 121 ˚C and 17 psi for 55 min and 0.25 ml of 3 M NaOH was added to the cooled oxidized extracts.

Plant available phosphorus (Olsen phosphorus)

Plant available phosphorus (P) was determined using 0.5 M $NaHCO_3$ extract when 5 g of fresh soil was suspended in 100 ml of $NaHCO_3$ after shaking for 30 min and filtered through Whatman no. 42 filter paper. Extracted Olsen P was determined using standard protocol on AA3 segmented flow analyser (SEAL Analytical Inc., Mequon, USA). Bulk density was determined as the weight of the dry soil per volume of the sampling rings with roots and stones bigger than 2 mm subtracted.

2.2 Soil microbial characteristics

Microbial biomass C and N content

Microbial biomass C and N content was determined by modified liquid fumigation method (Fierer & Schimel, 2003). In brief, for unfumigated and fumigated samples, 5 g of dry weight equivalent of fresh soil was weighed and 25 ml of pH 6.8 – 7.0 adjusted 0.5 M K_2SO_4 was added to each sample. Then, 0.5 ml of chloroform was added to fumigated samples only and both samples were shook on an orbital shaker at 300 rpm for 2 hours, followed by centrifugation at 3000 rpm for 10 minutes and filtering through Whatman no. 1 filter paper. Extracts were bubbled by air for 20 minutes. TOC and N in the extracts was determined using a TOC analyser (Shimadzu, Milton Keynes, UK).

Bacterial and fungal community composition

The sequencing including PCR amplifications and DNA extraction was performed in the Biochemistry lab at the Centre for Ecology and Hydrology (Wallingford, UK) employing standard protocols which were used in the lab at the time when the sequencing work was carried out. Due to the changes in the standard protocols from time to time in the lab, the protocol used in Chapter 3 differed from protocols used in the other chapters (Chapter 4, 5 and 6) as specified below.

Soil DNA extraction

Soil DNA was extracted using PowerSoil (Qiagen, Crawley, UK) (Chapter 3) or Quick-DNA Fecal/Soil Microbe 96 Kit (D6011, ZYMO Research, Irvine, USA) (Chapters 4, 5, 6) following manufactures instructions.

Target sequence amplification

Table 2.1 Temperature conditions for 16S and ITS PCR reactions.

	Chapter 3	**Chapter 4, 5, 6**
	One-step reaction	***Two-step reaction***
Initial denaturing	95 °C for 30 s	95 °C for 120 s
PCR cycles		
Number of cycles	25 (16S)/30 (ITS)	25 (16S, ITS) – step 1 8 (16S, ITS) – step 2
Denaturing	95 °C for 30 s	95 °C for 15 s
Primer annealing	52 °C for 30 s	55 °C for 20 s
Primer extension	72 °C for 120 s	72 °C for 30 s
Final extension	72 °C for 10 min	72 °C for 10 min

Chapter 3

Method listed in de Vries et al. (2018) was used. Separate bacterial and fungal PCRs were performed as a one-step procedure. Amplicons were generated using 20 ng of template DNA and Q5 Taq high-fidelity DNA polymerase (New England Biolabs) in PCR reactions specified in Table 2.1. The procedure was performed by personel of the Centre for Ecology and Hydrology (Wallingford, U.K.).

Chapters 4, 5 and 6

Separate bacterial and fungal PCRs were performed as a two-step procedure. Conditions of PCR reactions are specified in Table 2.1. For the first step, 16S rRNA PCRs for each sample contained: 0.25 µl of Taq polymerase (Merck KGaA, Germany), 5 µl buffer (10xPCR buffer), 0.5 µl BSA, 1 µl dNTPs, 0.1 µl of each primer (modified at 5' end with the addition of Illumina pre-adapter and Nextera sequencing primer sequences, primer details in each research chapter), 2 µl of DNA and 41.05 µl of molecular grade water; and ITS PCRs for each sample contained: 0.25 µl of Q5 high-fidelity DNA polymerase (New England Biolabs, USA), 5 µl buffer (5xPCR buffer), 5 µl buffer (5xhighGC), 0.5 µl dNTPs, 0.05 µl of each primer (modified at 5' end with the addition of Illumina pre-adapter and Nextera sequencing primer sequences, primer detailed in each research chapter), 1 µl of DNA and 13.15 µl of molecular grade water). For the second step, bacterial and fungal PCRs contained: 0.25 µl of Taq polymerase (Sigma-Aldrich), 5 µl buffer (10xPCR buffer), 0.5 µl BSA, 1 µl dNTPs, 5 µl of index primer (MiSeq adapters and 8nt dual-indexing barcode sequences), 1 µl of step 1 PCR product and 37.75 µl of molecular grade water. After each step, the products were quantified on NanoDrop 8000 (Thermo Fisher Scientific) and verified a 1.5 % agarose gel and cleaned using ZR-96 shallow well kit (D4018, ZYMO Research, Irvine).

Step 2 PCR product was pooled together in equimolar concentrations employing the following procedure. Amplicon sizes were determined using an Agilent 2200 TapeStation system. Libraries were normalized using SequalPrep Normalization Plate Kit (Thermo Fisher Scientific), quantified using Qubit dsDNA HS kit (Thermo Fisher Scientific) and pooled together at equal concentrations. The pooled library was diluted to achieve 400 pM in a 40 µl volume after denaturation and neutralisation. Denaturation was achieved with 4 µl 2 N NaOH for 5 minutes followed by neutralisation with 4 µl 2 N HCl. The prepared library was then diluted to the concentration of 12 pM with HT1 Buffer and 5% denatured PhiX control library. A final denaturation was performed by heating to 96°C for 2 minutes followed by cooling on ice.

Sequencing

The pooled PCR products were then analysed on an Illumina MiSeq instrument using TG-142-3003 - TG MiSeq® Reagent Kit v3 (600 cycle) Flow cell (Illumina, San Diego, USA) generating 2 x 300 bp reads. Sequencing was performed by personnel of the Centre for Ecology and Hydrology (Wallingford, U.K.).

Sequencer output processing and taxonomy assignment

Chapter 3

Bioinformatics pipelines as described in de Vries et al. (2018) were used for processing the data from the sequencer. For the bacterial sequences this involved joining paired sequence ends, quality filtering, length filtering with the minimum length of 300 bpsm, checking presence of PhiX and adaptors and their removal and removal of chimeras and singletons. Remaining sequences were clustered into operational taxonomic units (OTUs) at 97 % sequence identity and representative sequences for each OTU were taxonomically assigned by RDP Classifier with bootstrap threshold 0.8 or greater using the Greengenes database. Fungal sequences were analysed using the PIPIT pipeline with default parameters (Gweon et al., 2015) which involved quality filtering and clustering at 97 % similarity, chimeras removal and taxonomically assignment of representative OTUs using the UNITE database. The procedure was performed by personnel of the Centre for Ecology and Hydrology (Wallingford, U.K.).

Chapters 4, 5 and 6

Raw sequences, the output from Illumina with primers removed, were processed using DADA2 pipeline (Callahan et al., 2016). For bacteria and fungi separately, the sequences were filtered and trimmed to remove low quality ends for bacteria (where average quality scores dropped below Q30) and to the length of 250 nucleotides for fungi, dereplicated, sample inference performed using model learning error rates, forward and reverse reads were merged (using mergePairs function) to produce amplicon sequence variants (ASVs), sequence table constructed and chimeras removed (using removeBimeraDenovo fnction with default settings). Taxonomy was assigned using RDP classifier trained on the GreneGenes v13.8 (DeSantis et al., 2006) and Unite v7.2 (Kõljalg et al., 2005). The procedure was performed by personnel of the Centre for Ecology and Hydrology (Wallingford, U.K.).

2.3 Plant characteristics

Biomass yield

Biomass yield was determined after oven drying the plant material at 65 ˚C for 3 days.

Biomass C and N

Dried biomass samples (as described for biomass yield) were analysed using an automated Dumas procedure on a Vario EL analyser (Elementar, Stockport, UK).

Biomass P

Dried biomass samples (as described for biomass yield) by analysis of modified Kjeldahl digests on AA3 segmented flow analyser following standard method (SEAL Analytical Inc., Mequon, USA).

2.4 Soil extracellular enzyme potential activity

Extracellular enzyme assays (EEAs) were used for measurement of potential activity of hydrolytic extracellular enzymes related to C, N, P, and sulphur cycles (Table 2.2). A method based on Marx et al. (2001) and modified according to Puissant et al. (2015) was followed. Briefly, 1.5 g of fresh soil was mixed with 20 ml of dH_2O and agitated on a reciprocal shaker at 400 rpm (tubes places horizontally) at laboratory temperature for 20 min to obtain a homogenous soil solution. The soil solution (30 µl) was added to a 96-well microplate with 300 µM of flourimetric substrate (170 µl) including 4-MUB (4-methylumbelliferone) linked substrate for all enzymes apart from LAP which included 7-AMC (7-amino-4-methyl coumarin) linked substrate. The plates were then immediately incubated in dark at 28 ˚C for 3 hours using BioSpa 8 Automated Incubator and flourimetric measurements were performed every 30 minutes using Cytation 5 Cell Imaging Multi-Mode Reader (both instruments, BioTek, Winooski, USA). The excitation wavelength was 330 and 342 nm and emission wavelength was 450 and 440 nm for the 4-MUB and the 7-AMC substrate, respectively. The number of replicates and microplate templates were the same as Puissant et al. (2015) including three methodological replicates (sample + buffer + substrate) and a quenched standard (sample + buffer + 4-MUB or 7-AMC) for each sample and a control comprising the 4-

MUB- or 7-AMC-linked substrate and the buffer solution alone for each substrate. The plates were prepared and loaded into the instrument, the instrument was run, data were acquired from the instrument and enzyme kinetics were calculated by personnel of the Centre for Ecology and Hydrology (Wallingford, U.K.).

Table 2.2 Soil extracellular enzymes assayed for potential activity.

	EC number	Enzyme	Function
C cycle			
AG	3.2.1.20	α-D-glucopyranoside	Releases glucose during starch degradation. Able to degrade microbial cell wall sugars.[1)]
BG	3.2.1.21	β -D-glucopyranoside	Completes cellulose degradation, releases glucose.
BX	3.2.1.37	β-xylosidase	Degrades hemicellulose, target short xylan oligomers, releases xylose.
ACE	3.1.1.6	esterase	Catalyses the cleavage and formation of ester bonds.
C and N cycle			
CHIN	3.2.1.52	β-1,4-N-acetylhexosaminidase	Hydrolyses N-acetyl-β-glucosamine of fungal chitin and bacterial peptidoglycan and also cleaves amino sugar N-acetyl-β-D-galactosamine.
N cycle			
LAP	3.4.11.1	leucine-amino-peptidase	Targets peptides and release N-terminal (hydrophobic) amino acids, preferentially leucine.
P cycle			
PHO	3.1.3.1	alkaline phosphatase	P mineralization.
Sulphur cycle			
SUL	3.1.6	sulphatase	Hydrolyses ester-bonds of aryl-sulphate-esters releasing sulphate.

[1)] Gude et al. (2012)

2.5 Data analysis

2.5.1 Multivariate analysis of microbial communities

Multivariate generalised linear models (M-GLMs)

Generalised linear model (GLM) framework from MVABUND 3.10.4 package in R (Wang et al., 2012) was used (e.g. Sheldrake et al., 2017). This framework represents a model-based approach to the analysis of multivariate abundance data traditionally performed by distance-based analyses and it showed to have better power properties than the former approach (Warton et al., 2012). The model was built with *manyglm* function fitting a separate GLM to each taxonomic unit (based on taxonomic resolution). Model assumptions (i.e. mean variance assumption, and relationship between mean abundance and environmental variables) were checked by plotting the model's residuals vs. fitted values using function *plot*. The family selected was "negative binomial" in all cases. Treatment effect was tested using *anova*. Statistical power of the model was calculated by bootstrapping at 1000 permutations combined with "Wald" test statistics (models at species taxonomic resolution were assessed by "score" test statistics). Model argument *cor.type* was used in order to account for correlation between taxa whereby *cor.type* = "shrink" was set for phylum, class an order taxonomic resolution and *cor.type* = "I" was used for species analysis. Block was fit in the model formula as random factor (excluding species taxonomic resolution where block was not used in the model formula). Post hoc pairwise comparison was performed using *pairwise.comp* argument of *manyglm.anova* function.

Indicator species analysis

Indicator species analysis was used to assess response of microbial community at the species taxonomic level. Indicator value index (Dufrêne & Legendre, 1997) was calculated for each OUT/ASV using function *multipat* (R package indicspecies) and it is the product of the relative frequency and relative average abundance within each treatment. Significance of the test was assessed by bootstrapping at 1000 permutations. For the selection of OTUs/ASVs marked as indicator species, parameters A (specificity) and B (fidelity) were set to 0. Abundance of the selected indicator species (with $P < 0.05$) for each land management history category was grouped at order taxonomic resolution. The results were expressed as proportion (%) of indicator species to overall relative abundance of the order on all tested sites.

Chapter 3: **Land management history effect on soil aggregation, organic C distribution within soil structure and soil bacterial and fungal communities in calcareous grassland ecosystem.**

3.1 Introduction

Ecosystems deliver a variety of services important for humans with short term benefits (e.g. provisioning of food and goods) as well as long term benefits (i.e. regulating services including for instance climate regulation) (Alcamo et al. 2003). Moreover, indirect benefits are delivered by supporting services through their role in overall ecosystem functioning such as for instance soil formation and nutrient cycling. Maximising food and goods provisioning services, delivered through conversion of natural ecosystems to cropland (land use change) and further intensification of agriculturally managed land, can trade-off with delivery of other ecosystem services (Zhang et al., 2007; Power, 2010). This ecosystem services trade-off can negatively impact human well-being as well as ecosystem functioning and affect future delivery of ecosystem services, at a regional and global scales (Alcamo et al. 2003; Foley et al., 2005). Therefore agriculture has been identified as a key driver of many global threats such as climate change, biodiversity loss and degradation of land and freshwater (Alcamo et al. 2003; Foley et al., 2005; Power, 2010) and is a major force driving the environment beyond the 'planetary boundaries' (Rockström et al., 2009). Pressure on food production systems are expected to be further increased by the predicted population growth and rise of global consumption as well as by climate change (Godfray et al., 2010; Foley et al., 2011). Thus, it is necessary to change the way we manage our agricultural land in order to enhance food and goods provisioning services and sustainability and enhance delivery of other ecosystem services in agroecosystems (Foley et al., 2005, 2011; Power et al., 2010) through understanding of ecosystem responses to land use (Zhang et al., 2007).

3.1.1 Soil organic C storage mechanism

Formation of microaggregates and turnover of macroaggregates are key processes for OC stabilization in the soil and resulting build-up of SOC stock (J. Six et al., 2000). They are controlled by factors including soil animals (earthworms, termites, microorganisms), roots, inorganic binding agents (such as oxides and calcium) and environmental cycles (such as freeze-thaw cycles, dry-wet cycles and fire) (J. Six et al., 2004). Additionally, land use change and land management practise can also have a strong impact on SOC stocks (e.g. Six et al., 1998; Guo and Gifford, 2002).

Soil OC stock is a function of inputs of organic matter into the soil and its loss through processes such as microbial mineralization but also other processes including leaching of organic compounds or erosion. The OC storage is controlled by a multitude of interactive factors affecting ecosystem processes and operating at different scales (Wiesmeier at al., 2019). Climate and vegetation are strong factors which operate at a global scale (Jobbagy and Jackson, 2000) while land use and management, soil texture and microbial community drive SOC at a regional scale (Wiesmeier at al., 2019). Furthermore, the latter two together with soil physico-chemical characteristics control OC dynamics and storage within mineral soil structure at each location as they are related to OC storage mechanism.

In mineral soils, SOC can be stabilized by three distinct mechanisms (Six et al., 2002; Lutzow et al., 2006): inherent chemical composition, physical protection within soil aggregate structure and

organo-mineral interactions. The latter two are thought to be the key mechanisms for SOC long term storage while chemical composition can result only in a short-term protection, since all organic matter is essentially degradable under the right conditions (Schmidt et al., 2011). The majority of the SOC stock in mineral soils located within 'stable' pools such as organo-mineral interactions pool contains relatively labile compounds (Schmidt et al., 2011; Lehmann and Kleber, 2015). These compounds are largely of microbial origin delivered through processing of primary input compounds and microbial loop re-processing already stabilized organic compounds after their destabilization (Gleixner, 2013). The proportion of plant-derived and microbial-derived compounds would depend on relative importance of different paths of primary input degradation such as '*ex vivo*' processing through extracellular enzyme degradation or '*in vivo*' processing through microbial uptake and assimilation (Liang et al., 2017). Therefore SOC pool size and chemistry are related to the capacity of the soil to slow down its degradative processes through protective mechanisms (Dungait et al., 2012) and as such the persistence of OC in the soil can be characterized as an 'ecosystem property' rather than chemical composition of primary input chemistry (Schmidt et al., 2011).

Physical protection of OC through soil aggregation represents an important mechanism of OC stabilization in the soil (Tisdal and Oades, 1982; Six et al., 2000a; Six et al., 2004; O'Brien and Jastrow, 2013). Soil aggregation is characterized by spatial arrangement of soil particles and affects positioning of POM such as within or outside of soil aggregates. This positioning of POM results in its decomposition rate when the decomposition rate of organic matter within soil aggregates is lower than when it is situated outside of aggregates (Besnard et al., 1996; Angers et al., 1997). Furthermore, aggregation also offers protection from decomposition for OC stabilized by organo-mineral interactions because these particles are essentially building blocks of the aggregates. Most of the SOC can be in fact found within aggregates (Jastrow et al., 1996). Aggregates not only physically protect OC but also influence other important soil characteristics which then affect SOC dynamics (Six et al., 2004) such as microbial community (Mummey et al., 2006) and oxygen diffusion (Sexstone et al., 1985).

Aggregates are classified according to their size into macroaggregates and microaggregates when 250 µm diameter is size limit between them (Tisdall and Oades, 1982). The lower size limit for microaggregates is determined by the size limit for silt soil fraction, which can be between 50 – 63 µm, depending on regional soil texture classification system (Totsche et al., 2018). However, it has been shown that soil particles lower than 50 µm, which are typically classified as silt, and even smaller particles classified as clay, can also contain very small aggregate structures (Chenu and Plante, 2006; Virto et al., 2008, 2010). Thus, microaggregates can be further divided into larger microaggregates (250 – 50 um) and smaller microaggregates (< 50 um).

Formation and maintenance of soil aggregate structure is a dynamic process. It is characterized by a concept of aggregate hierarchy whereby microaggregates are understood to be formed inside of macroaggregates and different binding agents act at different hierarchical stages of aggregation (Tisdal and Oades, 1982; Oades, 1984; Six et al. 2000; Six et al., 2004). The hierarchical nature of aggregate formation also applies to microaggregates (Totsche et al., 2018). Microaggregates are held together by persistent binding agents including organo-mineral interactions and cementing and gluing agents which provide stronger protection from breaking up when compared to macroaggregates, which are on the other hand held together by temporary (root and hyphae entanglement) and transient (organic compounds acting as a glue) binding agents (Oades, 1984; Totsche et al., 2018). Microaggregats can be formed by associations of organic compounds with mineral surfaces (Edwards and Bremner, 1967; Lehmann et al., 2007) and encrustation of particulate OC by fine mineral particles (Oades and Tisdall, 1982). Mineral-mineral interactions can also initiate accumulation of building units and process of microaggregate formation (Totsche et

al., 2018). At the lower size range (< 50 μm), concept of aggregation also includes colloids and organo-mineral associations (Tisdal and Oades, 1982; Totsche et al. 2018).

As a result of the different binding agents acting at different scales (the concept of hierarchy), stability of microaggregates is higher than stability of macroaggregate (Oades, 1984; Six et al., 2004). This is reflected in their turnover rate when macroaggregates turn over faster than microaggregates such as 30 vs. 88 days (De Gryze et al., 2006). However, the material contained within the aggregates does not reflect the turnover of the aggregates (Six et al., 2004) when aggregates typically contain OC of much higher age that aggregate turnover. Moreover, macroaggregate turnover rate was found to be linked to microaggregate formation within them and consequently to stabilization of SOC in microaggregates, when too rapid turnover does not result in microaggregates being formed and stabilized (Six et al., 2000). Overall, the hierarchical nature of aggregate formation is reflected in POM flow through the soil structure when POM is first incorporated into macroaggregates and then is incorporated in microaggregates (Angers et al., 1997). Primary input organic material is progressively degraded over time as it 'flows' through soil structure into smaller microaggregates, and this results in SOC pool characterized as a mixture of organic matter at different stages of decomposition (Lehman and Kleber, 2015).

Microaggregates contain significant amount of total soil OC and microaggregate within slaking resistant macroaggregate fraction was shown to be an important diagnostic fraction for the negative effect of tillage in croplands (Johan Six & Paustian, 2014). It has been widely documented that OC associated with this fraction explained a large portion of the difference of soil OC stocks between conventional tillage and no-tillage practises (Six et al., 2014). This fraction stored the highest OC pool in a never tilled prairie soil (O'Brien and Jastrow, 2013; Scott et al., 2017). They further showed that cropland cultivation including conventional tillage redistributed microaggregate mass and associated OC pools from slaking resistant-macroaggregates to free microaggregates. Therefore, microaggregate within macroaggergate-associated OC represent an important pool for long term OC storage.

Food production through converting natural (e.g. native forest, prairie) or managed (e.g. pasture) ecosystems to cropland was shown to result in a reduction of SOC stock (Guo and Gifford, 2002; Poeplau and Don, 2013). Furthermore, land management practise can also affect SOC stock. This has been widely demonstrated for tillage whereby conventional tillage practise showed lower SOC stock when compared to no-tillage practise (Six et al., 2014). Similar for grasslands, management practise can also affect SOC stock. High intensity management was shown to decrease SOC stock when compared to grasslands with less intensive practises (Ward et al., 2016). Poor grassland management can result in SOC loss (Soussana et al., 2004; Smith, 2014) which has been associated with ploughing and re-seeding (Carolan & Fornara, 2016). However, tillage may not always cause reduction of SOC as other factors may have stronger impact on OC dynamics and OC stock such as climatic conditions (Singh et al., 2015) or bulk density (Carolana and Fornara, 2016). Similarly, grassland intensification can also enhance SOC (Barto et al., 2010) through, for instance, an improvement of their soil nutrient status and increased plant inputs to the soil. This was demonstrated by Ward et al. (2016) who found that intermediate intensity grassland management had higher SOC than less intensive practise. Grassland management can negatively affect soil aggregation through effect on arbuscular mycorrhizal fungi, compaction and animal trampling (Barto et al., 2010; Duchicela et al., 2012). On the other hand, increased aggregation may also reduce aggregate turnover over the point required for stabilization of new OC into microaggregates (Barto et al., 2010).

Study of grassland regeneration chronosequence on former intensively managed agricultural sites can give an important insight into OC stabilization mechanism (O'Brien and Jastrow, 2013). Most of

such studies targeted restored grasslands established on former arable land through reseeding (Baer et al., 2010; Hernández et al., 2013; O'Brien and Jastrow, 2013; Rosenzweig et al., 2016; Barber et al., 2017) and few of them targeted secondary successional development on abandoned land (Kuramae et al., 2010; Lozano et al., 2014). Typically, SOC stocks increased after the cessation of arable land management, which showed a linear relationship with age (e.g. Matamala et al., 2008; Barber et al., 2017). Nevertheless, older restored grasslands showed reduction of the initial OC increase compared to younger restoration sites (Jie et al., 2011; O'Brien and Jastrow, 2013; Spohn et al., 2016). Interestingly, Kuramae et al. (2010) and Baer et al. (2010) showed no changes of SOC stock during grassland restoration. Modelling of soil OC dynamics revealed that centuries will be need for SOC to reach the native grassland SOC stock (Matamala et al., 2008; Rosenzweig et al., 2016).

Within 33-year restored grassland chronosequence, O'Brien and Jastrow (2013) suggested that the initial linear increase of soil aggregate fractions and their overall soil pools may start to plateau in the nearest future, as was shown for the iMicroaggregate fraction. They further separated the main aggregate fractions into their building units including clay, silt and POM. This increased the insight into OC dynamics and silt fraction was found the most important fraction for overall soil OC stock. Moreover, silt OC pools within individual aggregate fractions plateaued or were predicted to plateau below the native prairie levels for both most important fractions, microaggregates and iMicroaggregates silt fractions. They speculated that OC recovery might go through several steady states before reaching the final steady state of the native ecosystem due to slower recovery of all protective mechanisms within the soil. There can be a time lag for delivery of organic matter of the right size or origin for all stabilization mechanisms even if the overall soil input is in surplus (O'Brien and Jastrow, 2013). They also suggested that silt size fraction might not be a homogeneous fraction and its sub-fraction(s) might continue to increase the OC content through time. Indeed, slaking resistant silt fraction, which was found to store over 50 % of total SOC, consisted of small microaggregates (Virto et al., 2008; Virto et al., 2010). This idea was followed in the present research (Hypothesis 1).

3.1.2 Microbial community relationship with soil aggregation and SOC stabilization

Microorganisms are involved in both, decomposition as well as stabilization of OC compounds (e.g. Schimel and Shaefer, 2012; Lehman and Kleber, 2015; Liang et al., 2017). Microbial contribution to the latter is due to their role in soil aggregation (Six et al., 2004). Microorganisms were found more important for soil aggregation than other soil organisms such as worms (Caruso et al., 2018). Their contribution to soil aggregation can be understood at two different scales. At the macroaggregate scale, where fungal hyphae network together with plant roots contributes to macroaggregate formation by transient forces of entanglement of soil particles (Miller and Jastrow, 2000; Oades, 1993; Leifheit et al., 2014; Lehmann and Rillig, 2015). At the microaggregate scale, production of mucilage and extracellular polysaccharides by bacteria and fungi, as well as by roots and soil fauna, stabilize soil structure (Oades, 1993). Fungi will also impact on microaggregate formation through stabilization of macroaggregate structures within microaggregates are formed. Bacteria were originally thought to play a role only during stabilization of microaggregates, however Lehman et al. (2017) found recently, that they are also important for stabilization of macroaggregates. Nevertheless, the relative importance of microbial role in aggregation and SOC stabilization was found to be determined by soil type (Denef and Six, 2005).

Microbial contributions to OC stabilization can be also related to their role in processing of OC compounds in the soil which directly affects amount and composition of OC available for stabilization. Efficiency by which substrates are processed by soil degraders affects quantity of the primary productivity inputs remaining in the soil (Cotrufo et al., 2013) as well as already stabilized

OC during microbial re-processing through the microbial loop (Gleixner et al. 2013). Moreover, mycorrhizal fungi, and also generally other fungal groups, can potentially influence soil aggregation at different levels through effects on plant communities, plant roots (individual host), and effects mediated by the fungal mycelium itself (Rillig and Mummey, 2006).

The relationship between the microbial community structure with aggregation and SOC stabilization is less understood (Six et al., 2004). Soils contain a broad diversity of microbial taxa (Tedersoo et al., 2014; Fierer, 2017). At a larger scale, bacterial community composition was shown to be strongly affected by soil pH (R. I. Griffiths et al., 2011), but also by SOC content, soil oxygen, redox status, moisture availability, N and P availability, soil texture and structure, and temperature (Fierer, 2017). Fungal community patterns were best predicted by climatic factors (mean annual precipitation), edaphic factors (soil calcium concentration and pH) and spatial patterns (Tedersoo et al., 2014). The relative importance of factors affecting microbial community changes with scale, and while soil pH is a strong factor, other factors become important in structuring bacterial community at smaller scale with constant soil pH, such as soil P status (Kuramae et al., 2011). Moreover, at single location, various conditions can be found in the microscale of the soil matrix which determine cell distribution and activity, their potential to interact and evolution and maintenance of biodiversity and function (Vos et al., 2013). Soil aggregation strongly contributes to spatial differentiation of soil conditions at the microscale through compartmentalization by aggregate structure as well as through creation of pore network (Totsche et al., 2018) modifying interactions of soil organisms through providing refuge sites for prey against predation (Young et al., 1998) or reducing competitive interaction through moisture variation increasing diversity (Carson et al., 2010). Soil aggregate sizes were found to be associated with different bacterial phyla (Davinic et al., 2012; Bach et al., 2018). Furthermore, spatial separation, such as through microaggregation, is speculated to promote microbial evolutionary processes inside of the aggregates resulting in increased diversity (Rillig et al., 2017). Soil aggregation thus create a scene where different microbial communities can coexist and thrive, thus promoting soil microbial diversity (Totsche et al., 2018), however microbes are to a large extent responsible for soil aggregation.

Understanding of microbial role in soil functioning through study of ecosystem secondary succession

Secondary succession following a temporary disturbance is a process which tends to re-establish a community similar to the original undisturbed one (Horn, 1974). It has received attention recently through an effort for restoration of degraded ecosystems negatively impacted by land use change and land management (Barber et al., 2017). Secondary successional development is initiated on ex-agricultural land through restoration practices of various intensities targeting end point plant communities (e.g. Martiny et al., 2005). It is assumed that through restoration of aboveground the belowground will follow (top down approach) (Strickland et al., 2017).

Studies related to such successional development can be an important tool to elucidate important ecosystem mechanisms (Harris, 2009). Latest studies in this area are increasingly involve the microbial community as a strong component of restoration effort which can serve as facilitator of ecosystem recovery or a monitoring tool to determine its success (Gellie et al., 2017; van der Bij et al., 2018). Such studies can give valuable information on shifts of microbial communities and consequently on contributions to ecosystem functioning (Hannula et al., 2017).

Microbial community biomass typically increases with age during ecosystem restoration by conversion to grasslands (e.g. Jangid et al., 2010). This has also been found in a global meta-analysis of grasslands and forests successional sequences (Zhou et al., 2017). Its initial increase can be attributed to a change of plant inputs to the soil after perennial vegetation establishment (Baer et al., 2002). However, microbial biomass was also observed constant within restoring grasslands of 7 to 30 years (Hannula et al., 2017).

Microbial community composition followed different paths during secondary succession including relatively fast divergence from arable community after the conversion (Kuramae et al., 2010; Barber et al., 2018) or delayed community change (Jangid et al., 2010; Lozano et al., 2014). The later suggests lasting effect of arable land use (Lozano et al., 2014). Natural ecosystems are understood to be characterized with wider fungal:bacterial ratio than agricultural ecosystems. Increase of the fungal:bacteria ratio typically follows immediately after arable management cessation (Scott and Blair, 2017), however it may not increase further after this initial change (e.g. Jangid et al., 2010; Hannula et al. 2017), although Zhou et al. (2017) showed an increase of fungal:bacterial ratio with age of restoring ecosystem in a meta-analysis of 85 successional sequences. Studies comparing microbial community structure of restoring grasslands with target native ecosystem showed that those communities were similar within less than 27 years (Barber et al., 2018) or still different within over 85 years (e.g. Jangid et al., 2010).

3.2 Hypotheses

Grasslands represent an important terrestrial ecosystem providing essential ecosystem services which include for instance soil OC storage (Conant et al., 2001) and food provisioning among others. They can be also important for maintaining biodiversity within agricultural landscape for instance such as field margins (Schulte et al., 2017), extensively managed permanent grasslands or within a cropping system, which would also positively impact on soil aggregation through their root system (Jastrow et al., 1998). Thus, studies aiming at understanding of soil functioning within grasslands would be beneficial for development of better management strategies for improved ecosystem services delivery from grasslands as well as for restoration of degraded sites. Traditional restoration strategies focus at aboveground component with expectations that other components

of the ecosystem will follow, however such approach might overlook important agents of ecosystem assembly and drivers of ecosystem processes related to delivery its function (i.e. soil microbial community) (Harris, 2009; Strickland et al., 2017). The present research addressed knowledge gap related to lack of detailed understanding of development of belowground ecosystem component (abiotic as well as biotic) during its restoration.

Restoration chronosequence of calcareous grasslands was studied. Such ecosystem is known for its large floral and faunal diversity (Redhead et al., 2014). The surveyed sites were grasslands taken from intensive land management practise at a time spanning on average from 6 to 136 years ago: Recent regenerating grasslands (≈ 6 years old), Medium regenerating grasslands (≈ 50 years old) and Old regenerating grasslands (≈ 136 years old). This chronosequence potentially represents the best-case scenario of natural regeneration (Redhead et al., 2014). Studied sites also involved nearby arable fields in order to represent the most intensive land management practise (i.e. frequent tillage, fertilization and row-cropping).

Soil aggregation and OC pools were studied within this chronosequence. It is understood that intensive land management negatively impact soil aggregation and OC stock and that after cessation of this management, these parameters are changing towards their levels in a native ecosystem.
Monitoring these parameters during ecosystem regeneration might further elucidate mechanism of SOC storage and their controls in order to facilitate recovery of the degraded C stocks (e.g. Jastrow et al., 1998; O'Brien and Jastrow, et al. 2013; Hannula et al., 2018). It was hypothesized that (1) land use will affect soil aggregation and bulk SOC concentration whereby intensive arable crop production will show lower soil aggregation as well as SOC concentration than extensively managed grasslands, and that (2) grasslands with the most recent history of intensive agricultural practise will show lower bulk SOC concentration than sites with the longest regenerating status.

Restoration of soil organic C stock was predicted to take centuries (Matamala et al., 2008) and increase of OC in different soil C pools was shown or predicted to plateau relatively soon after restoration initiation (O'Brien and Jastrow, 2013). Organic C associated with silt fraction represents an important C pool in the soil (Virto et al., 2008, 2010). O'Brien and Jastrow (2013) argued that silt fraction may consists sub-fractions containing OC pools of different dynamics which may still be sequestering C while other soil OC fractions might be saturated. The idea of silt sub-fractions was followed in the present research and silt fraction was separated into coarse and fine silt fractions. It was further hypothesized that (3) silt-size fraction will be the most important fraction in relation to OC storage and it will consist of two sub-fractions of different sizes which can be characterized as two distinct fractions based on aggregation and/or OC concentration.

Furthermore, successional change of soil microbial community was also studied. (4) Microbial communities of both bacteria and fungi will differ between arable and Recent regenerating grasslands as well as between Recent regenerating grasslands and Old regenerating grasslands. (5) Copiotrophic bacterial species will show a trend of decreasing their relative abundance while oligotrophic bacterial species will show opposite trend from arable and Recent regenerating grasslands towards Old regenerating grassland.

3.3 Methodology

Site description

The study area was located on calcareous grasslands within the Defence Training Estate Salisbury Plain (DTE SP, Wiltshire, UK) and surrounding arable sites. Incorporation of land into the DTE at various times since late 1890s resulted in a mosaic of grasslands without significant agricultural influence spanning a range from 6 to over 150 years. Redhead et al. (2014) classified these grasslands into three distinct categories based on overlay of historical land uses from six time periods (1840s, 1880s, 1930s, 1967, 1985 and 1996) including Old grasslands (grasslands from 1840 to 1880, ≈ 136 years old), Medium grasslands (grasslands from 1930 to 1967, ≈ 50 years old) and Recent grasslands (grasslands from 1985 to 1996, ≈ 6 years old) (Figure 3.1). Mapped grasslands within the DTE are likely to be agriculturally unimproved prior to late 1930s, however any earlier agricultural influence up to post-medieval period is possible (Redhead et al., 2014). The sites have history of disturbance from military activity, burning and shrub encroachment, however without evidence of systematic bias of these disturbance variables in relation to grassland age (Redhead et al., 2014). Current management includes extensive grazing introduced recently to prevent shrub encroachment characterised by high grazing density for limited time period during the year complying with suggested stocking density for UK lowland calcareous grasslands (Woodcock et al., 2005).

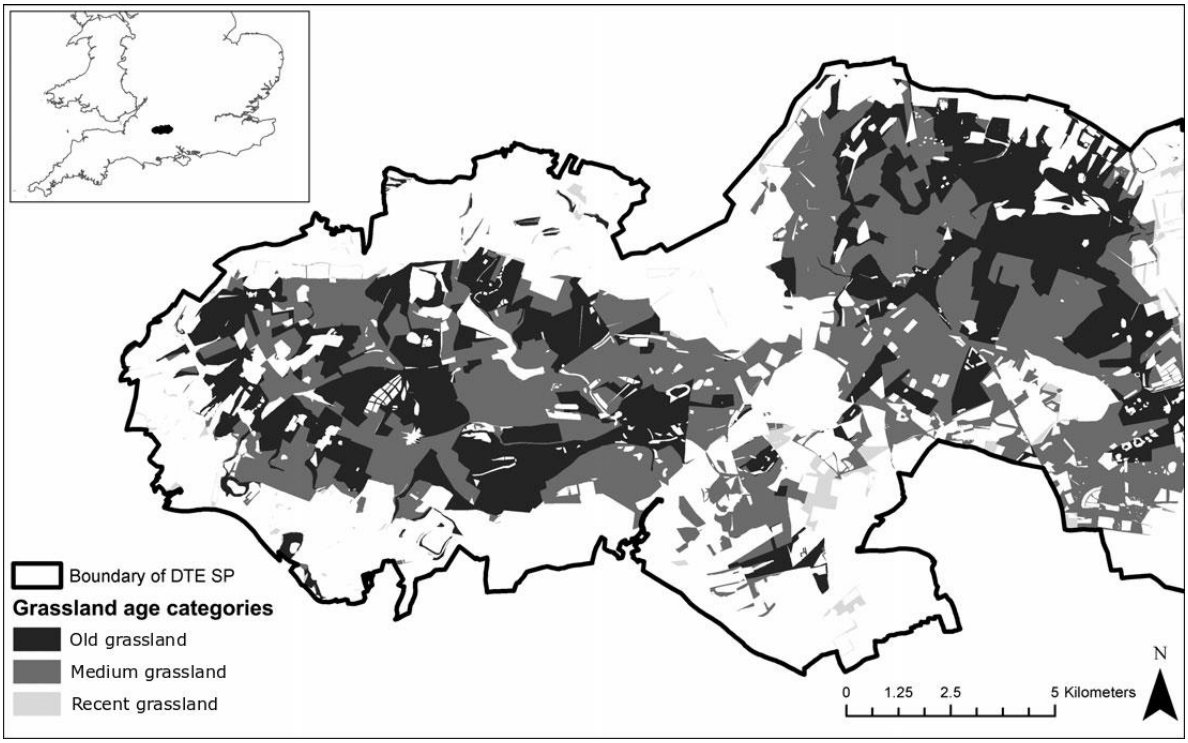


Figure 3.1 Estimated grassland age on western and central Defence Training Estate, Salisbury Plain. Sourced from (Redhead et al., 2014). Unshaded areas within the Defence Training Estate, Salisbury Plain are those lost to improvement, lacking historic data or those that are not grassland (forest, built up, etc.). Inset map shows location of Defence Training Estate, Salisbury Plain in southern UK.

Experimental design

Regenerating grasslands were selected from the DTE SP to comprise all three categories of sites with different land use history including Recent (13 sites), Medium (13 sites) and Old (14 sites) grasslands. Only sites without recent military activity were selected. The study also included 12

arable fields located in the same area (outside of the DTE SP) which had wheat crop in the year of sampling. Studied grasslands formed a chronosequence of sites with different times without significant agricultural influence (i.e. ecosystem regeneration) (Figure 3.2).

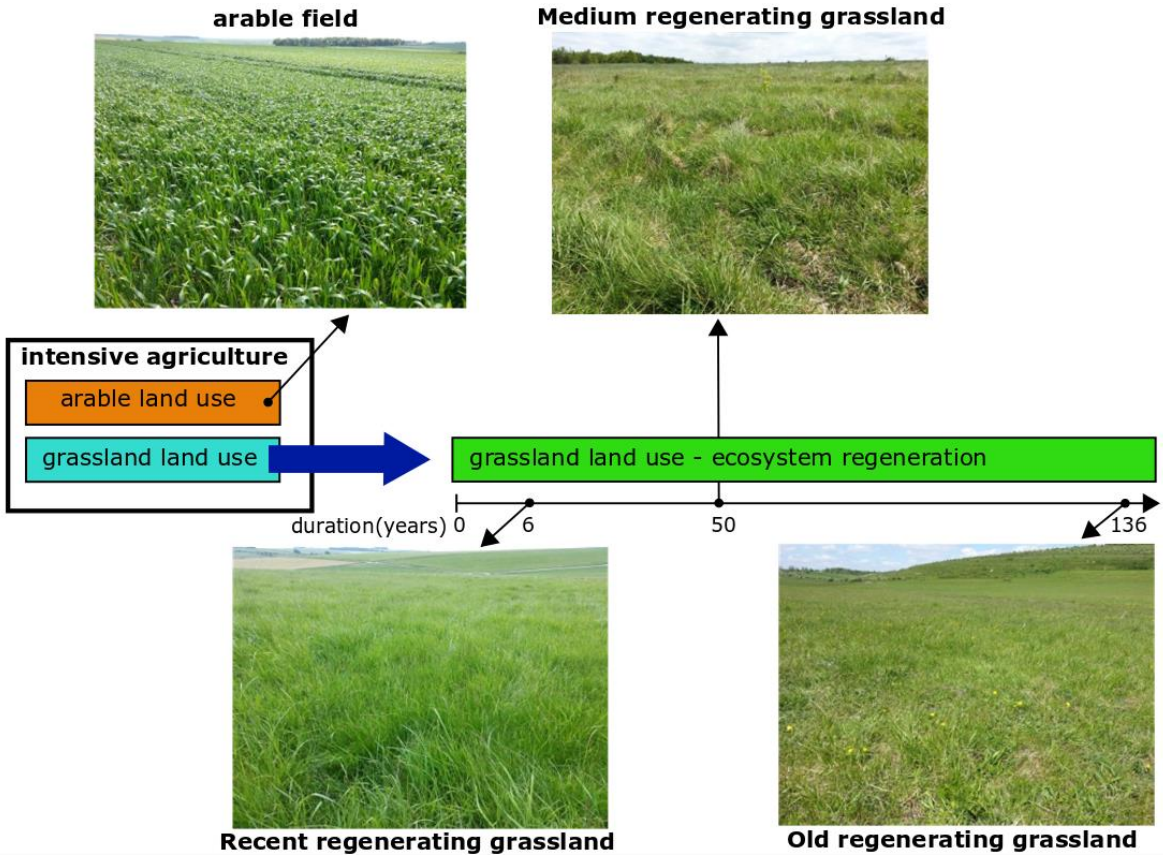


Figure 3.2 Studied ecosystems including (A) arable field, (B) recent grassland, (C) medium grassland and (D) old grassland. Grassland sites located within Defence Training Estate Salisbury Plain (DTE SP, Wiltshire, UK) and arable sites located in the nearest area within and outside the DTE. Pictures taken in the day of sampling (early June, 2014).

Soil sampling

Soil was sampled in early June 2014 on a transect of five sampling points positioned approximately 5 m apart. The transect was located in the inner section of the field and away from any visible vehicle trails. Soil cores were taken from a depth of 20 cm using plastic pipes of 5 cm diameter and soil corer of 2.5 cm diameter. The soil sampled by soil corer was used for basic soil characteristics and was stored at 4 ˚C until analysis. Soil sampled using plastic pipe was used for soil fractionation and soil microbial community analysis. For each sample, soil was gently taken out of the pipes and homogenized, a subsample was taken for microbial analysis, and both were stored at – 20 ˚C until used.

Basic soil characteristics analysis

For each sample, soil from all five sampling points within the transect was passed through a 2 mm sieve to remove stones, roots and homogenize the soil. Gravimetric soil moisture was determined on fresh soil. Measurement of pH was performed using 6 g of fresh soil suspended in 15 ml of deionised H_2O by shaking for 30 minutes followed by settling for 1 hour. A subsample was dried at

105 ˚C, ball-milled and analysed to determine organic and inorganic C and N contents. Each sample was analysed untreated to determine total soil C and N and after Cate removal treatment to determine organic C and N. Inorganic C and N was calculated by subtraction.

Soil aggregate fractions separation and analysis

Soil samples were gently defrosted, and a subsample was taken for the fractionation procedure which was passed through a 6 mm sieve. The subsample was air-dried. A three-step procedure based on modified O'Brien and Jastrow (2013) method was followed to separate organic matter pools from the soil sample (Figure 3.4). In brief, soil is separated into non-aggregated (NA) matter, free microaggregates (m) and macroaggregates, while macroaggregates are further separated into intra macroaggregate matter (intra-M/iM) and microaggregates within macroaggregates (iM-microaggregates/iM-m).

Wet sieving

The first step separated soil into water stable macroaggregates, microaggregates and non-aggregated soil matter. Soil sample (40 – 80 g) was slaked in deionized water for 5 minutes (2-mm sieve was placed into plastic wet-sieving bowl, deionized water was poured to the bowl to reach level of approximately 1 – 1.5 cm above the mesh of the sieve and the weighed soil sample was gently put into the partially submerged sieve). The sieve was gently agitated in order to wet all the soil aggregates. Weight of the sample used depended on the yield of non-aggregated fractions which was lower for Old and Medium grasslands. After the slaking, floating litter was collected by aspiring it away. The soil was manually wet sieved through a series of sieves in an order from 2 mm, 1 mm, 710 µm, 425 µm, 250 µm, 180 µm, 106 µm, 50 µm and 20 µm openings in a sequential procedure when the soil passing sieve with wider opening was gently poured to the next smaller sieve. For each sieve, sample was wet sieved for 2 minutes at 25 oscillations $minute^{-1}$ by lifting the sieve out of the liquid by approximately 1 – 2 cm. This process was followed for any other wet sieving procedure later in the process. Soil remaining on each sieve was pre-dried at 105 ˚C for 30 minutes, gently transferred into an aluminium tray, dried at 65 ˚C and weighed. Remaining soil material in the rinse water from wet sieving (< 20 µm fraction) was extracted via centrifugation at 3500xg for 10 minutes (after addition of 1 mL of 2.5 M $CaCl_2$ per litre of the rinse water to flocculate clay), dried at 65 ˚C and weighed.

Detailed soil fractionation

Five samples from each land management history category were selected for detailed soil fractionation (Figure 3.3). For each sample, aggregate fractions from wet sieving were pooled to achieve aggregate size distribution of > 250 µm (macroaggregates), 250 – 50 µm (free microaggregates) and 50 – 20 µm (non-aggregated fine silt and clay).

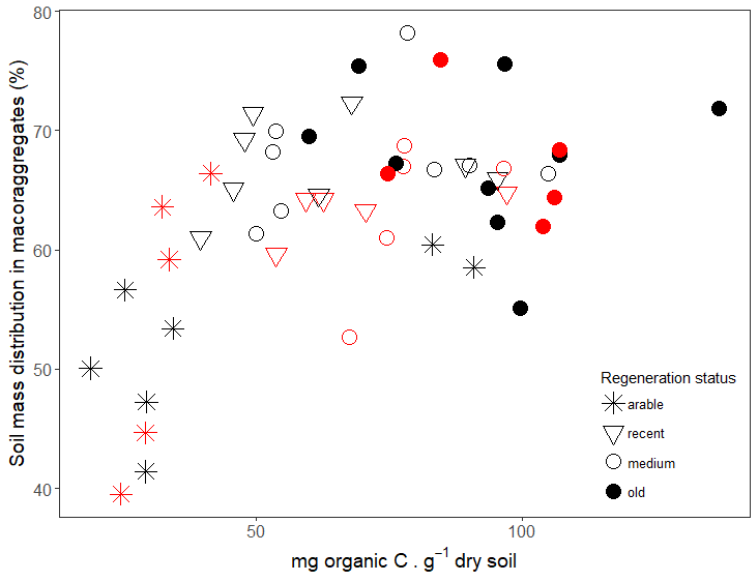


Figure 3.3 Soil total OC and distribution of soil mass in macroaggregates for sites with different land management history. Sites selected for detailed soil fractionation are marked by red colour.

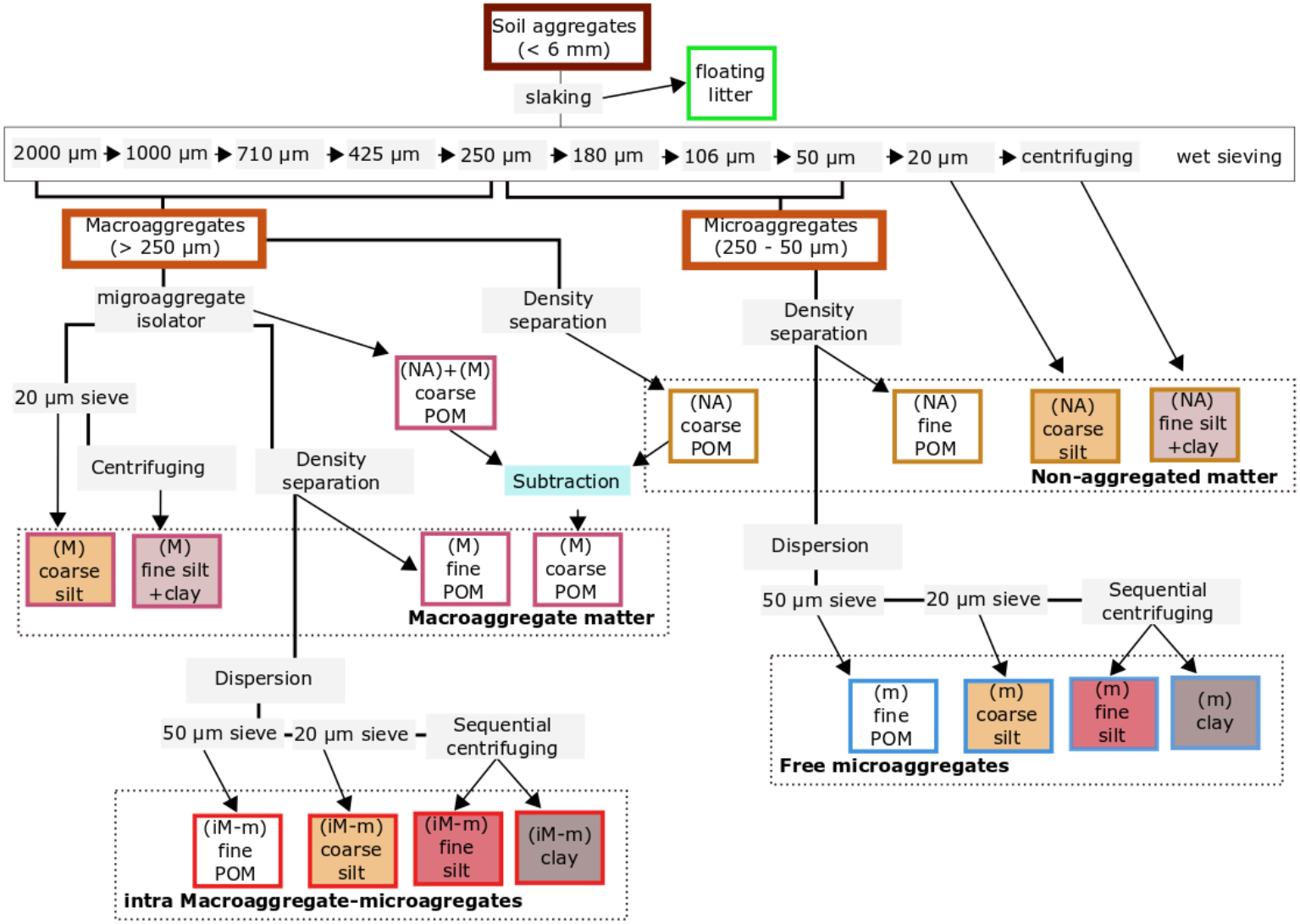


Figure 3.4 Fractionation scheme.

The second step of fractionation procedure included separation of microaggregates from within macroaggregates using microaggregate isolator which was constructed (in a workshop at Lancaster University) based on the design of Six et al. (2000) (Figure 3.5). The isolator disrupts macroaggregates while leaving microaggregates from within the macroaggregates intact. A macroaggregate sample (10 g) was slaked in 20 mL of water for 10 minutes and poured into microaggregate isolator chamber containing 50 metal balls (4 mm diameter) on a 250 µm mesh. The water level was set at approximately 1.5 cm above the mesh and stable water flow was maintained from the top of the apparatus. Water passing the mesh was then passed through a 50 µm sieve and collected in a large bowl. The isolator chamber was mounted on a reciprocal shaker and the disruption of the macroaggregates was achieved by energy of the moving metal balls in the chamber at 186 oscillations minute^{-1}. The isolation was completed when clear water was running from the isolator chamber and no visible soil aggregates were remaining in the chamber. The oscillation speed was increased when macroaggregates were still remaining in the chamber after few minutes.

Rinse water passing the 50 µm sieve was wet sieved through 20 µm sieve. Material collected on each sieve was pre-dried at 100 ˚C for 30 minutes, transferred to aluminium dish, dried at 65 ˚C and weighed. Remaining soil material in the rinse water from wet sieving (< 20 µm fraction) was extracted via centrifugation at 3500xg for 10 minutes (after addition of 1 mL of 2.5 M $CaCl_2$ per litre of the rinse water to flocculate clay), dried at 65 ˚C and weighed. Material collected on the sieves was pre-dried at 105 ˚C for 30 minutes, gently transferred into aluminium tray, dried at 65 ˚C and weighed. Microaggregates within macroaggregates fraction was collected on the 50 µm sieve, macroaggregate coarse silt fraction was collected from 20 µm sieve and combined coarse POM from macroaggregates and non-aggregated fraction was collected from 250 µm sieve.

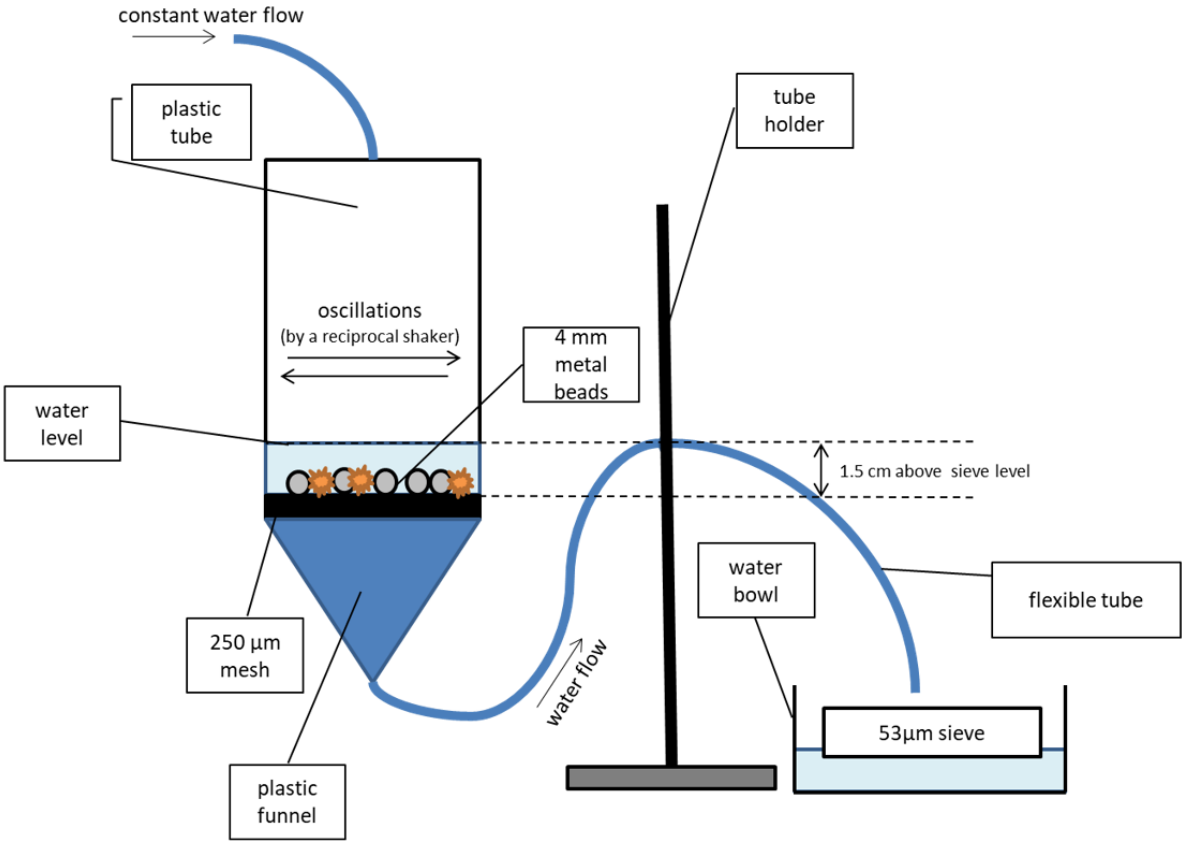


Figure 3.5 Microaggregate isolator set up. *Based on design of Six et al. (2000).*

Density separation was used to separate the combined coarse POM associated with non-aggregated soil matter. Sample of the macroaggregate fraction (5 - 10 g depending on POM amount) were gently inverted in 45 ml of sodium iodide (density of 1.6 g cm^{-3} based on testing different densities for best separation of mineral and POM fraction) in a 50 ml tube and subjected to a vacuum for 5 minutes. The sample was then centrifuged at 1173×g for 30 minutes, the POM was collected using aspiration and rinsed on a 50 µm mesh sieve to remove the chemical. The sample was pre-dried at 105 ˚C for 30 minutes, gently transferred into aluminium tray and dried at 65 ˚C.

The third step of the fractionation dispersed microaggregates into their components including fine POM, coarse silt, fine silt and clay. The microaggregate sample (5 g) was subjected to density fractionation (without the vacuum step) to remove fine POM associated with non-aggregated soil matter (free microaggregates sample) and macroaggregates (microaggregates within macroaggregates sample), washed to remove the density separation solution and dispersed by shaking with 20 metal balls (4 mm diameter) at 150 oscillations $minute^{-1}$ for 24 hours. The dispersed soil was subsequently wet sieved through 50 µm and 20 µm sieves. The rinse water was subjected to sequential centrifuging procedure (centrifugation at 270×g for 3 minutes to isolate fine silt followed by addition of 1 mL 2.5 M $CaCl_2$ per litre of the rinse water to flocculate clay and centrifugation at 3500×g for 10 minutes to isolate clay). All separated fractions were dried at 65 ˚C.

All mineral soil fractions (coarse silt, fine silt, clay and combined fine silt and clay fraction) were subjected to Cate removal procedure following modified procedure of Ramnarine et al. (2011). A sample (0.07 g) was weighed into a 2 ml plastic tube, 100 µl of 5 % hydrochloric acid was added to the sample and the tube was placed into a glass desiccator with 100 mL of concentrated acid in a beaker placed underneath the samples on the bottom of the desiccator. The desiccator was vacuum sealed and samples were exposed to acid fumes for a period of 16 hours. The beaker was then removed, the samples were subjected to three repeated vacuum excavations of 20 minutes each to remove remaining acid, oven dried at 65 ˚C until a constant weight was achieved. Samples were then disrupted by ball milling and analysed for C and N content using an automated Dumas procedure on Vario EL analyser (Elementar, Stockport, UK). The C and N content was recalculated for the mass loss of Cates during the Cates removal procedure.

Calculations

Relative mass of each fraction separated from the soil was calculated in relation to mass of recovered weight of soil portion from which it was separated (this assumes that mass losses would occur for each fraction separated at the same rate although it was not tested experimentally). Aggregation and relative abundance of aggregate fraction was expressed on the whole soil basis and also per aggregate fractions distributed according to aggregate hierarchy such as (non-aggregated, free microaggregates, microaggregates within macroaggregates and intra-macroaggregate non-aggregated matter). OC was expressed as a concentration per each fraction separated which was analysed (not all fractions were analysed) and also on the whole soil basis. Mean weight diameter (MWD) was calculated using following formula:

$$\text{MWD} = \sum_{i=1}^{n} x_i w_i,$$

where x_i is the mean diameter of individual size range of aggregates separated by sieving, and w_i is the relative weight of aggregates in that size range.

Soil microbial characteristics

Bacterial and fungal community composition was determined employing 16S and ITS amplicon sequencing respectively. For bacteria, the V3 – V4 hypervariable regions of 16S rRNA gene was amplified using 341F - 806R primers (De Vries et al., 2018) and for fungi, the ITS2 region was amplified using fITS1f-ITS4r (Ihrmark et al., 2012) primer sequences. Extraction of DNA, amplification of target gene sequences, sequencing and sequences processing and taxonomy assignment was performed as specified in method chapter (Chapter 2).

Sequencing including preparation of amplicon libraries and processing of output data from the sequences (as detailed in Chapter 2) were performed by personel of the Centre for Ecology and Hydrology (Wallingford, U.K.). Samples were rarefied to the lowest sequencing depth of 13,249 and 8,165 reads for bacteria and fungi respectively to account for differences in sequencing depth among samples. The rarefied data were used for all the subsequent analysis. Microbial diversity indices including richness (number of OTUs/ASVs), Simpson's and Shannon indices were calculated (R package vegan).

Data analysis

Effects of land management history on basic soil characteristics, soil fractions obtained from fractionation procedure and data from microbial DNA sequencing were analysed using one-way ANOVA tests and differences between the all levels of the factor were tested using Tukey HSD post hoc comparison with Bonferroni correction for multiple comparisons. Basic soil characteristics included OC and total N and their ratio, moisture and pH. Soil fractions from fractionation included distribution of soil mass, concentration of soil C in the soil fractions and soil C pools in the soil fractions. The microbial sequencing data tested included diversity indexes and relative proportions of microbial taxonomic groups including phylum, class and order. For each fractionation category and microbial taxonomic group, results of ANOVA analysis were corrected for multiple comparisons using Benjamini-Hochberg correction procedure. Models that violated assumptions of normality and homoscedasticity received ln(y) transformation. Non-parametric Kruskal-Wallis test and Dunn's pairwise comparison with Benjamini-Hochberg correction for multiple comparisons were used if ln(y) transformation did not improve model fit.

Response of microbial community to land management history was assessed by multivariate generalised linear models (M-GLMs) using GLM framework from MVABUND 3.10.4 package in R (Y. Wang et al., 2012). Data rarefied to the lowest total sequence reads were used for the analysis. Details of the analysis are listed in method chapter (Chapter 2). Argument 'offset' was not used in the model.

To identify OTUs/ASVs significantly associated with each land management history category, indicator species analysis (R package indicspecies) was used. Details of the analysis are listed in method chapter (Chapter 2). Abundance of the selected indicator species (with $P < 0.05$) for each land management history category was grouped at order taxonomic resolution. The results were expressed as proportion (%) of indicator species to overall relative abundance of the order on all tested sites.

All analyses were conducted in R of version 3.5.0 (R Core Team, 2018).

3.4 Results

3.4.1 Soil abiotic characteristics

Overall soil characteristics

Land management history affected both, concentrations of soil OC (Kruskal-Wallis, $chi^2_{3,38}$ = 25.3, P < 0.001) and total N (Kruskal-Wallis, $chi^2_{3,48}$ = 25, P < 0.001) within the chronosequence whereby arable sites showed lower values than grasslands including all grassland sites for OC and Medium and Old grasslands for total N (post hoc comparisons P < 0.05) (Table 3.1). Moreover, organic C and total N on Recent grasslands were lower than on Old grasslands (post hoc comparisons P < 0.05). Ratio of soil OC:total N concentrations was lower on arable sites compared to all grassland regeneration sites (Kruskal-Wallis, $chi^2_{3,48}$ = 13.8, P < 0.01) (Table 3.1). Soil pH did not differ among sites ($chi^2_{3,48}$ = 6.1, P = 0.11). Soil moisture was affected by land management history (ANOVA, $F_{3,48}$ = 24.9, P < 0.001) (Table 3.1).

Table 3.1 Soil characterization of arable and grassland regeneration sites.

	Sites				One-way ANOVA		
	Arable field	Grassland regeneration status			df	F	P
		Recent	Medium	Old			
Soil organic C (mg.g^{-1} dry soil) [1)]	39.4 ± 6.6 [a]	64.7 ± 5.3 [b]	74.1 ± 4.9 [bc]	93.7 ± 5.2 [c]	3	25.3 [2)]	***
Soil total N (mg.g^{-1} dry soil [1)]	4.7 ± 0. [a]	6.3 ± 0.4 [ab]	8.6 ± 0.6 [bc]	10.2 ± 0.5 [c]	3	27.3 [2)]	***
Soil organic C:total N ratio [1)]	9.0 ± 0.2 [a]	10.2 ± 0.4 [b]	9.7 ± 0.2 [b]	9.7 ± 0.1 [b]	3	13.8 [2)]	**
Soil pH [1)]	7.8 ± 0.1	7.8 ± 0	7.7 ± 0.1	7.7 ± 0.1	3	6.1 [2)]	0.11
Soil moisture (%)	15.9 ± 2.0 [a]	25.1 ± 1.5 [b]	27.3 ± 1.8 [b]	33.0 ± 1.3 [c]	3	24.9	***

Differences in mean values (± standard error). Letters indicate significant differences at P < 0.05 (Tukey HSD post hoc test or Dunn's test if Kruskal-Wallis was used). *** P < 0.001, ** P < 0.01 [1)] Kruskal-Wallis test was used [2)] Chi-squared value

Soil mass distribution within soil water stable aggregates

Distributions of soil mass within macroaggregate- and microaggregate-sized aggregates and non-aggregated matter were affected by land management history (ANOVA, $F_{3,34}$ = 9.7, P < 0.001, $F_{3,34}$ = 9.5, P < 0.001 and $F_{3,34}$ = 5.3, P < 0.01 respectively). Arable sites showed higher distribution of soil mass in microaggregates and lower distribution of soil mass in macroaggregates than all three different grassland sites (post-hoc comparisons P < 0.05), while the grassland sites were similar with each other (post-hoc comparisons P > 0.05) (Figure 3.6). Non-aggregated soil matter in arable sites was higher than that of Medium and Old grasslands (post-hoc comparisons P < 0.05) (Figure 3.2).

Distribution of soil mass among nine size classes of water stable aggregates within an overall range of < 20 – 2000 µm aggregate diameter sizes was affected by land management history for all aggregate sizes but 20 – 50 µm, 50 – 106 µm and 425 – 710 µm (ANOVA/Kruskal-Wallis, details in Table 3.2). Soil mass distribution within aggregate classes did not differ among grassland sites of different regeneration status (post-hoc comparisons P > 0.05) while it differed between arable sites and grassland sites (post-hoc comparisons P < 0.05) (Table 3.2). Arable sites showed higher soil mass distribution than that of grasslands for smaller aggregate size classes including < 20 µm, 106 – 180 µm, 180 – 250 µm and 250 – 425 µm aggregate size classes, and arable sites further showed lower soil mass distribution than that of grasslands for two highest aggregate size classes determined including 710 – 1000 µm and 1000 – 2000 µm aggregate size classes (post-hoc comparisons P < 0.05) (Figure 3.6).

Mean weight diameter of water stable soil aggregates was affected by land management history (ANOVA, $F_{3,34} = 12.4$, $P < 0.001$) and it was lower for arable sites when compared to each grassland site (post-hoc comparisons $P < 0.05$) while the grasslands sites did not differ among themselves (post-hoc comparisons $P > 0.05$) (data not shown).

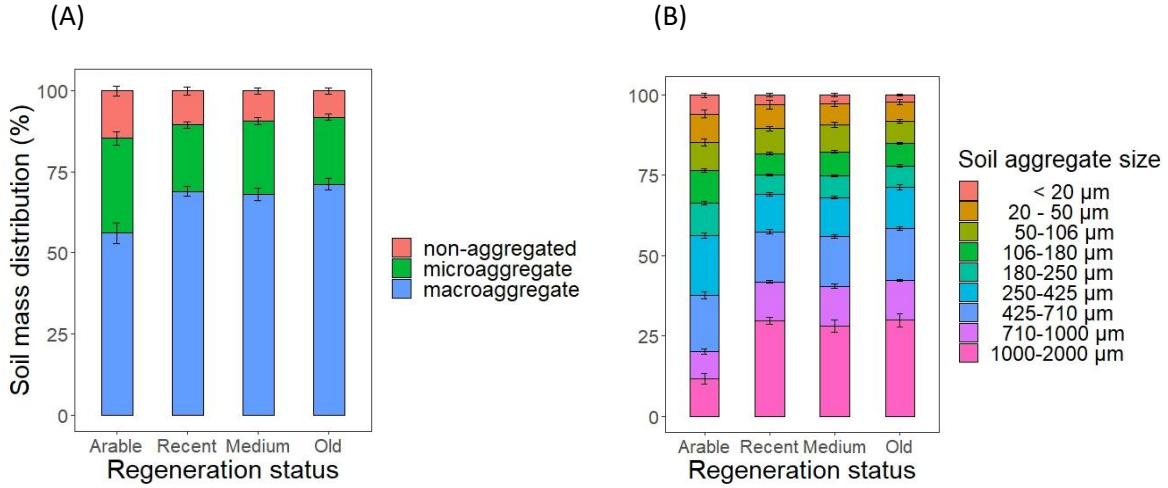


Figure 3.6 Distribution of soil mass (A) in aggregates of macroaggregate and microaggregate sizes and non-aggregated soil matter and (B) in soil aggregate or particle categories of 9 sizes within < 20 – 2000 µm range extracted from soil by wet sieving. Divisions within bars represent cumulative means of proportion of soil mass in each size category. Error bars are standard errors of the means of proportion of soil mass in each size category.

Table 3.2 Effect of land management history on soil mass distribution among soil aggregates and particles extracted from soil by wet sieving.

Aggregate size (µm)	df	F	P adjusted
1000 - 2000	3	28.0	***
710 - 1000 [1)]	3	16.6 [2)]	**
425 - 710	3	1.4	0.32
250 - 425 [3)]	3	20.8	***
180 - 250	3	26.9	***
106 - 180	3	12.6	***
50 - 106 [3)]	3	0.9	0.48
20 - 50 [3)]	3	1.2	0.35
< 20	3	10.0	***

Tested by one-way ANOVA or Kruskall-Wallis test. P adjusted using Benjamini-Hochberg transformation.
*** $P < 0.001$, ** $P < 0.01$ [1)] Kruskal-Wallis test used [2)] chi-squared value [3)] ln (y) transformed

Soil mass distribution within soil hierarchical aggregates – selected sites

Five sites from each land management history class (Figure 3.4) were selected for detailed fractionation according to aggregate hierarchy concept (Tisdall and Oades, 1982; Six et al., 2000a).

Land management history affected proportion of overall soil mass distribution in intra-Macroaggregate-microaggregates (Kruskal-Wallis, $chi^2_{3,16} = 12.4$, $P < 0.5$), free-microaggregates (Kruskal-Wallis, $chi^2_{3,16} = 11.2$, $P < 0.5$) and non-aggregated soil matter fractions (Kruskal-Wallis, $chi^2_{3,16} = 10.0$, $P < 0.5$) but intra-Macroaggregate matter (Kruskal-Wallis, $chi^2_{3,16} = 1.2$, $P = 0.35$) (Figure 3.7). Arable sites showed lower soil mass distribution within iM-microaggregates and higher

soil mass distribution in free-microaggregates when compared to grassland sites of each land management history (post-hoc comparisons $P < 0.05$). For non-aggregated soil matter, arable sites showed higher distribution of soil mass in this fraction when compared to Recent grasslands only (post-hoc comparison $P < 0.05$). Land management history did not affect soil mass distribution within overall mass of microaggregates (sum of free-microaggregates and iM-microaggregates) (Kruskal-Wallis, $chi^2_{3,16} = 6.2$, $P = 0.10$).

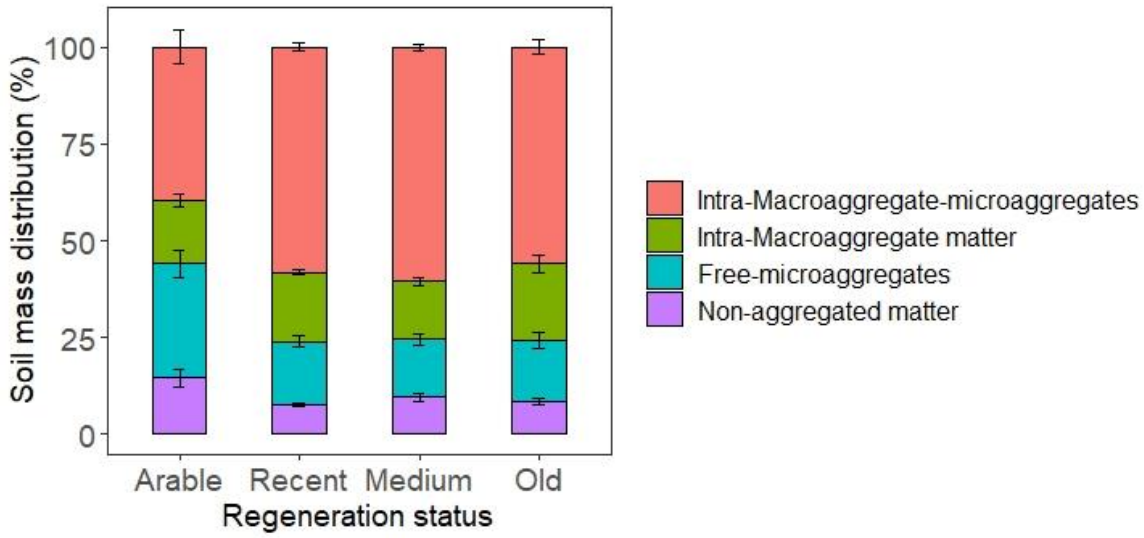
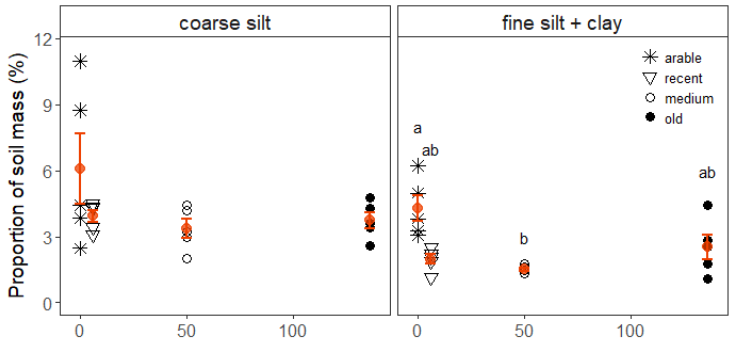


Figure 3.7 Redistribution of soil mass within hierarchical aggregates and non-aggregated matter for sites with different land management history. Error bars represent standard errors of the means for soil aggregate fractions within each land management history category.

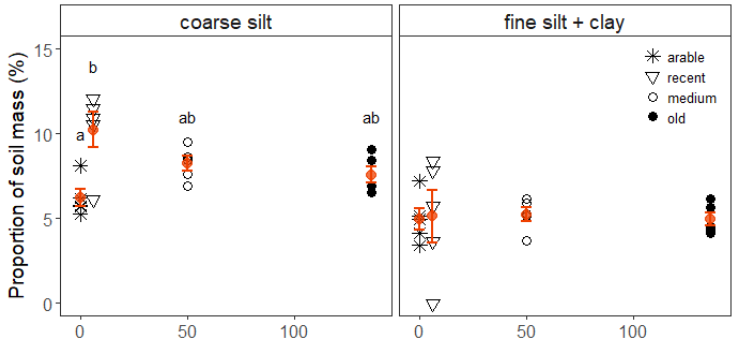
Hierarchical soil aggregates and non-aggregated matter were further fractionated into its components (Figure 3.8, Figure 3.9, Table 3.3). For non-aggregated matter, land management history affected fine silt + clay fraction (Kruskal-Wallis, $chi^2_{3,18} = 11.2$, $P < 0.05$), whereby it was higher on arable sites than on Medium grasslands (post- hoc comparisons $P < 0.05$). For i-Macroaggregate matter, land management history affected coarse silt (Kruskal-Wallis, $chi^2_{3,16} = 9.5$, $P < 0.05$) whereby it was lower for arable sites when compared to recent grasslands (post-hoc comparisons $P < 0.05$). For free-microaggregates, land management history affected fine silt as well as clay fractions (Kruskal-Wallis, $chi^2_{3,16} = 11.4$, $P < 0.05$ and $chi^2_{3,16} = 14.8$, $P < 0.05$) whereby both fractions were lower for all grasslands when compared to arable sites (post-hoc comparisons $P < 0.05$). For iM-microaggregates, land management history affected coarse silt fraction (Kruskal-Wallis, $chi^2_{3,16} = 14.3$, $P < 0.05$) whereby it increased for Medium and Old grasslands when compared to arable sites (post-hoc comparisons $P < 0.05$). Land management history affected fine silt fraction (Kruskal-Wallis, $chi^2_{3,16} = 10.0$, $P < 0.05$) whereby it was higher for all grasslands sites when compared to arable sites (post-hoc comparisons $P < 0.05$).

Land management history affected overall proportion of plant litter and most POM fractions associated with hierarchical soil aggregates and non-aggregated matter apart from POM in free-microaggregates (Table 3.3) (Figure 3.9). Litter was higher on all grassland sites when compared to arable sites (ANOVA, $F_{3,16} = 24.5$, $P < 0.001$; post-hoc comparisons $P < 0.05$), and the same was true for non-aggregated coarse POM fraction (ANOVA, $F_{3,16} = 26.2$, $P < 0.001$; post-hoc comparisons $P < 0.05$). Non-aggregated fine POM was higher for Old grasslands when compared to arable and Recent grasslands (ANOVA, $F_{3,16} = 10.4$, $P < 0.05$; post-hoc comparisons $P < 0.05$). Fine POM associated with i-Macroaggregate matter and iM-microaggregates was higher for all grassland sites when compared to arable sites (ANOVA, $F_{3,16} = 11.5$, $P < 0.05$ and $F_{3,16} = 11.3$, $P < 0.05$ respectively; post-hoc comparisons $P < 0.05$).

(A)



(B)



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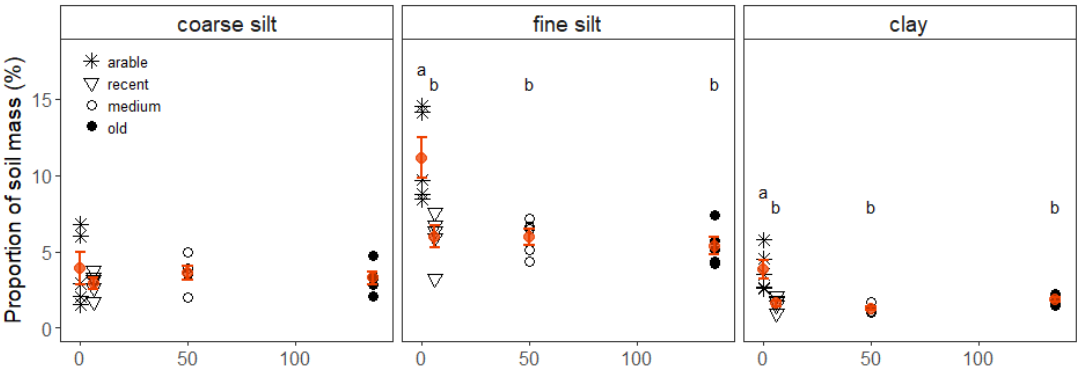


(D)

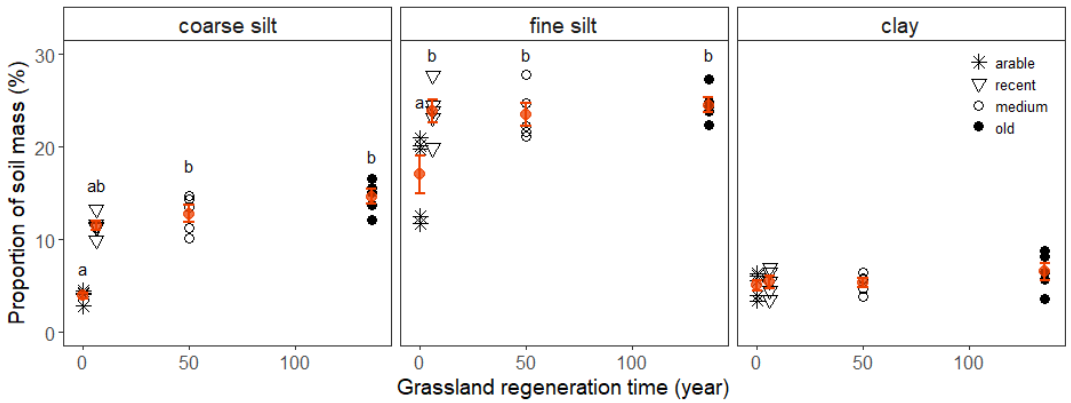


Figure 3.8 Response of soil mass associated with (A) non-aggregated soil matter, (B) macroaggregates, (C) microaggregates and (D) microaggregates within macroaggregates to land management history. One-way ANOVA (or Kruskal-Wallis test) testing an effect of land management history on soil mass distribution. Orange point represent mean and error bar is standard error of the mean for land management history category. Letters indicate significant differences at $P < 0.05$ (Tukey HSD post hoc test or Dunn's test if Kruskal-Wallis test was used).

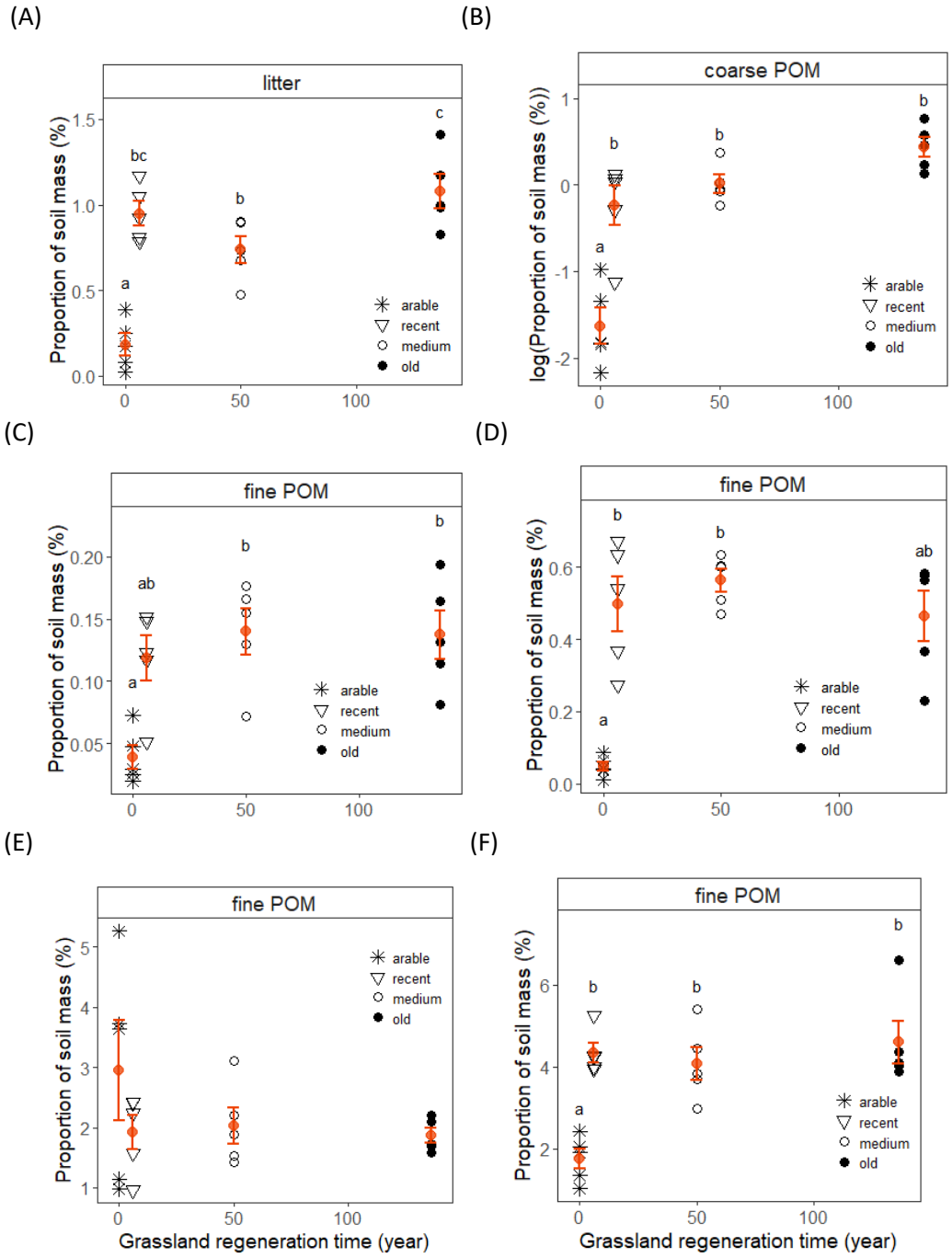


Figure 3.9 Response of POM associated with (A) litter (B-C) non-aggregated soil matter, (D) macroaggregates, (E) microaggregates and (F) microaggregates within macroaggregates to land management history. One-way ANOVA (or Kruskal-Wallis test) testing an effect of land management history on soil mass distribution. Orange point represent mean and error bar is standard error of the mean for land management history category. Letters indicate significant differences at $P < 0.05$ (Tukey HSD post hoc test or Dunn's test if Kruskal-Wallis test was used).

Table 3.3 Effect of land management history on distribution of total soil mass in mineral and POM fractions within aggregated and non-aggregated soil matter and in litter.

		df	F	P adjusted
Mineral soil fraction	Non-aggregated			
	Coarse silt [2)]	3	2.9 [3)]	0.55
	Fine silt + clay [2)]	3	11.2 [3)]	*
	Macroaggregate			
	Coarse silt [2)]	3	9.5 [3)]	*
	Fine silt + clay [2)]	3	0.6 [3)]	0.90
	Free microaggregates			
	Coarse silt [2)]	3	1.3 [3)]	0.84
	Fine silt [2)]	3	11.4 [3)]	*
	Clay [1)]	3	15.8	***
	Microaggregates within macroaggregate			
	Coarse silt [2)]	3	14.3 [3)]	*
	Fine silt [2)]	3	10.0 [3)]	*
	Clay	3	1.0	0.55
POM soil fraction	Non-aggregated			
	Coarse POM [1)]	3	26.2	***
	Fine POM [2)]	3	10.4 [3)]	*
	Macroaggregate			
	Fine POM [2)]	3	11.5 [3)]	*
	Free microaggregates			
	Fine POM [2)]	3	0.8 [3)]	0.90
	Microaggregates within macroaggregate			
	Fine POM [2)]	3	11.3 [3)]	*
Litter	Litter	3	24.5	***

Tested by one-way ANOVA or Kruskall-Wallis test. P adjusted using Benjamini-Hochberg transformation. *** $P < 0.001$, $P < 0.05$, [1)] ln (y) transformed [2)] Kruskal-Wallis test used [3)] chi-squared value

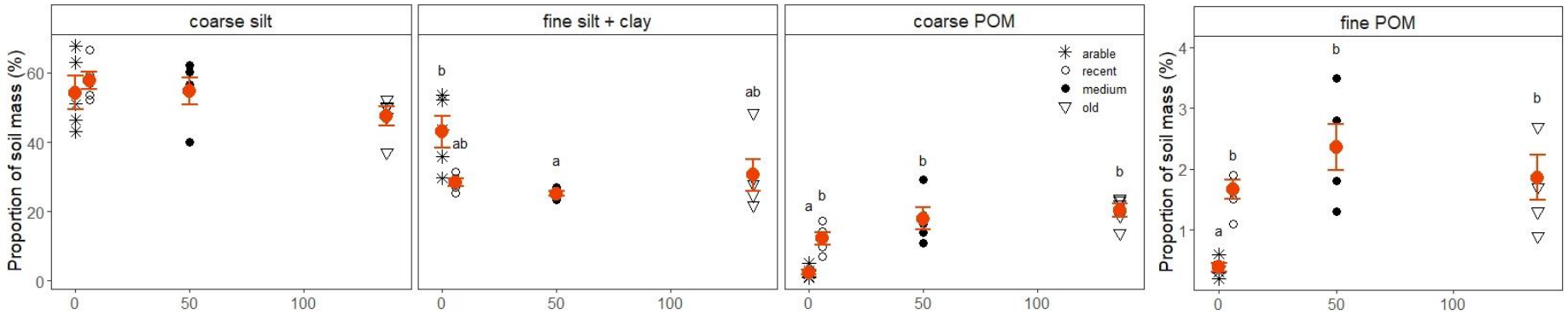
For each aggregate category, relative mass distribution of its components was recalculated (Figure 3.10). Coarse silt relative distribution within individual aggregate fractions was affected by land management history only for free-microaggregates (Kruskal-Wallis, $chi^2_{3,16} = 11.1$, $P < 0.05$) and iM-microaggregates (Kruskal-Wallis, $chi^2_{3,16} = 18.3$, $P < 0.001$) whereby it was higher for most of the grassland sites when compared to arable sites (post-hoc comparisons $P < 0.05$) but not within free-microaggregates on Recent grasslands (Table 3.4). Fine silt + clay fraction was affected by land management history only for non-aggregated matter (Kruskal-Wallis, $chi^2_{3,16} = 10.5$, $P < 0.05$) when it was lower on medium grasslands when compared to arable sites (post-hoc comparisons $P < 0.05$). Fine silt was affected only for iM-microaggregates (ANOVA, $F_{3,16} = 4.3$, $P < 0.05$) when it was lower on Old grassland sites than on arable sites (post-hoc comparisons $P < 0.05$) while clay was affected only for free-microaggregates (Kruskal-Wallis, $chi^2_{3,16} = 12.8$, $P < 0.05$) when it was lower for Medium grassland sites than arable sites (post-hoc comparisons $P < 0.05$). POM was affected for non-aggregated matter (ANOVA, coarse POM: $F_{3,16} = 29.2$, $P < 0.001$; fine POM: $F_{3,16} = 23.1$, $P < 0.001$) and iMacroaggregate matter (ANOVA, fine POM: $chi^2_{3,16} = 11.4$, $P < 0.05$) but not for any of the microaggregate fractions. When POM was affected by land management history, it was allways lower on arable sites compared to grassland sites (post-hoc comparisons $P < 0.05$).

Table 3.4 Effect of land management history on proportion of soil mineral fractions and POM within water stable aggregates and non-aggregated soil matter.

	df	F	P adjusted
Non-aggregated			
Coarse silt	3	1.5	0.26
Fine silt + clay [2)]	3	10.5 [3)]	*
Coarse POM [1)]	3	29.2	***
Fine POM [1)]	3	23.1	***
Macroaggregate			
Coarse silt [2)]	3	2.6 [3)]	0.46
Fine silt + clay [2)]	3	4.4 [3)]	0.33
Fine POM [2)]	3	11.4	*
Free microaggregates			
Coarse silt [2)]	3	11.1 [3)]	*
Fine silt [1)]	3	3.1	0.08
Clay [2)]	3	12.8 [3)]	*
Fine POM [1)]	3	1.2	0.34
Microaggregates within macroaggregate			
Coarse silt	3	18.3	***
Fine silt [1)]	3	4.3	*
Clay [2)]	3	7.9 [3)]	0.06
Fine POM [2)]	3	4.7 [3)]	0.07

Tested by one-way ANOVA or Kruskall-Wallis test. P adjusted using Benjamini-Hochberg transformation. *** P < 0.001, ** P < 0.01, * P < 0.05, [1)] ln (y) transformed [2)] Kruskal-Wallis test was used [3)] chi-squared value

(A)



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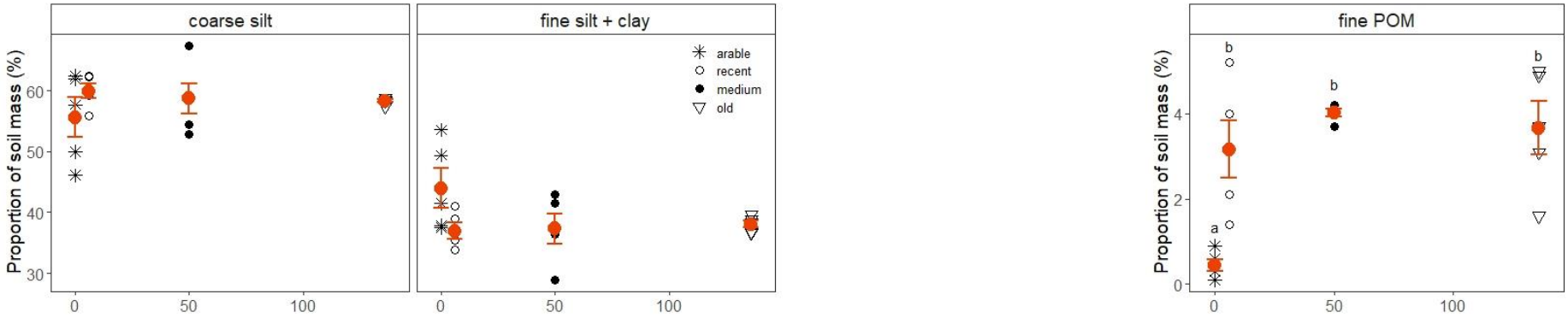

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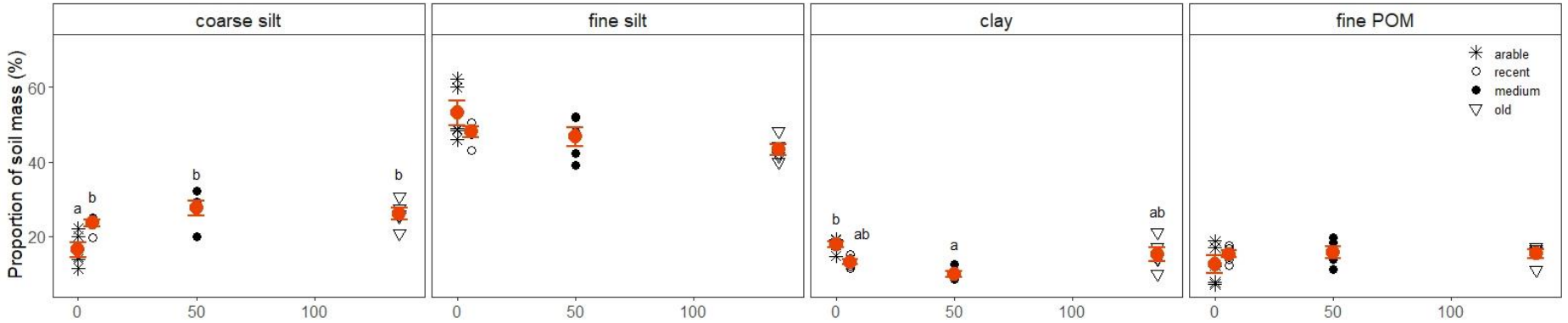

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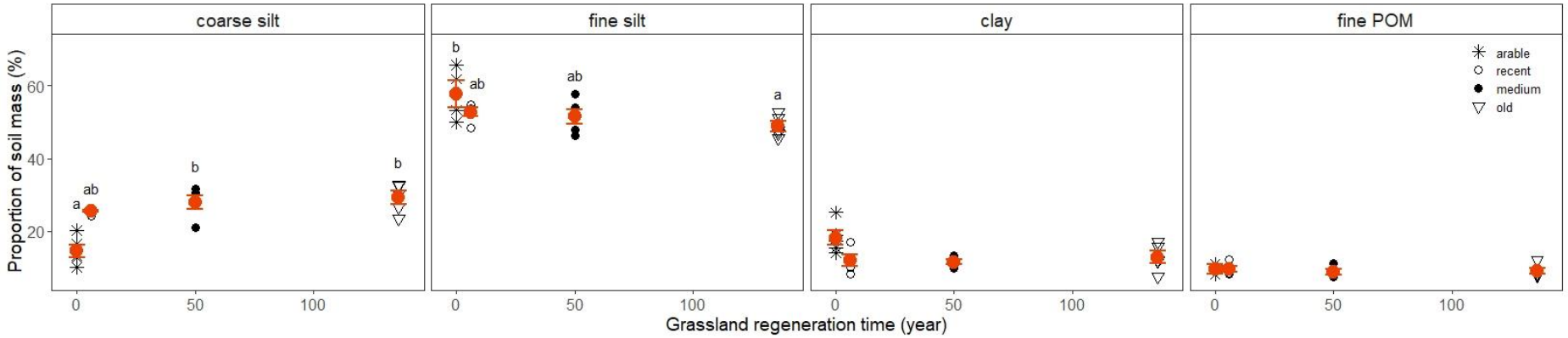


Figure 3.10 Response of components including mineral fraction and associated POM of (A) non-aggregated soil matter, (B) macroaggregate matter, (C) free microaggregates and (D) intra-macroaggregate microaggregates to land management history. One-way ANOVA (or Kruskal-Wallis test) testing an effect of land management history on soil mass distribution. Orange point represent mean and error bar is standard error of the mean for land management history category. Letters indicate significant differences at $P < 0.05$ (Tukey HSD post hoc test or Dunn's test if Kruskal-Wallis test was used).

Difference in contribution of individual microaggregate components to overall aggregate mass between free microaggregates and iM-microaggregates was calculated (Table 3.5). Most of the microaggregate components have similar relative contribution to overall aggregate mass for free microaggregates and iM-microaggregates, apart from fine POM on Recent and Medium grassland sites when fine POM had higher relative contribution to overall aggregate mass for free-microaggregate than iM-microaggregates (ANOVA, $F_{1,8} = 21.8$, $P < 0.05$ and $F_{1,8} = 16.6$, $P < 0.05$ resp.).

Table 3.5 Difference in contribution of individual microaggregate components to overall aggregate mass between free microaggregates and iM-microaggregates.

Microaggregate component	Land management history	dF	F value	$P_{unadjusted}$	$P_{adjusted}$
Coarse silt	Arable	8	0.5	0.49	0.58
	Recent grasslands	8	4.4 [1)]	* [2)]	0.09 [2)]
	Medium grasslands	8	0 [1)]	0.92 [2)]	0.92 [2)]
	Old grasslands	8	1.7	0.23	0.34
Fine silt	Arable	8	2	0.19	0.31
	Recent grasslands	8	7.2	*	0.09
	Medium grasslands	8	2.1	0.18	0.31
	Old grasslands	8	8.3	*	0.08
Clay	Arable	8	0	0.83	0.89
	Recent grasslands	8	0.5	0.51	0.58
	Medium grasslands	8	3.2	0.11	0.22
	Old grasslands	8	0.9	0.36	0.48
Fine POM	Arable	8	4.8	0.06	0.13
	Recent grasslands	8	21.8	**	*
	Medium grasslands	8	16.6	**	*
	Old grasslands	8	5.8 [1)]	* [2)]	0.08 [2)]

Tested by one-way ANOVA or Kruskall-Wallis test. P adjusted using Benjamini-Hochberg transformation.
** $P < 0.01$, ** $P < 0.05$ [1)] Kruskal-Wallis test used [2)] chi-squared value

C concentration in hierarchical soil aggregate fractions

Only mineral and POM fractions, which were determined for C and N concentrations for all five replicates, were statistically analysed for OC and total N concentrations in soil fractions and soil pools.

Land management history affected concentration of OC in all mineral fractions separated from the aggregates that were statistically analysed (Table 3.6) (Figure 3.11). C concentration in coarse silt fraction was higher for Old and Medium grasslands when compared to arable sites for free-microaggregates and iM-microaggregates (Kruskal-Wallis, $chi^2_{3,16}$ = 12.3, P < 0.01 and $chi^2_{3,16}$ = 12.2, P < 0.01 respectively, post-hoc comparisons P < 0.05). Furthermore, Old grasslands showed higher coarse silt C concentration than Recent grasslands for free-microaggregates (post-hoc comparison P < 0.05) but not for iM-microaggregates. Land management history affected fine silt C concentration for both free-microaggregates and iM-microaggregates (Kruskal-Wallis, $chi^2_{3,16}$ = 11.8, P < 0.05 and $chi^2_{3,16}$ = 12.5, P < 0.01) whereby they were higher on Old and Medium grassland sites than on arable sites (post-hoc comparison P < 0.05). Clay C concentration was analysed only for free-microaggregates and it showed higher concentration on all grassland sites when compared to arable sites (ANOVA, $F_{3,16}$ = 8.5, P < 0.01, post-hoc comparison P < 0.05). For free microaggregates, differences between C concentrations among the microaggregate mineral building blocks (i.e. clay, fine and coarse silt) were determined and clay fractions showed higher C concentration than that in fine or coarse silt fraction regardless of land management history site (ANOVA, $F_{2,53}$ = 16.2, P < 0.001, post-hoc comparison P < 0.05).

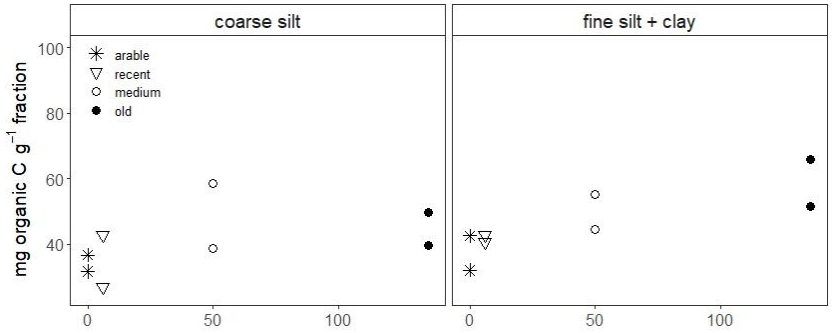
Land management affected C concentration of litter (Kruskal-Wallis, $chi^2_{3,16}$ = 13.5, P < 0.01) and un-aggregated coarse POM (ANOVA, $F_{3,16}$ = 24.8, P < 0.01), however with opposite patterns, whereby C concentration was higher for litter on Old grasslands than on arable and Recent grassland sites, while C concentration of non-aggregated coarse POM was lower for all grasslands sites when compared to arable sites (post-hoc comparisons P < 0.05) (Figure 3.12).

Table 3.6 Effect of land management history on concentration of C in soil mineral and POM fractions within soil aggregates, non-aggregated matter and litter.

		df	F	P adjusted
Mineral soil fraction	Free microaggregates			
	Coarse silt [2)]	3	12.3 [3)]	**
	Fine silt [2)]	3	11.8 [3)]	*
	Clay	3	8.5	**
	Microaggregates within macroaggregate			
	Coarse silt [2)]	3	12.2 [3)]	**
	Fine silt [2)]	3	12.5 [3)]	**
POM soil fraction	Non-aggregated			
	Coarse POM [1)]	3	24.8	***
	Fine POM [2)]	3	3.2 [3)]	0.40
	Macroaggregate			
	Fine POM [2)]	3	2.8 [3)]	0.43
Litter	Litter [2)]	3	13.5 [3)]	**

Tested by one-way ANOVA or Kruskall-Wallis test. P adjusted using Benjamini-Hochberg transformation. *** P < 0.001, ** P < 0.01, * P < 0.05, [1)] ln (y) transformed [2)] Kruskal-Wallis test used [3)] chi-squared value

(A)



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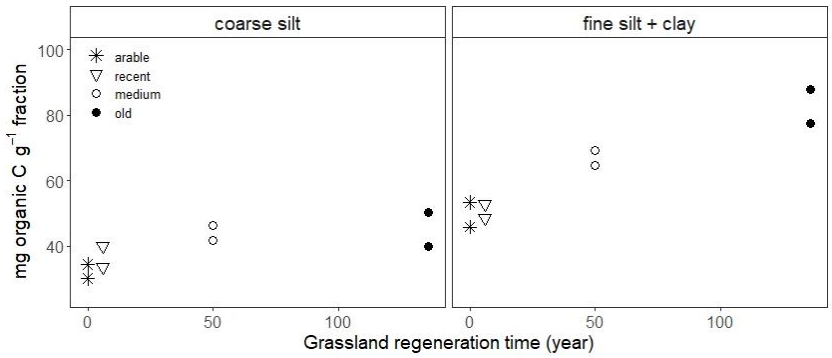

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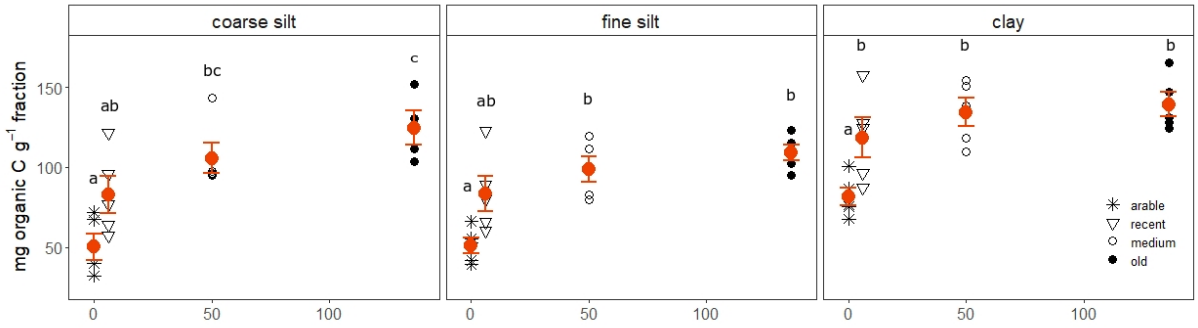

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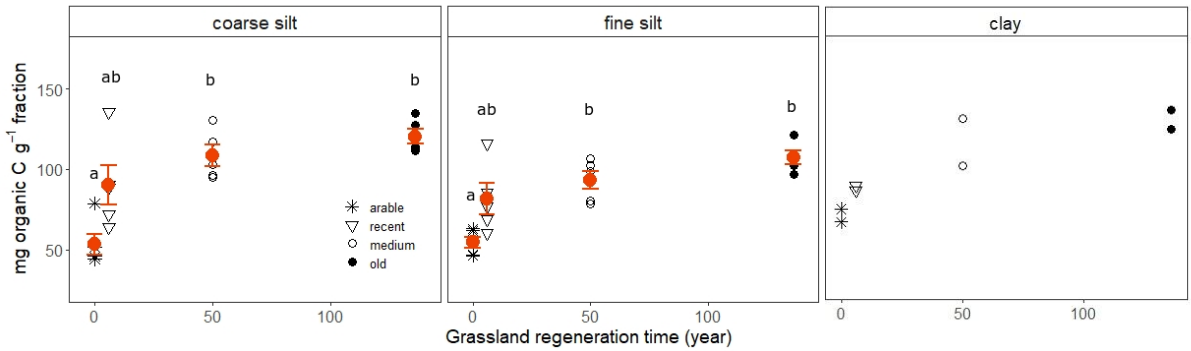


Figure 3.11 Concentration of SOC in mineral fractions of (A) non-aggregated soil matter, (B) macroaggregates (C) free microaggregates and (D) microaggregates within macroaggregates extracted from sites of different land management history. Only land management history categories for which soil C was determined for all 5 replicates were tested using one-way ANOVA (or Kruskal-Wallis test) for an effect of land management history on soil C concentration. Orange point represent mean and error bar is standard error of the mean for land management history category. Letters indicate significant differences at $P < 0.05$ (Tukey HSD post hoc test or Dunn's test if Kruskal-Wallis test was used).

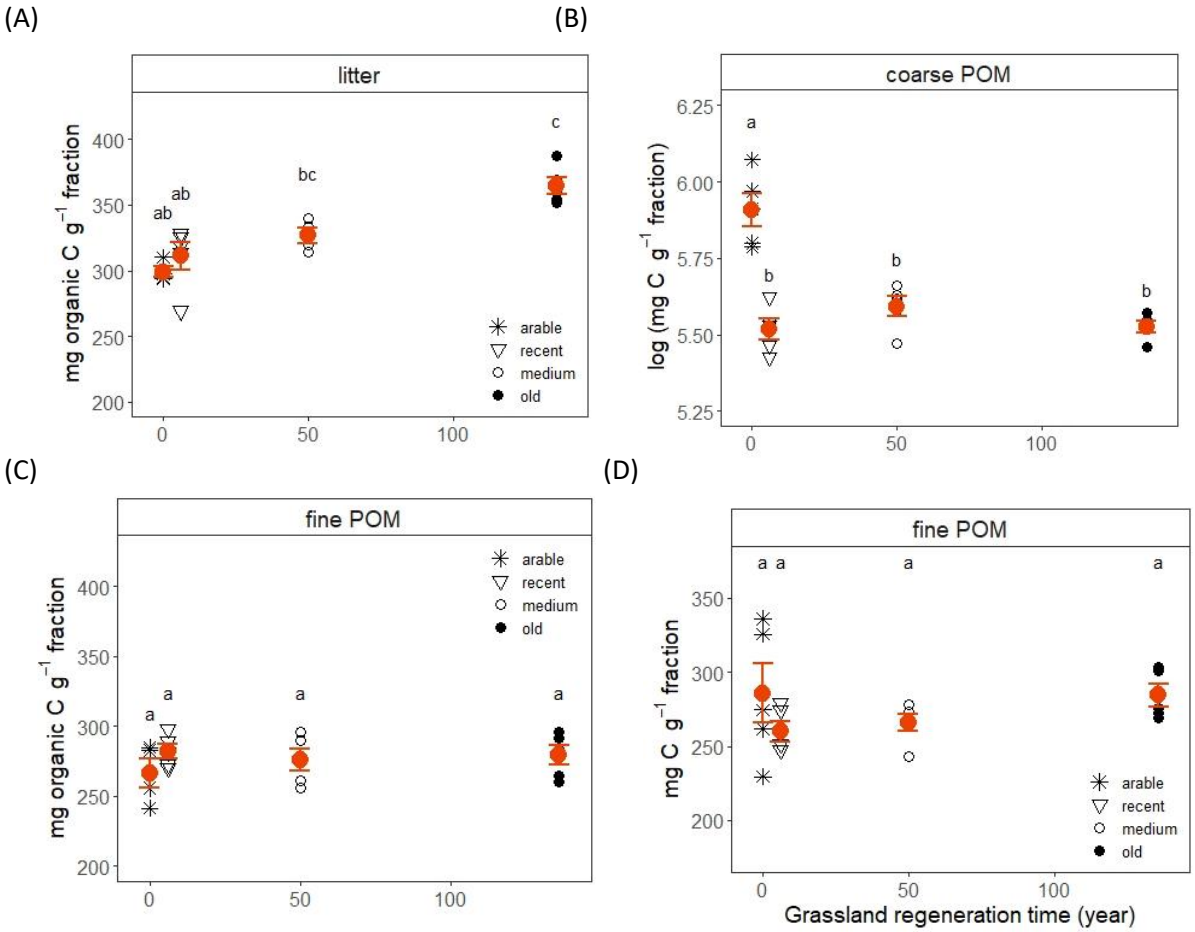


Figure 3.12 Concentration of SOC in POM of (A) litter (B-C) non-aggregated soil matter, (D) macroaggregates extracted from sites of different land management history. Tested using one-way ANOVA (or Kruskal-Wallis test) for an effect of land management history on soil C concentration. Orange point represent mean and error bar is standard error of the mean for land management history category. Letters indicate significant differences at $P < 0.05$ (Tukey HSD post hoc test or Dunn's test if Kruskal-Wallis test was used).

When differences of C concentration of microaggregate components between free- and iM-microaggregates were tested, such as for coarse silt and fine silt components, no differences were found (ANOVA, $F_{1,34} = 0.15$, $P = 0.70$ and $F_{1,35} = 0.11$, $P = 0.74$ respectively).

Land management history affected ratio of OC:total N concentrations of tested mineral and POM soil fractions only for non-aggregated coarse POM fraction (Kruskal-Wallis, $chi^2_{3,16} = 12.8$, $P < 0.05$) whereby it was higher on Old grasslands than on arable and Recent grassland sites (post-hoc comparisons $P < 0.05$) (Table 3.7).

C pools in hierarchical soil aggregate fractions

Land management affected C pool of coarse silt and clay in free-microaggregates (ANOVA, $F_{3,16} = 3.6$, $P < 0.05$ and $F_{3,16} = 3.9$, $P < 0.05$ respectively) and coarse silt and fine silt in iM-microaggregates (Kruskal-Wallis, $chi^2_{3,16} = 14.3$, $P < 0.01$ and $chi^2_{3,16} = 13.8$, $P < 0.01$ respectively) (Table 3.8) (Figure 4.13). However, pairwise comparisons revealed differences only for mineral pools in iM-microaggregates whereby C pool associated with coarse silt was higher on Old and Medium grasslands when compared to arable sites and C pool associated with fine silt was higher on Old grasslands than on arable sites (post-hoc comparisons $P < 0.05$).

Table 3.7 Effect of land management history on C:N ratio of soil fractions in the soil.

		df	F	P adjusted
Mineral soil fraction	Free microaggregates			
	Coarse silt [2)]	3	1.4 [3)]	0.82
	Fine silt [2)]	3	2.8 [3)]	0.76
	Clay [2)]	3	3.0 [3)]	0.76
	Microaggregates within macroaggregate			
	Coarse silt [2)]	3	0.8 [3)]	0.84
	Fine silt [2)]	3	1.5 [3)]	0.82
POM soil fraction	Non-aggregated			
	Coarse POM [2)]	3	12.8 [3)]	*
	Fine POM [2)]	3	3.3 [3)]	0.76
	Macroaggregate			
	Fine POM [2)]	3	1.3 [3)]	0.82
Litter	Litter [2)]	3	5.1 [3)]	0.74

Tested by one-way ANOVA or Kruskall-Wallis test. P adjusted using Benjamini-Hochberg transformation. * $P < 0.05$, [2)] Kruskal-Wallis [3)] chi-squared

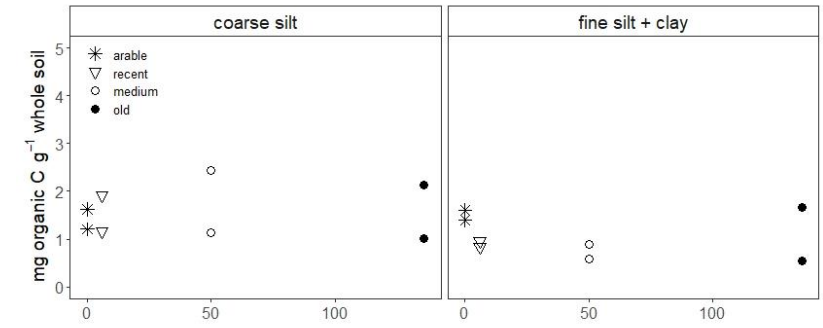
Land management affected all measured C pools associated with litter and POM (Kruskal-Wallis, litter: $chi^2_{3,14} = 24.9$, $P < 0.001$, non-aggregated coarse POM: $chi^2_{3,16} = 10.3$, $P < 0.05$, non-aggregated fine POM: $chi^2_{3,16} = 15.2$, $P < 0.01$ and macroaggregate matter fine POM: $chi^2_{3,16} = 11.7$, $P < 0.01$) (Figure 3.14). All grasslands sites showed higher C pools than arable sites for litter and macroaggregate matter fine POM, and furthermore for litter only, Old grassland C pool was higher than that of Medium grassland, while for non-aggregated coarse POM, Old grassland C pool was higher than that of Recent grasslands (all post-hoc comparisons $P < 0.05$).

Table 3.8 Effect of land management history on C pools in soil mineral and POM fractions within aggregated and non-aggregated soil matter and in litter.

		df	F	P adjusted
Mineral soil fraction	Free microaggregates			
	Coarse silt	3	3.6	*
	Fine silt [2)]	3	1.1 [3)]	0.78
	Clay	3	3.9	*
	Microaggregates within macroaggregate			
	Coarse silt [2)]	3	14.5 [3)]	**
	Fine silt 2)	3	13.8 [3)]	**
POM soil fraction	Non-aggregated			
	Coarse POM [2)]	3	10.3 [3)]	*
	Fine POM [2)]	3	15.2 [3)]	**
	Macroaggregate			
	Fine POM [2)]	3	11.7 [3)]	*
Litter	Litter	3	24.9	***

Tested by one-way ANOVA or Kruskall-Wallis test. P adjusted using Benjamini-Hochberg transformation. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, [1)] ln (y) transformed [2)] Kruskal-Wallis test was used [3)] chi-squared value

(A)



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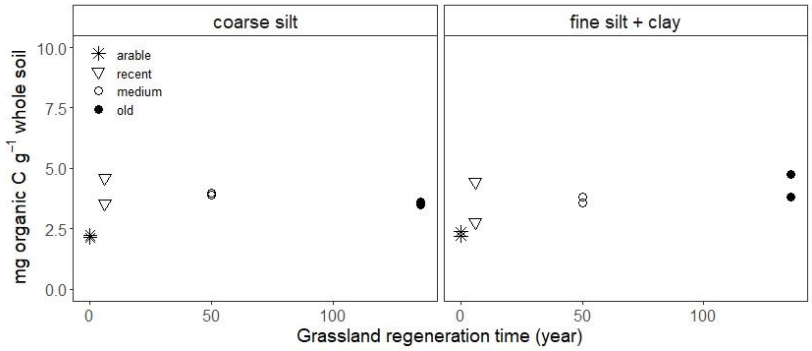

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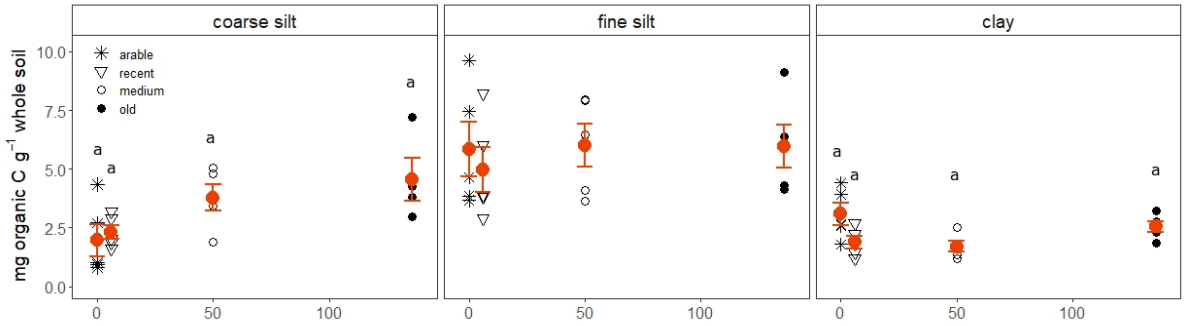

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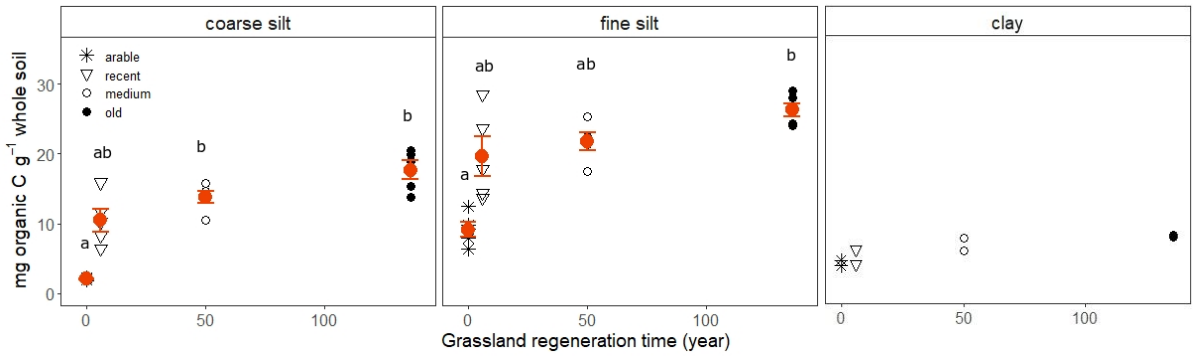


Figure 3.13 OC pool content in mineral fractions associated with (A) non-aggregated soil matter, (B) macroaggregates, (C) microaggregates and (D) microagregates within macroaggregates. Tested using one-way ANOVA (or Kruskal-Wallis test) for an effect of land management history on soil C concentration. Orange point represent mean and error bar is standard error of the mean for land management history category. Letters indicate significant differences at $P < 0.05$ (Tukey HSD post hoc test or Dunn's test if Kruskal-Wallis test was used).

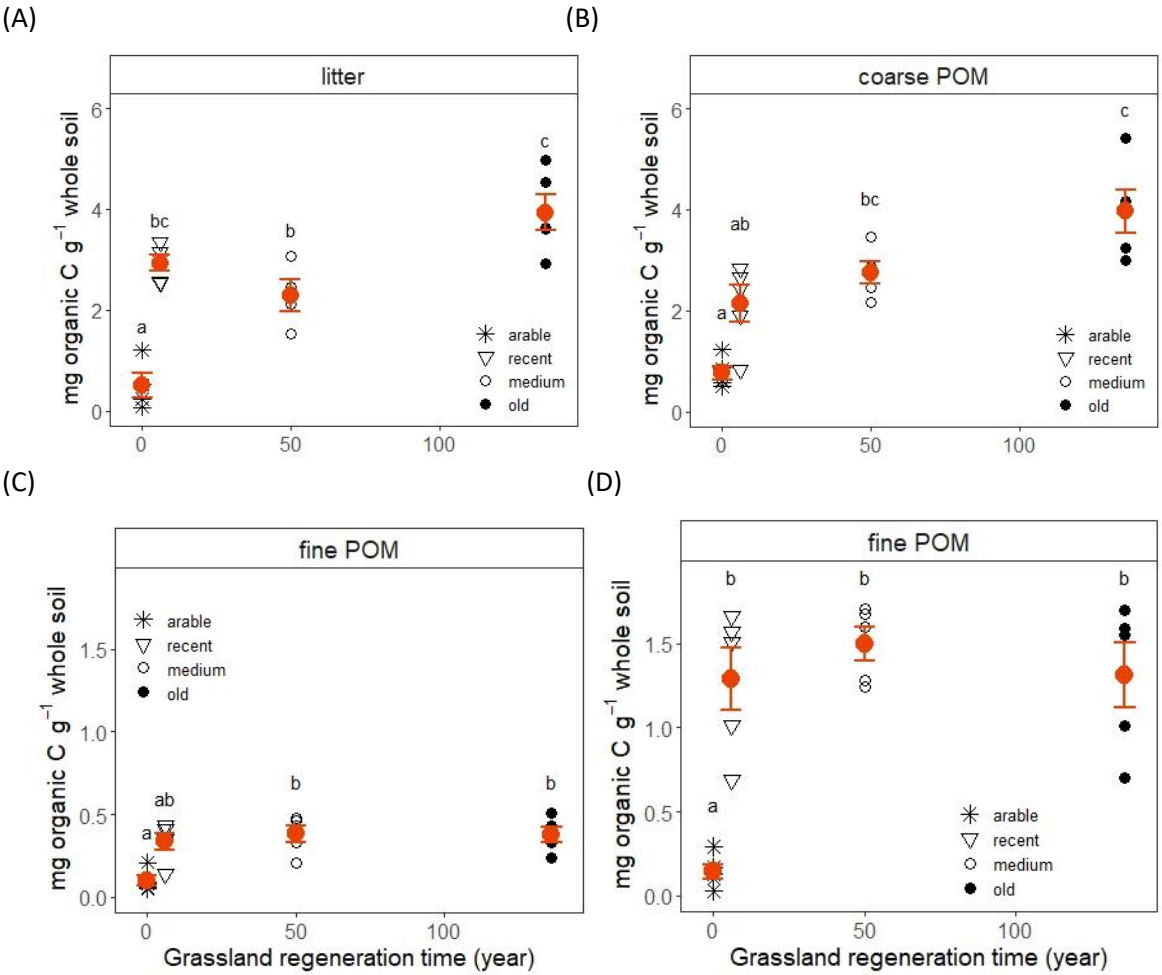


Figure 3.14 OC pool content in POM fractions associated with (A) litter, (B-C) non-aggregated soil matter and (D) macroaggregates. Tested using one-way ANOVA (or Kruskal-Wallis test) for an effect of land management history on soil C concentration. Orange point represent mean and error bar is standard error of the mean for land management history category. Letters indicate significant differences at $P < 0.05$ (Tukey HSD post hoc test or Dunn's test if Kruskal-Wallis test was used).

3.4.2 Soil biotic characteristics

<u>Soil microbial community</u>

Sequencing and overall diversity

Sequencing yielded 1,403,358 and 922,169 reads for bacteria and fungi respectively. Phylogenetic identification of the DNA amplicons was on average higher than 90 % at phylum, class and order taxonomic resolution for bacteria while it was lower than 90 % for fungi at these taxonomic resolutions.

Both, Simpson's, Shannon, bacterial community diversity indices responded to land management history (ANOVA, $F = 35.4$, $P < 0.001$ and $F = 35.4$, $P < 0.001$ respectively) whereby the indices were lower on Medium and Old grasslands when compared to arable sites (post hoc comparisons $P < 0.05$) (Table 3.9). The opposite pattern to the bacterial community was seen for fungal community whereby only richness (number of unique sequences) was affected by land management history

(ANOVA, F = 16.3, P < 0.001) whereby it was higher on grasslands in comparison to arable sites (post hoc comparisons P < 0.05) (Table 3.9).

Table 3.9 Microbial community diversity on sites of different land management history.

Microbial community	Arable	Grassland regeneration status			ANOVA		
Diversity		Recent	Medium	Old	df	F	P
Bacteria							
Richness	3594 ± 248	2953 ± 217	3372.5 ± 231	3125.25 ± 173	3	0.76	0.17
Simpson's index [1)]	0.9969 ± 0.0004 [a]	0.9935 ± 0.0004 [b]	0.9906 ± 0.001 [bc]	0.9841 ± 0.0017 [c]	3	35.4 [2)]	***
Shannon index [1)]	6.94 ± 0.04 [a]	6.52 ± 0.04 [b]	6.44 ± 0.05 [bc]	6.17 ± 0.06 [c]	3	33.6 [2)]	***
Fungi							
Richness [1)]	360 ± 14 [a]	457 ± 29 [b]	498 ± 38 [b]	509 ± 25 [b]	3	16.3 [2)]	***
Simpson's index [1)]	0.9647 ± 0.0038	0.953 ± 0.0093	0.951 ± 0.0139	0.9673 ± 0.006	3	1.8 [2)]	0.62
Shannon index [1)]	4.27 ± 0.07	4.34 ± 0.15	4.46 ± 0.18	4.57 ± 0.10	3	6.0 [2)]	0.11

1) Kruskal-Wallis test 2) chi squared. *** P < 0.001. Letters denotes differences at P < 0.05.

Bacterial community

Bacterial community was affected by land management history (M-GLMs, Wald =107013, P < 0.001). Pairwise comparison revealed that bacterial communities differed between all pairs of land management histories (post hoc comparisons P < 0.05) (Table 3.10).

Proteobacteria, *Verrucomicrobia*, *Actinobacteria* and *Acidobacteria* represented the most abundant phyla (Figure 3.15). Land management history did not affect relative abundance of phylum *Proteobacteria* (ANOVA, F = 1.0, P = 0.34) but affected that of *Actinobacteria* (ANOVA, F = 11.7, P = 0.34), *Verrucomicrobia* (ANOVA, F = 31.6, P = 0.34), *Acidobacteria* (ANOVA, F = 1.0, P = 11.9), *Bacteroidetes* (ANOVA, F = 1.0, P = 28.2) and *Chloroflexi* (ANOVA, F = 1.0, P = 17.5) (Table 3.11) (Figure 3.16). Arable land use promoted relative abundance of *Acidobacteria, Bacteroidetes* and *Chloroflexi* when compared to all regenerating grasslands (apart from Recent grasslands for *Acidobacteria*) while it decreased relative abundance of *Verrucomicrobia* when compared to all regenerating grasslands (post hoc comparisons P < 0.05). Arable land use also negatively affected relative abundance of *Actinobacteria* but only for Recent and Medium regenerating grasslands (post hoc comparisons P < 0.05). Within regenerating grassland land use, relative abundance of *Acidobacteria* and *Chloroflexi* decreased with Old grasslands and relative abundance of *Verrucomicrobia* increased with Old grasslands when compared to Recent regenerating grasslands (post hoc comparisons P < 0.05).

Table 3.10 Pairwise comparison of soil microbial communities on different land management history categories.

Pairwise comparison of land management categories [1)]	Bacteria		Fungi	
	Statistics [2)]	P [3)]	Statistics [2)]	P [3)]
Arable - Recent	36439	***	8818	***
Arable - Medium	33209	***	9821	***
Arable - Old	43737	***	11319	***
Medium - Recent	21427	*	3988	+
Medium - Old	23435	*	5699	**
Old - Recent	30972	**	6977	***

[1)] Recent, Medium and Old represent age of regenerating grasslands which was 6, 50 and 136 years respectively; [2)] Wald test statistics was used; [3)] adjusted P by Holm's step down transformation

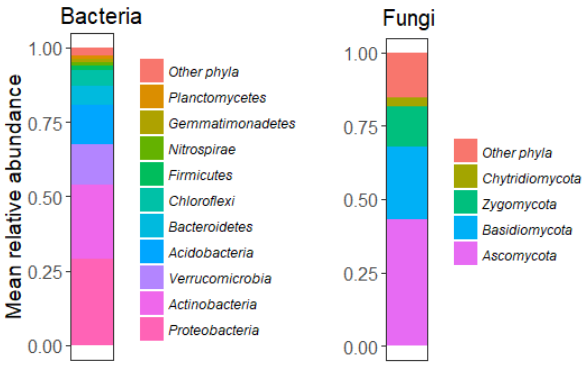


Figure 3.15 Microbial community overall mean relative abundance at phylum taxonomic resolution.
Other phyla represent unassigned taxa and phyla with total mean relative abundance lower than 0.99 %

Table 3.11 Effect of land management history on relative abundance of microbial community taxa at phylum resolution.

Microbial community	Arable	Grassland regeneration status			ANOVA	
Phylum		Recent	Medium	Old	F	P adjusted
Bacteria						
Proteobacteria	28.33 ± 0.92	28.73 ± 0.89	29.19 ± 0.98	30.20 ± 0.54	1.0	0.34
Actinobacteria	19.67 ± 0.91 [a]	29.39 ± 1.23 [c]	26.85 ± 1.86 [bc]	23.12 ± 1.31 [ab]	11.7	***
Verrucomicrobia [3)]	2.15 ± 0.07 [a]	2.41 ± 0.05 [b]	2.66 ± 0.07 [b]	3 ± 0.07 [c]	31.6	***
Acidobacteria	15.37 ± 0.61 [a]	13.55 ± 0.56 [ab]	12.6 ± 0.58 [bc]	10.9 ± 0.44 [c]	11.9	***
Bacteroidetes [3)]	2.32 ± 0.07 [a]	1.62 ± 0.05 [b]	1.62 ± 0.11 [b]	1.57 ± 0.06 [b]	28.2	***
Chloroflexi [3)]	1.88 ± 0.06 [a]	1.63 ± 0.07 [b]	1.56 ± 0.05 [b]	1.27 ± 0.05 [c]	17.5	***
Firmicutes	1.64 ± 0.18	1.26 ± 0.13	1.49 ± 0.25	1.42 ± 0.17	0.9	0.34
Nitrospirae	1.26 ± 0.10 [ab]	1.44 ± 0.12 [b]	1.38 ± 0.18 [ab]	1.01 ± 0.07 [a]	2.9	+
Gemmatimonadetes [3)]	0.72 ± 0.07 [a]	-0.17 ± 0.07 [b]	-0.36 ± 0.15 [b]	-0.05 ± 0.08 [b]	28.9	***
Planctomycetes	0.85 ± 0.12	0.96 ± 0.09	1.00 ± 0.10	1.16 ± 0.10	1.6	0.19
Fungi						
Ascomycota [1)]	58.18 ± 3.27 [a]	36.12 ± 5.04 [b]	39.62 ± 3.6 [b]	39.11 ± 3.39 [b]	13.7 [2)]	**
Basidiomycota [1)]	10.73 ± 1.24 [a]	24.43 ± 3.6 [b]	31.55 ± 4.46 [b]	31.92 ± 3.45 [b]	24.6 [2)]	***
Zygomycota [1)]	15.61 ± 2.18	15.5 ± 2.16	10.1 ± 1.34	13.46 ± 1.09	6.1 [2)]	0.13
Chytridiomycota [1)]	2.59 ± 0.62	3.76 ± 0.85	2.92 ± 0.53	3.14 ± 0.44	1.5 [2)]	0.69
Glomeromycota [3)]	0.37 ± 0.14 [a 4)]	0.57 ± 0.09 [b]	0.36 ± 0.06 [ab]	0.3 ± 0.06 [ab]	3.8	*

*** P < 0.001, ** P < 0.01, * P < 0.05, + P < 0.1, Effect of land management history tested using ANOVA or Kruskal-Wallis test when heteroscedasticity observed. P values adjusted using Benjamini-Hochberg transformation within bacteria and fungi separately. Differences between means were post hoc tested using Tukey or Dunn's test (when Kruskal-Wallis was used). Letters denote differences between means at P < 0.05. [1)] Kruskal-Wallis test [2)] chi squared [3)] ln(y) transformed [4)] an outlier removed from the analysis

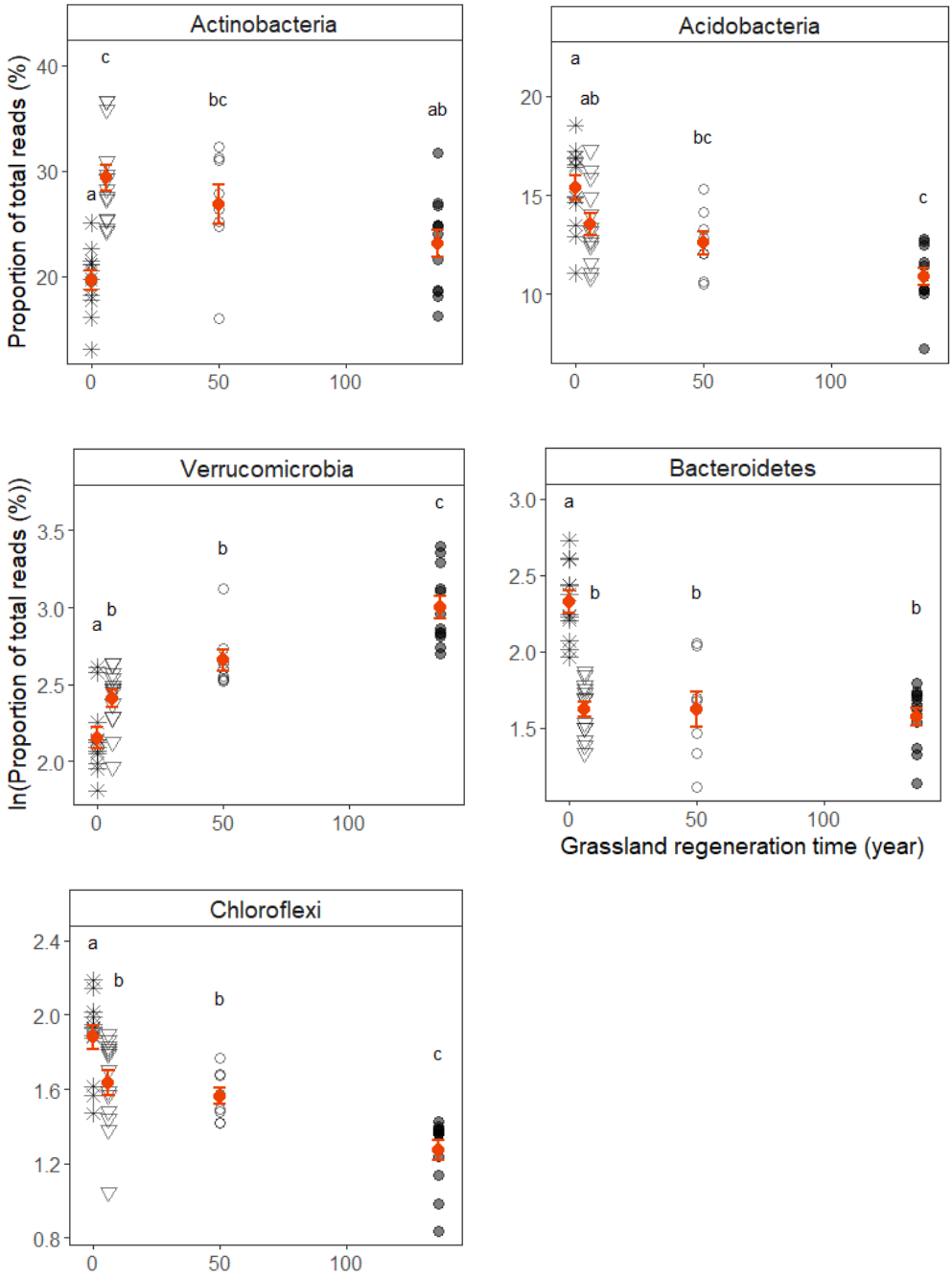


Figure 3.16 Abundance of bacterial phyla at sites of different management history. Grassland regeneration time represent sites of different management history: 0 year is arable sites, 6 years is recent grassland, 50 years is medium grassland and 136 years is old grassland. Orange point represent mean and error bar is standard error of the mean for land management history category. Differences in mean values tested using one-way ANOVA (or Kruskal-Wallis test). Letters indicate significant differences at $P < 0.05$ (Tukey HSD post hoc test or Dunn's test if Kruskal-Wallis test was used).

For the classes of overall relative abundance greater than 0.5 % (Table 3.12), classes of *Acidobacteria* and *Bacteroidetes* showed similar trend as the response of the phyla, while for *Acidobacteria*, only some classes showed similar trend as the response of the phyla. For *Verrucomicrobia*, only the most abundant class showed similar trend as the response of the phyla while the other, less abundant, classes showed an opposite trend. For *Proteobacteria*, the most abundant classes showed

contracting responses to the land management history to each other resulting in no response of the phyla (Figure 3.17).

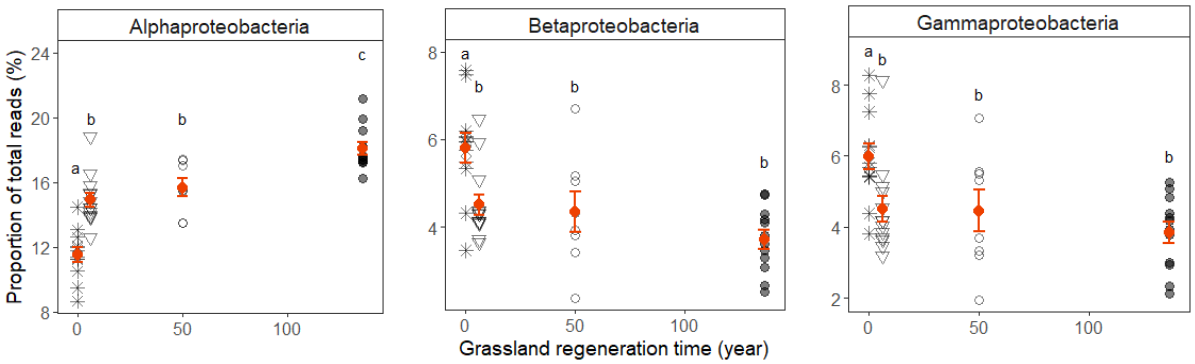


Figure 3.17 Abundance of bacterial classes of *Proteobacteria* at sites of different management history. Grassland regeneration time represent sites of different management history: 0 year is arable sites, 6 years is recent grassland, 50 years is medium grassland and 136 years is old grassland. Orange point represent mean and error bar is standard error of the mean for land management history category. Differences in mean values tested using one-way ANOVA (or Kruskal-Wallis test). Letters indicate significant differences at $P < 0.05$ (Tukey HSD post hoc test or Dunn's test if Kruskal-Wallis test was used).

A trend typical for most bacterial orders (with overall relative abundance higher than 0.1 %) (Table 3.13) included response to the land management history of the most abundant bacterial order(s) within class which was similar to the response of the class while the remaining orders within the class showed opposite or no response: *Alphaproteobacteria*, *Betaprotebacteria*, *Gammaproteobacteria*, *Verrucomicorbia*, *Acidobacteria* and *Actinobacteria*. Bacterial phyla including orders showing similar trend in their response as the overall phyla included *Bacteroidetes* and *Chloroflexi*. Other trend in the response to land management history included class without the response while its most abundant orders showed a contrasting response which was the case of *Deltaproteobacteria*.

Table 3.12 Relative abundance of bacterial phyla and classes at different land management history sites.

Phylum	Class	Arable	Grassland regeneration status			ANOVA	
			Recent	Medium	Old	F	P [4]
Proteobacteria	*Alphaproteobacteria*	11.56 ± 0.45 [a]	14.94 ± 0.43 [b]	15.7 ± 0.56 [b]	18.12 ± 0.39 [c]	38.6	***
	Gammaproteobacteria	5.98 ± 0.37 [a]	4.5 ± 0.36 [b]	4.46 ± 0.59 [b]	3.84 ± 0.3 [b]	5.8	**
	Deltaproteobacteria	4.93 ± 0.13	4.72 ± 0.09	4.64 ± 0.19	4.47 ± 0.09	2.9	+
	Betaproteobacteria [2]	5.8 ± 0.33 [a]	4.51 ± 0.23 [b]	4.35 ± 0.46 [b]	3.71 ± 0.21 [b]	16.8 [3]	**
Actinobacteria	*Thermoleophilia*	7.37 ± 0.53 [a]	13.38 ± 0.64 [b]	12.6 ± 1.14 [b]	10.92 ± 0.73 [b]	14.0	***
	Actinobacteria [2]	6.54 ± 0.21 [a]	6.64 ± 0.3 [ab]	6.23 ± 0.39 [ab]	5.79 ± 0.32 [b]	7.0 [3]	+
	Acidimicrobiia	4.08 ± 0.23 [a]	7.09 ± 0.39 [c]	6.34 ± 0.49 [bc]	5.05 ± 0.43 [ab]	12.8	***
	MB-A2-108	1.35 ± 0.07 [a]	2.01 ± 0.12 [b]	1.44 ± 0.11 [a]	1.03 ± 0.09 [a]	18.7	***
Verrucomicrobia	*[Spartobacteria]* [2]	4.86 ± 0.77 [a]	8.81 ± 0.53 [ab]	12.36 ± 1.39 [b]	18.96 ± 1.59 [c]	33.7 [3]	***
	[Pedosphaerae] [1]	0.8 ± 0.08 [a]	0.42 ± 0.06 [b]	0.21 ± 0.11 [bc]	0.09 ± 0.07 [c]	16.6	***
	Verrucomicrobiae	1.24 ± 0.07 [a]	0.74 ± 0.05 [b]	0.68 ± 0.1 [bc]	0.47 ± 0.05 [c]	25.5	***
Acidobacteria	*Acidobacteria-6*	8.34 ± 0.34 [a]	8.58 ± 0.33 [a]	7.54 ± 0.51 [a]	5.48 ± 0.42 [b]	14.3	***
	[Chloracidobacteria]	4.44 ± 0.23 [a]	2.69 ± 0.2 [b]	3.04 ± 0.28 [b]	3.16 ± 0.23 [b]	11.7	***
	Solibacteres [2]	0.64 ± 0.04 [ab]	0.55 ± 0.02 [a]	0.62 ± 0.05 [ab]	0.85 ± 0.11 [b]	7.6 [3]	+
	iii1-8	0.64 ± 0.06	0.53 ± 0.06	0.46 ± 0.06	0.47 ± 0.05	1.9	0.17
Bacteroidetes	*[Saprospirae]*	4.77 ± 0.33 [a]	2.25 ± 0.14 [b]	2.45 ± 0.26 [b]	2.48 ± 0.13 [b]	29.4	***
	Cytophagia [2]	2.76 ± 0.28 [a]	1.47 ± 0.07 [b]	1.59 ± 0.22 [b]	1.29 ± 0.12 [b]	20.1 [3]	***
	Sphingobacteriia [2]	1.61 ± 0.14 [a]	0.7 ± 0.04 [b]	0.59 ± 0.07 [b]	0.6 ± 0.05 [b]	27.6 [3]	***
	Flavobacteriia [2]	1.31 ± 0.23 [a]	0.66 ± 0.06 [b]	0.64 ± 0.11 [ab]	0.49 ± 0.06 [b]	14.9 [3]	**
Chloroflexi	*Ellin6529*	1.52 ± 0.14 [ab]	1.91 ± 0.16 [a]	1.69 ± 0.12 [ab]	1.38 ± 0.09 [b]	3.1	+
	Anaerolineae	2.85 ± 0.19 [a]	1.1 ± 0.14 [b]	1.33 ± 0.14 [b]	0.94 ± 0.07 [b]	37.9	***
	S085	0.7 ± 0.06 [a]	0.63 ± 0.05 [ab]	0.45 ± 0.06 [bc]	0.33 ± 0.05 [c]	11.1	***
Firmicutes	*Bacilli* [1]	0.37 ± 0.09	0.11 ± 0.11	0.27 ± 0.16	0.24 ± 0.12	0.9	0.54
Nitrospirae	*Nitrospira*	1.26 ± 0.10 [a]	1.44 ± 0.12 [ab]	1.38 ± 0.18 [ab]	1.01 ± 0.07 [b]	2.9	+
Gemmatimonadetes	*Gemmatimonadetes* [3]	-0.02 ± 0.08 [a]	-1.14 ± 0.1 [b]	-1.32 ± 0.22 [b]	-0.68 ± 0.12 [b]	22.1	***
	Gemm-1 [3]	-0.35 ± 0.09 [a]	-0.99 ± 0.1 [b]	-1.23 ± 0.2 [b]	-1.16 ± 0.09 [b]	12.5	***
Planctomycetes	*Planctomycetia* [1]	0.26 ± 0.04 [a]	0.41 ± 0.05 [a]	0.44 ± 0.05 [ab]	0.71 ± 0.08 [b]	12.8	***
Cyanobacteria	*Chloroplast* [2]	1.13 ± 0.38 [a]	0.04 ± 0.01 [b]	0.03 ± 0.01 [b]	0.04 ± 0.01 [b]	7.5 [3]	***

Data are relative abundances (%). *** P < 0.001, ** P < 0.01, * P < 0.05, + P < 0.1, Effect of land management history tested using ANOVA or Kruskal-Wallis test when heteroscedasticity observed. P values adjusted using Benjamini-Hochberg transformation within bacteria and fungi separately. Differences between means were post hoc tested using Tukey or Dunn's test (when Kruskal-Wallis was used). Letters denote differences between means at P < 0.05. [1] Kruskal-Wallis test [2] chi squared [3] ln(y) transformed [4] an outlier removed from the analysis

A relatively high proportion of indicator species (Table 3.14) for arable sites included orders *Sphingomonadales*, *Burkholderiales* (both *Alphaproteobacteria*), *Myxococcales* (*Deltaproteobacteria*), *Xanthomonadales* (*Gammaproteobacteria*), *Actinomycetales* (*Actinobacteria*), *[Pedosphaerales]* (*[Pedosphaerae*]), *iii1-15* (*Acidobacteria-6*), *RB41* (*[Chloracidobacteria]*), *[Saprospirales]* ([*Saprospirae*]) and *Cytophagales* (*Cytophagia*). Recent grasslands included relatively high proportion of indicator species in orders *Rhizobiales*, *Rhodobacterales* (both *Alphaproteobacteria*), *Gaiellales*, *Solirubrobacterales* (both *Thermoleophilia*), *Actinomycetales* (*Actinobacteria*), *Acidimicrobiales* (*Acidimicrobiia*), *0319-7L14* (*MB-A2-108*), *iii1-15* (*Acidobacteria-6*). Medium grasslands included indicator species in orders *Gaiellales* (*Thermoleophilia*) and *Acidimicrobiales* (*Acidimicrobiia*) and old grasslands in orders *Rhizobiales*, *Rhodobacterales* (both *Alphaproteobacteria*), *Syntrophobacterales* (*Deltaproteobacteria*), *Solirubrobacterales* (*Thermoleophilia*), *Actinomycetales* (*Actinobacteria*) and *[Chthoniobacterales]* (*[Spartobacteria]*). Also other orders included indicator species for the particular land management history categories but these orders were of relatively low abundance or the indicator species comprised relatively low proportion of the particular orders.

Table 3.13 Relative abundance of bacterial orders at different land management history sites.

Phylum / Class	Order	Arable	Grassland regeneration status			ANOVA	
			Recent	Medium	Old	F [3]	P [4]
Proteobacteria							
Alphaproteobacteria	*Rhizobiales* [2]	5.13 ± 0.27 [a]	8.94 ± 0.26 [b]	9.39 ± 0.63 [b]	10.54 ± 0.38 [b]	30.1	***
	Rhodospirillales [2]	2.11 ± 0.18 [a]	3.09 ± 0.17 [b]	3.27 ± 0.28 [bc]	4.19 ± 0.21 [c]	25.6	***
	Sphingomonadales [2]	2.87 ± 0.34 [a]	1.63 ± 0.25 [b]	1.65 ± 0.29 [b]	1.77 ± 0.15 [b]	13.7	**
	Rhodobacterales	0.59 ± 0.04	0.59 ± 0.04	0.56 ± 0.04	0.51 ± 0.05	0.7	0.56
	Caulobacterales [2]	0.39 ± 0.07 [a]	0.2 ± 0.02 [b]	0.24 ± 0.04 [ab]	0.38 ± 0.03 [ac]	16.4	**
	Ellin329 [1]	-1.83 ± 0.1 [a]	-1.76 ± 0.09 [a]	-1.61 ± 0.13 [ab]	-1.2 ± 0.17 [b]	5.4	*
Betaproteobacteria	*Burkholderiales* [2]	2.99 ± 0.21 [a]	2.35 ± 0.18 [ab]	2.39 ± 0.33 [ab]	1.71 ± 0.16 [b]	15.4	***
	SC-I-84	0.51 ± 0.06 [a]	0.53 ± 0.05 [a]	0.53 ± 0.06 [ab]	0.8 ± 0.07 b	5.1	**
	Ellin6067 [1]	-0.74 ± 0.1	-0.7 ± 0.08	-0.79 ± 0.12	-0.79 ± 0.07	0.3	0.72
	MND1	0.41 ± 0.06 [a]	0.26 ± 0.03 [a]	0.19 ± 0.03 [ab]	0.11 ± 0.02 [b]	18.4	***
Deltaproteobacteria	*Myxococcales* [2]	2.4 ± 0.14 [a]	1.55 ± 0.09 [b]	1.43 ± 0.06 [b]	1.54 ± 0.09 [b]	21.3	***
	Syntrophobacterales	0.93 ± 0.04 [a]	1.14 ± 0.05 [b]	1.3 ± 0.04 [bc]	1.44 ± 0.08 [c]	16.6	***
	NB1-j	0.45 ± 0.05	0.6 ± 0.05	0.7 ± 0.13	0.56 ± 0.06	2.1	0.15
	[Entotheonellales]	0.5 ± 0.06 [a]	0.74 ± 0.04 [b]	0.64 ± 0.1 [ab]	0.37 ± 0.05 [a]	7.3	***
	Bdellovibrionales	0.22 ± 0.02	0.26 ± 0.03	0.23 ± 0.03	0.22 ± 0.02	1.0	0.49
	Desulfuromonadales	0.07 ± 0.01	0.18 ± 0.05	0.14 ± 0.03	0.11 ± 0.03	1.8	0.20
Gammaproteobacteria	*Xanthomonadales* [2]	4.54 ± 0.33 [a]	3.47 ± 0.29 [b]	3.43 ± 0.45 [ab]	2.86 ± 0.24 [b]	13.2	**
	Thiotrichales	0.36 ± 0.03 [a]	0.34 ± 0.03 [a]	0.29 ± 0.04 [ab]	0.18 ± 0.02 [b]	8.3	***
	Pseudomonadales	0.39 ± 0.09	0.21 ± 0.03	0.25 ± 0.06	0.21 ± 0.03	2.6	+
	Legionellales	0.22 ± 0.02	0.19 ± 0.02	0.19 ± 0.03	0.22 ± 0.02	0.8	0.54
	Alteromonadales	-1.42 ± 0.14 [a]	-2.59 ± 0.12 [b]	-2.54 ± 0.19 [bc]	-2.07 ± 0.14 [c]	15.3	***
Actinobacteria							
Thermoleophilia	*Gaiellales*	4.41 ± 0.34 [a]	7.57 ± 0.35 [b]	6.67 ± 0.57 [bc]	5.27 ± 0.33 [ac]	14.9	***
	Solirubrobacterales	2.97 ± 0.24 [a]	5.81 ± 0.37 [b]	5.93 ± 0.72 [b]	5.64 ± 0.44 [b]	11.5	***
Actinobacteria	*Actinomycetales*	6.31 ± 0.2	6.21 ± 0.29	5.87 ± 0.36	5.64 ± 0.3	1.3	0.36
	Micrococcales [2]	0.23 ± 0.02 [ac]	0.43 ± 0.03 [b]	0.35 ± 0.04 [ab]	0.15 ± 0.04 [c]	21.6	***
Acidimicrobiia	*Acidimicrobiales*	4.08 ± 0.23 [a]	7.09 ± 0.39 [b]	6.34 ± 0.49 [bc]	5.05 ± 0.43 [ac]	12.8	***
MB-A2-108	*0319-7L14*	1.33 ± 0.07 [ab]	2.01 ± 0.12 [c]	1.44 ± 0.11 [a]	1.03 ± 0.09 [b]	18.7	***
Verrucomicrobia							
[Spartobacteria]	*[Chthoniobacterales]* [2]	4.86 ± 0.77 [a]	8.81 ± 0.53 [b]	12.36 ± 1.39 [bc]	18.96 ± 1.59 [c]	33.7	***
[Pedosphaerae]	*[Pedosphaerales]* [2]	2.3 ± 0.17 [a]	1.55 ± 0.1 [b]	1.29 ± 0.17 [bc]	1.12 ± 0.07 [c]	24.2	***
Verrucomicrobiae	*Verrucomicrobiales* [2]	1.24 ± 0.07 [a]	0.74 ± 0.05 [b]	0.68 ± 0.1 [bc]	0.47 ± 0.05 [c]	27.0	***
Acidobacteria							
Acidobacteria-6	*iii1-15*	7.81 ± 0.32 [a]	7.82 ± 0.31 [a]	6.72 ± 0.44 [a]	4.9 ± 0.37 [b]	16.5	***
	CCU21	0.53 ± 0.04 [a]	0.75 ± 0.05 [ab]	0.81 ± 0.11 [b]	0.58 ± 0.05 [ab]	4.5	*
[Chloracidobacteria]	*RB41*	3.84 ± 0.21 [a]	2.26 ± 0.19 [b]	2.56 ± 0.23 [b]	2.71 ± 0.21 [b]	11.5	***
	PK29	0.26 ± 0.03	0.26 ± 0.02	0.36 ± 0.05	0.31 ± 0.03	1.9	0.19
	11-24	0.21 ± 0.03	0.11 ± 0.02	0.07 ± 0.01	0.05 ± 0.01	10.9	***
Solibacteres	*Solibacterales* [2]	0.64 ± 0.04 [ab]	0.55 ± 0.02 [a]	0.62 ± 0.05 [ab]	0.85 ± 0.11 [b]	7.6	+

	iii1-8	*32-20*	0.33 ± 0.03	0.31 ± 0.04	0.33 ± 0.05	0.32 ± 0.03	0.1	0.98
		DS-18 2)	0.3 ± 0.04 [a]	0.21 ± 0.03 [ab]	0.12 ± 0.02 [b]	0.14 ± 0.04 [b]	12.7	**
Bacteroidetes								
	[Saprospirae]	*[Saprospirales]*	4.77 ± 0.33 [a]	2.25 ± 0.14 [b]	2.45 ± 0.26 [b]	2.48 ± 0.13 [b]	29.4	***
	Cytophagia	*Cytophagales* [2)]	2.76 ± 0.28 [a]	1.47 ± 0.07 [b]	1.59 ± 0.22 [b]	1.29 ± 0.12 [b]	20.1	***
	Sphingobacteriia	*Sphingobacteriales* [2)]	1.61 ± 0.14 [a]	0.7 ± 0.04 [b]	0.59 ± 0.07 [b]	0.6 ± 0.05 [b]	27.6	***
	Flavobacteriia	*Flavobacteriales* [2)]	1.31 ± 0.23 [a]	0.66 ± 0.06 [b]	0.64 ± 0.11 [ab]	0.49 ± 0.06 [b]	14.9	***
Chloroflexi								
	Anaerolineae	*SBR1031*	1.59 ± 0.11 [a]	0.75 ± 0.11 [b]	0.99 ± 0.13 [b]	0.74 ± 0.06 [b]	15.9	***
		H39	0.37 ± 0.07 [a]	0.1 ± 0.03 [b]	0.12 ± 0.04 [b]	0.06 ± 0.01 [b]	21.6	***
		CFB-26	0.37 ± 0.06 [a]	0.08 ± 0.01 [b]	0.07 ± 0.02 [b]	0.04 ± 0.01 [b]	27.8	***
Firmicutes								
	Bacilli	*Bacillales* [1)]	0.37 ± 0.09	0.11 ± 0.11	0.27 ± 0.16	0.24 ± 0.12	1.0	0.54
Nitrospirae								
	Nitrospira	*Nitrospirales*	1.26 ± 0.1 [a]	1.44 ± 0.12 [ab]	1.38 ± 0.18 [ab]	1.01 ± 0.07 [b]	2.9	+

Data are relative abundances (%). *** P < 0.001, ** P < 0.01, * P < 0.05, + P < 0.1, Effect of land management history tested using ANOVA or Kruskal-Wallis test when heteroscedasticity observed. P values adjusted using Benjamini-Hochberg transformation within bacteria and fungi separately. Differences between means were post hoc tested using Tukey or Dunn's test (when Kruskal-Wallis was used). Letters denote differences between means at P < 0.05. [1)] Kruskal-Wallis test [2)] chi squared [3)] ln(y) transformed [4)] an outlier removed from the analysis

Table 3.14 Bacterial indicator species.

Phylum	Class	Order	Relative abundance (%)					Proportion of indicator species (%)															
			Total	Arable	Grassland regeneration status			Arable indicator species				Grassland regeneration status indicator species											
												Recent				Medium				Old			
					Recent	Medium	Old	A	R	M	O	A	R	M	O	A	R	M	O	A	R	M	O
Proteobacteria																							
	Alphaproteobacteria	*Rhizobiales*	8.4	5.1	8.9	9.4	10.5	6	3	3	2	12	25	19	12	1	2	4	2	21	44	52	79
		Rhodospirillales	3.2	2.1	3.1	3.3	4.2	14	5	3	1	1	2	1	0	0	0	1	0	19	39	43	72
		Sphingomonadales	2.0	2.9	1.6	1.6	1.8	44	14	13	10					0	0	0	0	1	2	2	4
		Rhodobacterales	0.57	0.59	0.59	0.56	0.51	24	1	0	1	21	34	28	10	1	2	4	0	2	6	6	15
		Caulobacterales	0.31	0.39	0.20	0.24	0.38	64	6	8	12									31	35	47	72
		Ellin329	0.23	0.17	0.18	0.21	0.35					0	2	0	1	0	1	2	0	68	64	77	126
	Betaproteobacteria	*Burkholderiales*	2.4	3.0	2.4	2.4	1.7	32	9	6	5	0	0	0	0	2	2	5	4	0	0	0	1
		SC-I-84	0.60	0.51	0.53	0.53	0.80	19	6	2	1					0	1	1	0	15	20	14	49
		Ellin6067	0.49	0.50	0.52	0.48	0.47	24	9	7	11	1	3	1	2	14	34	44	35				
		MND1	0.25	0.41	0.26	0.19	0.11	91	25	23	6	3	4	0	0								
	Deltaproteobacteria	*Myxococcales*	1.8	2.4	1.5	1.4	1.5	29	7	6	7	2	10	7	4	0	1	2	1	1	2	2	12
		Syntrophobacterales	1.2	0.93	1.1	1.3	1.4	11	6	4	2					0	0	2	0	25	33	44	65
		NB1-j	0.57	0.45	0.60	0.70	0.56									0	0	0	0	3	5	4	12
		[Entotheonellales]	0.56	0.50	0.74	0.64	0.37					29	35	19	8	0	1	4	0				
		Bdellovibrionales	0.23	0.22	0.26	0.23	0.22					2	35	18	9	1	1	8	2				
	Gammaproteobacteria	*Xanthomonadales*	3.6	4.5	3.5	3.4	2.9	37	10	8	5	0	0	0	0	0	0	2	0	0	1	1	2
		Thiotrichales	0.29	0.36	0.34	0.29	0.18	25	19	10	5												
		Pseudomonadales	0.27	0.39	0.21	0.25	0.21	18	6	7	4					0	1	2	0				
		Legionellales	0.21	0.22	0.19	0.19	0.22	3	0	0	1					0	1	4	0	0	2	10	17
		Alteromonadales	0.15	0.28	0.082	0.089	0.14	98	13	16	4									6	14	28	52
Actinobacteria																							
	Thermoleophilia	*Gaiellales*	6.0	4.4	7.6	6.7	5.3	2	1	1	0	27	49	32	22	11	23	24	14	1	2	2	8
		Solirubrobacterales	5.0	3.0	5.8	5.9	5.6	3	1	1	0	10	23	13	7	0	1	3	1	5	11	17	32
	Actinobacteria	*Actinomycetales*	6.0	6.3	6.2	5.9	5.6	39	11	8	4	11	19	12	6	1	2	4	2	6	7	11	24
		Micrococcales	0.29	0.23	0.43	0.35	0.15					77	145	112	52	0	1	2	0				
	Acidimicrobiia	*Acidimicrobiales*	5.6	4.1	7.1	6.3	5.1	3	1	1	1	21	34	22	15	7	20	24	15	1	2	2	3
	MB-A2-108	*0319-7L14*	1.5	1.3	2.0	1.4	1.0	7	3	1	0	66	110	78	55	0	0	1	0	0	0	0	1
	Rubrobacteria	*Rubrobacterales*	0.28	0.31	0.26	0.23	0.32	51	19	10	1					0	0	1	0	12	21	32	74
Verrucomicrobia																							
	[Spartobacteria]	*[Chthoniobacterales]*	11.1	4.9	8.8	12.4	19.0	6	2	2	1	0	0	0	0	1	2	4	3	28	65	93	155
	[Pedosphaerae]	*[Pedosphaerales]*	1.6	2.3	1.6	1.3	1.1	83	43	34	23					0	1	2	1	0	1	1	3
	Opitutae	*Opitutales*	0.21	0.39	0.16	0.15	0.12	110	43	33	23												
	Verrucomicrobiae	*Verrucomicrobiales*	0.79	1.2	0.74	0.68	0.47	105	53	50	34	0	2	1	0	0	0	2	0				
Acidobacteria																							
	Acidobacteria-6	*iii1-15*	6.8	7.8	7.8	6.7	4.9	40	25	19	13	17	29	20	14	0	1	2	1	0	0	1	3
		CCU21	0.66	0.53	0.75	0.81	0.58					4	7	4	1	4	14	27	15	0	0	1	2
	[Chloracidobacteria]	*RB41*	2.9	3.8	2.3	2.6	2.7	79	29	31	26									1	2	3	7
		PK29	0.29	0.26	0.26	0.36	0.31									1	3	8	3	0	0	2	23
		11-24	0.11	0.21	0.11	0.066	0.053	161	75	42	35												
	Solibacteres	*Solibacterales*	0.66	0.64	0.55	0.62	0.85	24	11	8	6	3	5	3	3	1	1	4	3	14	18	22	59
	iii1-8	*32-20*	0.32	0.33	0.31	0.33	0.32	30	5	5	1					0	0	1	0				
		DS-18	0.20	0.30	0.21	0.12	0.14	3	0	0	0	1	14	2	3								
	BPC102	*MVS-40*	0.19	0.11	0.27	0.23	0.16					49	142	115	81	0	0	2	0				
	Sva0725	*Sva0725*	0.26	0.38	0.26	0.26	0.14	43	11	16	6					0	0	1	0				
Bacteroidetes																							
	[Saprospirae]	*[Saprospirales]*	3.0	4.8	2.3	2.4	2.5	93	32	34	28					0	0	0	0	1	2	4	10
	Cytophagia	*Cytophagales*	1.8	2.8	1.5	1.6	1.3	68	17	16	15					0	0	2	1	0	0	0	1
	Sphingobacteriia	*Sphingobacteriales*	0.90	1.6	0.70	0.59	0.60	88	33	27	21					1	1	3	0	0	1	0	2
	Flavobacteriia	*Flavobacteriales*	0.78	1.3	0.66	0.64	0.49	72	12	8	9	1	4	2	1	3	3	5	1				

Proportion of sum of indicator species at order level to the mean relative abundance of particular order (%): 100 0

Fungal community

The fungal community was affected by land management history (M-GLMs, Wald = 25216, P < 0.001). Pairwise comparisons revealed that fungal communities differed between all pairs of land management histories apart from Recent and Medium grasslands (P < 0.05) (Table 3.10). *Ascomycota*, *Basidiomycota* and *Zygomycota* represented the most abundant phyla (Figure 3.15).

Fungal phyla which reacted to land management history only reacted to the difference between land management categories (arable against grassland) but not to different regenerating grasslands (Table 3.11). The relative abundance of *Ascomycota* was higher while relative abundance of *Basidiomycetes* was lower on arable sites in comparison to the grasslands (ANOVA, F = 1.0, P < 0.05 and F = 1.0, P < 0.05 respectively, posthoc comparisons P < 0.05) (Figure 3.18). Relative abundance of *Zygomycota* and *Chytridiomycota* did not react to the land management history (Table 3.11).

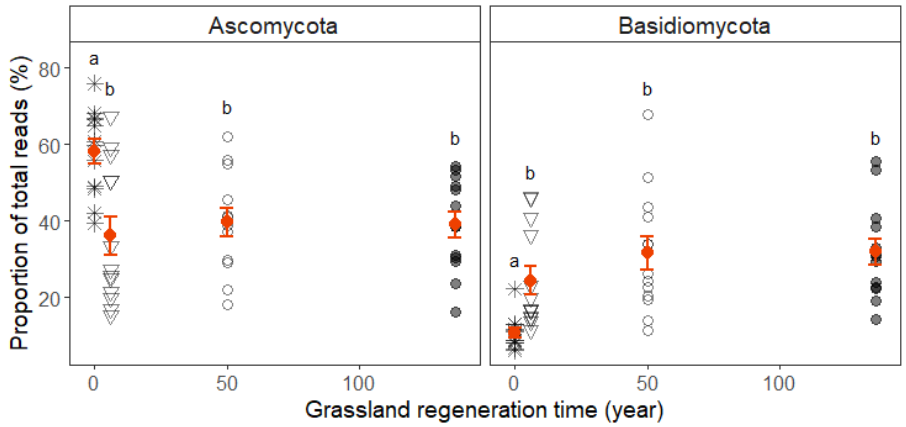


Figure 3.18 Abundance of fungal phyla at sites of different management history.
Grassland regeneration time represent sites of different management history: 0 year is arable sites, 6 years is recent grassland, 50 years is medium grassland and 136 years is old grassland. Orange point represent mean and error bar is standard error of the mean for land management history category. Differences in mean values tested using one-way ANOVA (or Kruskal-Wallis test). Letters indicate significant differences at P < 0.05 (Tukey HSD post hoc test or Dunn's test if Kruskal-Wallis test was used).

The most abundant fungal classes, *Sordariomycetes* (*Ascomycota*) and *Agaricomycetes* (*Basidiomycota*), showed response to land management history similar to the response of the phyla they belonged to. Same trend in the response was also observed for two other abundant classes of *Ascomycota* (*Pezizomycetes* and *Dothideomycetes*) and a minor class of *Basidiomycetes*, while other classes of both phyla showed different response than the phyla (Table 3.15).

For *Ascomycota* orders (Table 3.16), the classes with similar response of their orders as the phylum included *Sordariomycetes*, *Pezizomycetes* and *Dothideomycetes*. Class *Eurotiomycetes* included only most abundant order showing the same response as the class as well as the phylum. Similarly for *Basidiomycetes* (Table 3.16), classes which reacted in the same direction as the phylum included orders with the same trend of response (*Agaricomycetes* and *Ustilaginomycetes*) while other classes included orders with opposite, different or no response. Interestingly, fungal phyla with unassigned taxonomy which were excluded from statistical analysis showed a mean relative abundance of 16 % on arable sites while the abundance of less than 7.5 % on grassland sites.

Indicator species analysis (Table 3.17) showed that relatively high abundance fungal orders of *Hypocreales* (*Sordariomycetes*), *Helotiales* (*Leotiomycetes*), *Pezzizales* (*Pezizomycetes*) and *Pleosporales* (*Dothideomycetes*), all from phylum *Ascomycetes*, and *Mortierellales* (phylum *Zygomycota*) included a relatively high proportion of arable site indicator species. Recent grassland

indicator species were partially from the same fungal orders as arable indicator species including *Hypocreales* (*Sordariomycetes*), *Pleosporales* (*Dothideomycetes*) and *Mortierellales* (phylum *Zygomycota*) but also from *Sordariales* (*Sordariomycetes)* and *Agaricales* (*Agaricomycetes*, *Basidiomycetes*), however the indicator species for the recent grassland encompassed relatively lower total proportion of the particular orders than observed for arable sites. The relatively lowest number of orders with indicator species was seen for medium grassland including *Hypocreales and Sordariales* (both *Sordariomycetes)* and also *Agaricales* (*Agaricomycetes*). Old grassland showed a relatively high number of orders with indicator species and from the most abundant orders, relatively high proportion of indicator species were determined from *Hypocreales, Sordariales* (both *Sordariomycetes)*, *Helotiales* (*Leotiomycetes*), *Chaetothyriales* (*Eurotiomycetes*), *Pezzizales* (*Pezizomycetes*), *Agaricales* (*Agaricomycetes*) and *Mortierellales* (phylum *Zygomycota*). Similarly to bacterial indicator species, other orders included indicator species for the particular land management history categories but these orders were of relatively low abundance or the indicator species comprised relatively low proportion of the particular orders (Table 3.17).

Table 3.15 Fungal phyla and classes at different land management history sites.

Phylum	Class	Arable	Grassland regeneration status			ANOVA	
			Recent	Medium	Old	F	P [4]
	Sordariomycetes [2]	24.1 ± 2.4 [a]	12.67 ± 1.94 [b]	14.96 ± 2.38 [b]	10.49 ± 1.3 [b]	16.4 [3]	**
	Leotiomycetes [2]	5.12 ± 0.8 [ab]	3.7 ± 0.61 [a]	6.33 ± 0.69 [bc]	9.84 ± 1.49 [c]	14.2 [3]	**
	Eurotiomycetes [1]	0.32 ± 0.19 [a]	1.22 ± 0.19 [b]	1.36 ± 0.19 [b]	1.76 ± 0.13 [b]	11.1	***
Ascomycota	*Pezizomycetes* [2]	6.53 ± 1.94 [a]	3.23 ± 1.39 [ab]	2.57 ± 0.79 [ab]	2.07 ± 1.04 [b]	10.1 [3]	*
	Dothideomycetes [2]	4.12 ± 0.54 [a]	2.27 ± 0.41 [b]	2.41 ± 0.29 [b]	2.37 ± 0.41 [b]	9.2 [3]	*
	Incertae_sedis [2]	0.36 ± 0.11 [a]	2.03 ± 0.82 [a]	0.64 ± 0.35 [a]	0.02 ± 0.02 [b]	21.8 [3]	***
	Orbiliomycetes [2]	0.01 ± 0.01 [a]	0.73 ± 0.17 [b]	0.37 ± 0.09 [b]	0.08 ± 0.04 [a]	23.0 [3]	***
	Agaricomycetes [1]	0.49 ± 0.27 [a]	2.61 ± 0.21 [b]	3.01 ± 0.18 [b]	3.01 ± 0.16 [b]	32.7	***
	Tremellomycetes [1]	1.71 ± 0.15 [a]	1.06 ± 0.15 [b]	0.97 ± 0.15 [b]	1.27 ± 0.2 [ab]	3.3	*
Basidiomycota	*Microbotryomycetes* [2]	1.56 ± 0.26 [a]	0.49 ± 0.15 [b]	0.35 ± 0.09 [b]	0.56 ± 0.09 [b]	19.7 [3]	***
	Ustilaginomycetes [2]	0.07 ± 0.05 [a]	0.71 ± 0.18 [b]	0.49 ± 0.08 [b]	0.69 ± 0.2 [b]	18.1 [3]	**
	Wallemiomycetes[2]	0.03 ± 0.03	0.01 ± 0.01	0.06 ± 0.04	0.9 ± 0.43	5.8 [3]	0.14
Zygomycota	*Incertae_sedis* [2]	15.61 ± 2.18	15.5 ± 2.16	10.1 ± 1.34	13.45 ± 1.09	6.1 [3]	0.13
Chytridiomycota	*Chytridiomycetes* [1]	0.29 ± 0.32	0.47 ± 0.32	0.03 ± 0.27	-0.46 ± 0.31	1.8	0.19
Glomeromycota	*Glomeromycetes* [1]	0.37 ± 0.14	0.57 ± 0.09	0.36 ± 0.06	0.29 ± 0.05	2.5	+

*** P < 0.001, ** P < 0.01, * P < 0.05, + P < 0.1, Effect of land management history tested using ANOVA or Kruskal-Wallis test when heteroscedasticity observed. P values adjusted using Benjamini-Hochberg transformation within bacteria and fungi separately. Differences between means were post hoc tested using Tukey or Dunn's test (when Kruskal-Wallis was used). Letters denote differences between means at P < 0.05. [1] Kruskal-Wallis test [2] chi squared [3] ln(y) transformed [4] an outlier removed from the analysis

Table 3.16 Fungal phyla, classes and orders at different land management history sites.

Phylum		Arable	Grassland regeneration status			ANOVA	
Class	Order		Recent	Medium	Old	F [3]	P [4]
Ascomycota							
	Hypocreales [1]	2.5 ± 0.14 [a]	1.94 ± 0.14 [ab]	2.07 ± 0.21 [ab]	1.82 ± 0.15 [b]	3.3	+
Sordariomycetes	*Sordariales* [1]	0.95 ± 0.15	0.78 ± 0.28	0.91 ± 0.18	0.38 ± 0.17	1.6	0.24
	Microascales [2]	3.02 ± 0.44 [a]	0.18 ± 0.04 [b]	0.14 ± 0.05 [b]	0.07 ± 0.02 [b]	29.3	***
	Helotiales [1]	1.32 ± 0.18	0.99 ± 0.17	1.36 ± 0.11	1.16 ± 0.22	1.0	0.47
Leotiomycetes	*Incertae_sedis* [2]	0.68 ± 0.13 [a]	0.22 ± 0.09 [b]	1 ± 0.26 [a]	3.49 ± 0.88 [c]	22.7	***
	Chaetothyriales [2]	0.71 ± 0.24 [a]	1.68 ± 0.43 [ab]	2.12 ± 0.54 [ab]	3.5 ± 0.73 [b]	16.2	**
Eurotiomycetes	*Eurotiales* [1]	0.2 ± 0.11	0.34 ± 0.09	0.25 ± 0.06	0.4 ± 0.08	2.5	+
	Onygenales [1]	0.32 ± 0.11	0.27 ± 0.08	0.26 ± 0.05	0.34 ± 0.09	0.5	0.75
Pezizomycetes	*Pezizales* [2]	6.53 ± 1.94 [a]	3.22 ± 1.39 [ab]	2.56 ± 0.79 [ab]	1.84 ± 0.91 [b]	10.3	*
Dothideomycetes	*Pleosporales*	3.58 ± 0.51 [a]	1.7 ± 0.31 [b]	1.93 ± 0.26 [b]	1.8 ± 0.35 [b]	5.8	**
Basidiomycota							
Agaricomycetes	*Agaricales* [1]	-0.5 ± 0.3 [a]	2.3 ± 0.25 [b]	2.68 ± 0.23 [b]	2.67 ± 0.22 [b]	35.6	***
	Sebacinales [2]	0.06 ± 0.04 [a]	0.25 ± 0.06 [bc]	0.15 ± 0.03 [b]	0.96 ± 0.46 [c]	22.5	***
Tremellomycetes	*Tremellales* [1]	1.57 ± 0.19 [a]	0.95 ± 0.15 [ab]	0.82 ± 0.17 [b]	1.09 ± 0.24 [ab]	2.9	+
	Trichosporonales [2]	0.18 ± 0.08	0.18 ± 0.02	0.28 ± 0.05	0.23 ± 0.04	6.3	0.13
Microbotryomycetes	*Leucosporidiales* [1]	0.6 ± 0.1 [a]	0.07 ± 0.03 [b]	0.05 ± 0.02 [b]	0.05 ± 0.02 [b]	18.0	***
	Sporidiobolales [2]	0.15 ± 0.04 [ab]	0.27 ± 0.11 [ab]	0.15 ± 0.06 [b]	0.38 ± 0.07 [a]	9.2	*
Ustilaginomycetes	*Ustilaginales* [2]	0.07 ± 0.05 [a]	0.64 ± 0.18 [b]	0.36 ± 0.07 [b]	0.55 ± 0.18 [b]	16.2	**
Wallemiomycetes	*Geminibasidiales* [2]	0.03 ± 0.03	0.01 ± 0.01	0.06 ± 0.04	0.9 ± 0.43	5.8	0.16
Zygomycota							
Incertae_sedis	*Mortierellales* [2]	15.45 ± 2.19	15.18 ± 2.14	9.62 ± 1.2	12.68 ± 1.07	6.6	0.12
Incertae_sedis	*Kickxellales* [1]	0.11 ± 0.04	0.24 ± 0.06	0.21 ± 0.04	0.31 ± 0.09	2.4	0.11
Incertae_sedis	*Mucorales* [2]	0.01 ± 0 [a]	0.02 ± 0.01 [a]	0.21 ± 0.15 [ab]	0.25 ± 0.06 [b]	22.0	***
Chytridiomycota							
	Rhizophydiales [1]	0.75 ± 0.17	0.45 ± 0.14	0.37 ± 0.14	0.41 ± 0.09	1.7	0.23
Chytridiomycetes	*Spizellomycetales* [1]	0.15 ± 0.04 [ab]	0.49 ± 0.16 [a]	0.31 ± 0.08 [ab]	0.07 ± 0.03 [b]	6.6	**
	Olpidiales	0.29 ± 0.26	0.5 ± 0.46	0.11 ± 0.04	0.11 ± 0.07	0.5	0.69
Glomeromycota							
Glomeromycetes	*Glomerales* [2]	0.58 ± 0.35 [b]	0.7 ± 0.14 [c]	0.4 ± 0.07 [a]	0.33 ± 0.07 [ab]	7.5	+

*** P < 0.001, ** P < 0.01, * P < 0.05, + P < 0.1, Effect of land management history tested using ANOVA or Kruskal-Wallis test when heteroscedasticity observed. P values adjusted using Benjamini-Hochberg transformation within bacteria and fungi separately. Differences between means were post hoc tested using Tukey or Dunn's test (when Kruskal-Wallis was used). Letters denote differences between means at P < 0.05. [1] Kruskal-Wallis test [2] chi squared [3] ln(y) transformed [4] an outlier removed from the analysis

Table 3.17 Fungal indicator species.

Phylum	Class	Order	Relative abundance (%)					Proportion of indicator species (%)															
			Total	Arable	Grassland regeneration status			Arable indicator species				Grassland regeneration status indicator species											
												Recent				Medium				Old			
					Recent	Medium	Old	A	R	M	O	A	R	M	O	A	R	M	O	A	R	M	O
Ascomycota																							
	Sordariomycetes	*Hypocreales*	9.6	13.75	7.8	10.2	7.0	72	11	6	1	9	27	19	3	3	8	21	11	2	3	6	22
		Sordariales	2.8	2.98	3.4	3.0	1.8	18	2	3	1	5	34	22	6	10	14	38	14	0	11	19	27
		Microascales	0.81	3.02	0.18	0.14	0.071	353	8	3	1	0	4	0	0	1	6	8	2				
		Incertae_sedis	0.24	0.40	0.12	0.21	0.23													0	0	8	17
		Xylariales	0.18	0.33	0.048	0.12	0.24	22	0	5	0									0	0	0	4
		Coniochaetales	0.15	0.075	0.15	0.12	0.23													0	6	12	21
	Leotiomycetes	*Helotiales*	4.0	4.40	3.2	4.2	4.1	65	13	8	4	0	0	0	0	0	0	2	0	0	0	2	38
		Incertae_sedis	1.4	0.68	0.22	1.0	3.5	37	0	1	1									5	9	46	221
	Eurotiomycetes	*Chaetothyriales*	2.0	0.71	1.7	2.1	3.5									0	1	4	1	28	24	37	83
		Eurotiales	0.42	0.34	0.47	0.31	0.55													0	0	0	3
		Onygenales	0.42	0.48	0.37	0.32	0.49	4	0	0	0	0	41	16	5					3	2	5	11
	Pezizomycetes	*Pezizales*	3.5	6.53	3.2	2.6	1.8	125	38	7	0					0	1	1	0	0	1	3	27
	Dothideomycetes	*Pleosporales*	2.2	3.58	1.7	1.9	1.8	128	12	28	24	1	16	6	1					0	3	3	5
		Capnodiales	0.12	0.35	0.055	0.052	0.062	270	33	39	31												
	Geoglossomycetes	*Geoglossales*	0.074	0.010	0.10	0.067	0.11													0	1	15	55
	Archaeorhizomycetes	*Archaeorhizomycetales*	0.10	0	0.15	0.12	0.14					0	83	57	14					0	4	3	52
Basidiomycota																							
	Agaricomycetes	*Agaricales*	13.7	1.04	14.8	19.4	18.5					0	15	2	2	0	3	9	4	0	3	3	31
		Sebacinales	0.36	0.062	0.25	0.15	0.96													0	2	3	69
		Cantharellales	0.20	0.51	0.065	0.18	0.062	108	0	36	0									0	0	4	7
		Trechisporales	0.13	0.0051	0.078	0.24	0.19													0	3	3	56
	Tremellomycetes	*Tremellales*	3.9	5.77	3.0	2.7	4.2	12	2	1	0									0	0	0	1
	Microbotryomycetes	*Leucosporidiales*	0.26	0.92	0.075	0.048	0.055	290	6	1	0												
		Sporidiobolales	0.24	0.15	0.27	0.15	0.38													0	2	9	55
	Ustilaginomycetes	*Ustilaginales*	0.41	0.068	0.64	0.36	0.55													0	33	23	80
	Wallemiomycetes	*Geminibasidiales*	0.25	0.029	0.0075	0.056	0.90													9	3	21	340
Zygomycota																							
	Incertae_sedis	*Mortierellales*	13.2	15.45	15.2	9.6	12.7	47	19	7	1	7	52	16	29					4	8	11	22
	Incertae_sedis	*Kickxellales*	0.28	0.13	0.30	0.24	0.44					1	8	1	0								
	Incertae_sedis	*Mucorales*	0.12	0.0061	0.016	0.21	0.25													0	0	27	100
Chytridiomycota																							
	Chytridiomycetes	*Rhizophydiales*	0.89	1.53	0.82	0.67	0.59	62	0	2	0	0	17	0	0					0	0	1	8
		Spizellomycetales	0.44	0.17	1.1	0.42	0.079					0	235	90	9								
Glomeromycota																							
	Glomeromycetes	*Glomerales*	0.50	0.58	0.70	0.40	0.33					0	17	3	7	0	19	32	13				

Proportion of sum of indicator species at order level to the mean relative abundance of particular order (%): 100 0

3.5 Discussion

Soil OC was depleted in studied soils of cropland and grasslands with recent intensive agricultural practise when compared to grasslands with minimum of 136 years without intensive management suggesting benefits of improvement of management practise or ecosystem restoration for SOC storage recovery. Arable land management affected soil aggregation, reducing larger macroaggregates and resulting in an increase of relative proportion of smaller macroaggregates, free microaggregates and non-aggregated matter while no differences were observed within grasslands. Detailed fractionation revealed that grasslands contained larger mass of microaggregates within macroaggregates than cropland soils. Free microaggregates and microaggregates within macroaggregate showed similar relative contribution of their building blocks towards overall mass. Clay fraction contained large concentration of OC but silt-size fractions contributed the most towards overall OC pool.

Land use history strongly affected bacterial and fungal community structure whereby all sites showed differentiation (or tendency for differentiation in one case) among their microbial communities. Bacterial diversity followed different trends than fungal diversity whereby bacterial diversity indices (Simpson's and Shannon) decreased with land management extensification and time since extensive management implementation. On the other hand, fungal phylotype richness was higher in grasslands with extensive management than in cropland soils. Relative abundance of the main bacterial phyla or their classes showed gradual changes in soils of different land use history such as decrease of their relative abundance (*Acidobacteria*, *Bacteroidetes*, *Chloroflexi*, *Betaproteobacteria* and *Gammaproteobacteria*) or increase of their relative abundance (*Alphaproteobacteria*, *Verrucomicrobia*) from arable sites towards Old grasslands. Similarly for fungi, *Ascomycota* and *Basidiomycota* showed an opposite trend.

3.5.1 Effect of land management history on SOC

Effect of arable land management on SOC

Arable sites had lower concentration of OC in the soil than extensively managed grasslands with at least of 6 years without significant agricultural influence. This confirms the negative role of crop production on SOC which was found locally (e.g. Knops and Tilman, 2000; Baer et al., 2002; Allison et al., 2005; McLauchlan et al., 2006; Duchicela et al., 2013) and also confirmed at the global scale (e.g. Amundson et al., 2001; Guo and Gifford, 2002). Guo and Gifford (2002) showed that a change from land uses including forest and pasture to agricultural land use resulted in a loss of soil C stocks at a rate of 50 % or more. Loss of soil C of up to 89 % has been shown for agricultural land management when compared to a native ecosystem (Knops and Tilman, 2000). The results in the present study showed an average of 41 % reduction of SOC concentration in arable soils when compared to regenerating grasslands with on average of 136 years without intensive agricultural practise. It is likely that if these results were presented on OC stock basis then the difference between arable and grasslands will be lower due to generally higher bulk density with intensive arable practise than restoring grasslands or native ecosystems (e.g. Baer et al., 2002; Scott and Blair, 2017). Overall, the results show a substantial reduction of SOC in arable soils in comparison to less intensively managed ecosystems and thus a potential for SOC recovery after measures including land management practise improvement, land use change or ecosystem restoration to for instance species rich calcareous grassland.

Effect of grassland land management on SOC

Grasslands with a recent history of intensive management practise had on average of 31 % lower SOC concentration than grasslands with an average of 136 years without intensive agricultural practise. This confirms that intensity of land management practise is a strong factor in determining capacity of SOC storage in grasslands (McSherry and Ritchie, 2013; S. Wang et al., 2011; Ward et al. 2016).

Old regenerating grasslands may appear approaching a steady state of soil C. An overall steady state can be characterized by saturation of all soil C pools when those are unresponsive even under a surplus of C inputs to the soil (Six et al., 2002). Old grasslands showed higher litter mass and litter C pool than Medium grasslands suggesting higher organic matter input for Old grasslands. On the other hand, both grassland categories showed similar POM masses as well as SOC concentrations in bulk soil and soil fractions. Due to the higher litter inputs which were not reflected in higher OC pools, the Old grasslands might be approaching a steady state. It can also be that the OC is not at a steady state but is increasing only very slowly.

Furthermore, although the extensive management applied on the studied grasslands is very low (0.25 units ha^{-1}) and have been applied recently to stop shrub encroachment, such a low intensity grazing have been shown to have a negative impact on soil C in moist cool regions globally (Abdalla et al., 2018).

3.5.2 Effect of land management history on soil aggregation

Arable land use decreased soil aggregation. Proportion of soil mass within macroaggregates as well as proportion of soil mass within all separated aggregate classes classified as macroaggregates was lower for arable sites when compared to grasslands of all three regenerating categories. Negative effect of tillage on soil aggregation has been widely documented when tillage crop production systems were compared to the non-tillage systems (e.g. Six et al, 1998) or when arable crop production was compared to native vegetation soils (e.g. Jastrow et al. 1996; Scott and Blair, 2017). Furthermore, an increase of relative proportion of macroaggregates within soil mass was frequently observed in soils recovering from arable land use disturbance after perennial vegetation was re-established by sowing (Jastrow, 1987) or ex-arable land was left to natural succession (Duchicela et al., 2013).

Detailed fractionation of stable macroaggregates into iM-microaggregates and non-aggregated macroaggregate matter revealed that land use affected proportion of iM-microaggregates. Arable soil had lower relative proportion of this fraction within overall soil mass than soil of grasslands. This agrees with other studies where arable sites were compared to native grassland sites (O'Brien and Jastrow, 2013; Scott and Blair, 2017). Microaggregates are understood to be formed within macroaggregates (Oades, 1984; Six et al., 1999; Six et al., 2004). Under arable management, tillage leads to disruption of macroaggregates and increase their turnover resulting in interruption of microaggregate formation and stabilization and also lower amount of associated stabilized POM (Six et al., 1999, Six et al., 2000).

On the other hand, land management history did not affect overall aggregation in grassland soils. Intensively managed grasslands which were transformed into extensively managed grasslands ≈ 6 years ago showed the same aggregation status as grasslands with on average of 136 years without significant agricultural influence. In fact, grasslands at all three regenerating stages did not differ in proportion of soil mass in free microaggregates, macroaggregates and iM-microaggregates.

Aggregation status of Recent and Old grasslands was similar regardless of differences in their bulk SOC concentrations. This shows that soil aggregation is related only to certain soil C pools and not to overall soil C stock, at least at the scale of (hierarchical) aggregates larger than 50 µm diameter. This agrees with Jastrow et al. (1998) who showed that major improvement in aggregate stability can be achieved without significant increases in OC after land use change from arable cropland to restoring grassland. Soil aggregation was found to recover relatively quickly when tillage was ceased and perennial vegetation was re-established as determined by the proportion of soil mass within free microaggregates, iM-microaggregates and macroaggregate matter (O'Brien and Jastrow, 2013). While only encompassing a proportion of total SOC stock, these C pools important for aggregate stability might be the most vulnerable when aggregates are disrupted.

Both microaggregate classes (free microaggregates and iM-microaggregates) showed similar pattern of relative presence of their building blocks (coarse silt, fine silt, clay and POM) (Figure 3.10) as well as OC concentration in these building blocks within land management history. This suggests that these microaggregates, were formed and stabilized by the same mechanism within each land use type. This agrees with understanding that microaggregates are formed within macroaggregates and then released into the soil after macroaggregate disintegration (Oades, 1984; Six et al., 2000, 2004). Furthermore, arable land use should have fewer microaggregates due to tillage disruption of their formation within macroaggregates. However, sum of proportions of mass of both microaggregate classes (free microaggregates and iM-microaggregates) did not show a difference between arable and grassland soils. This may suggest that another mechanism is responsible for free microaggregate formation and stabilization under arable management. Microaggregates were originally considered relatively stable soil components (Oades, 1984), but it has been shown that their turnover is less than 100 days (De Gryze et al. 2006). These results imply that if microaggregates turn over regularly (Virto et al., 2010) but their primary formation mechanism (i.e. within macroaggregates) is inhibited by fast macroaggregate turnover under arable land use (Six et al., 2004), they must be formed by an alternative mechanism under arable land use in order to achieve same mass of whole microaggregate pool under arable land use and regenerating grasslands as shown in the present study.

3.5.3 Coarse and fine silt fractions

Land management history effect on coarse and fine silt mass

Land use (arable vs. grassland) affected relative mass of building blocks of microaggregates and specifically building blocks related to silt fraction such as coarse and fine silt. Grassland microaggregates showed a pattern of higher proportion of coarse silt (nearly all sites for both microaggregate classes) and lower proportion of fine silt (significant only for Old grasslands) when compared to the pattern in microaggregates under arable land use (Figure 3.10). Land use thus did not affect mass of overall microaggregate pool but affected microaggregate composition. Virto et al. (2008) showed that silt fractions (coarse as well as fine silt-sized fractions) extracted from fully dispersed mineral soil contained sub-fractions such as non-occluded micro-POM (< 50 µm diameter), non-occluded minerals (clay and sand) and stable silt-sized microaggregates. They dispersed microaggregates by a relatively mild procedure such as shaking with metal balls for four hours. The silt-sized microaggregates were further separated into mineral fraction (silt and clay) and micro-POM using higher disruption energy. It is likely that the silt fractions separated in the present research are in fact composite fractions containing non-occluded POM, non-occluded mineral fractions, and silt-sized microaggregates similar to Virto et al. (2008). It can be further speculated that the negative impact of arable land use on the mass of coarse silt sub-fraction is due to lower delivery of micro-POM to both silt sub-fractions. Tillage was found to reduce microaggregate formation (Six et al., 1998, 1999) and thus stabilization of fine POM fraction. Such increased

availability of fine POM to soil decomposers might result in its greater mineralization resulting in lower micro-POM mass. This micro-POM can be incorporation into silt fraction as occluded micro-POM (Virto et al., 2008, 2010). Thus lower coarse silt proportion within microaggregates might be related to lower delivery of micro-POM due to arable land use. Increased fine silt fraction observed in arable soil can be building blocks which would have been used for micro-POM stabilization resulting in coarse silt-sized aggregates but which were not utilized because of lower availability of micro-POM. These building blocks can be fine silt-sized microaggregates which do not contain micro-POM but are formed by mineral-OC associations (Lehman et al., 2007; Totsche et al., 2018).

It would also imply that due to the hypothetical lower delivery of micro-POM resulting from negative effect of arable land use, there would be less fine POM found within microaggregates as the micro-POM should originates from larger POM fractions. However, this was not the case and there was no difference in fine POM found after dispersion of microaggregates between different land uses (arable vs. grassland). Fine POM fraction was separated from microaggregates following method presented by O'Brien and Jastrow (2013) when microaggregates were fully dispersed by ball-shaking and fine POM was separated by wet sieving through 50 µm diameter sieve. Such fraction should also contain sand component but it was not separated and may have obscured differences between true fine POM mass associated with different land uses. Furthermore, arable soils contained more sand mass (0.25 – 1 mm diameter) associated with non-aggregated and intra-Macroaggregate matter than grasslands ($F_{3,16} = 14.5$, $p < 0.05$, post-hoc comparisons $p < 0.05$). It is likely that the fine POM fraction for arable soils might contain higher amount of mineral fraction in comparison to grasslands, and potentially resulting in lower fine POM in arable microaggregates than grasslands.

Land management history effect on coarse and fine silt OC concentration

Organic C concentration in coarse and fine silt fractions were higher for Medium and Old grasslands than for arable sites in free microaggregates and iM-microaggregates, and further for free microaggregates and coarse silt, Old grasslands were higher than Regenerating grasslands in their OC concentration. It must be pointed out that C concentration associated with mineral soil fractions was analysed only for microaggregates (free and i(M)- microaggregates), and it can be suggested that these fractions are the most important due to the highest soil mass allocated in these fractions (e.g. Jastrow and O'Brien, 2013). This lower amount of OC associated with coarse and silt fractions for arable land use might be related to the hypothesized lower amount of micro-POM in these fractions. POM associated with silt-sized fractions was shown to have higher OC concentration than OC associated with mineral fractions (Virto et al. 2010), thus its absence should lower overall OC concentration. On the other hand, it has been also shown that most of OC in silt-sized fractions is associated with mineral fractions (due to higher mass of mineral fraction than POM) and in particular clay fraction (Virto et al., 2010), thus OC associated with clay minerals might be relatively more important to differences between the land uses than OC associated with POM. In the present research, clay fraction OC concentration was analysed only for free microaggregates, and OC concentration was higher for clay than for coarse or fine silt sub-fractions. Thus the main reason for lower OC in arable soils might be due to lower OC associated with clay minerals such as those that are un-occluded and also those which are contained in the hypothesized silt-sized aggregates. Virto et al. (2010) further showed that clay within silt-sized aggregates contained higher OC concentration than un-occluded clay.

Organic C associated with clay minerals might be delivered from recent plant primary productivity through rhizodeposition (Hannula et al., 2018) and degradation of POM (Cotrufo et al., 2015). Such OC form can be associated with clay minerals through mechanisms including (i) incorporation via microbial activity and (ii) adsorption of soluble compounds (Virto et al., 2010). Adsorption of organic compounds onto mineral surfaces was proposed to be more important mechanism of

microaggregate formation than POM occlusion (Lehman et al., 2007). Thus, lower delivery of soluble C compounds from recent plant productivity, which can differ between different land uses, might affect stability of silt-sized aggregates, however this hypothesis warrants further investigation (Virto et al., 2010). It would also imply that delivery of organic compounds for stabilization might be affected by soil microorganisms, as they are largely responsible for transport of recent plant rhizodeposits (Hannula et al., 2018) and for transformations of OC in the soil including efficiency of this process (Malik et al., 2018).

3.5.4 Effect of land management history on microbial community

Microbial diversity

Diversity indices of soil bacterial and fungal community showed different dynamics in dependence of land management history. Bacterial diversity indexes (Simpson's, Shannon) were higher on arable sites in comparison to the grassland sites. Trivedi et al. (2017) found higher bacterial diversity (Shannon) on agricultural sites compared to natural ecosystems in temperate regions in a global meta-analysis. Barber et al. (2017) found decrease of bacterial diversity when a cropland was restored by reseeding plant species of a native prairie. Kuramae et al. (2011) found higher bacterial diversity in soils of arable land use and early successional grasslands than in older grasslands aged over 66 years since abandonment. Bacterial diversity increased after land use change from grassland management to cropland in a European survey (Szoboszlay et al., 2017). These studies together with the present result suggest a positive effect of arable management on bacterial diversity, however this effect may be only present in temperate and arid regions but not in continental and tropical regions (Trivedi et al., 2017). Upchurch et al. (2008) speculated that bacterial diversity in agricultural soils is promoted by higher seasonal and plant variability and increased immigration opportunities for air borne bacteria. Agricultural management promotion of soil bacterial diversity may be delivered through greater bio-physical and chemical heterogeneity in arable system than in natural systems, which is a result of high fluctuating environment in arable soils, characterized by high variable resource gradient (spatial and temporal changes in soil physical and chemical properties) (Trivedi et al., 2017). Contrary to the preceding, Jangid et al. (2011) showed that bacterial diversity did not change after arable cropland restoration to grassland or forest land use. Furthermore, Jangid et al. (2008) determined higher bacterial diversity in pasture soils than in highly disturbed cropland and less disturbed forest soils.

In contrast to diversity, bacterial richness (i.e. number of OTUs) was not affected by land management history. Similarly, French *et al.* (2017) showed no difference in bacterial richness of arable sites in comparison to never tilled unimproved grasslands and restored grasslands (Oxfordshire). Barber et al. (2017) found lower bacterial richness, while Kuramae et al. (2010) found higher bacterial richness for older regenerating grasslands in comparison to arable fields. Bacterial richness was partially related to changes in soil nitrate, P and soil pH (Kuramae et al., 2010). Karimi et al. (2019) showed an increase of bacterial richness as well as diversity (Shannon) with increasing agricultural intensity forest < grassland < cropland = vineyards based on sampling around whole France.

Fungal richness was lower in arable soils compared to grassland soils, while diversity (Simpson's, Shannon) did not change. Similarly to the present research, French et al. (2017) found lower fungal taxonomic richness on arable fields in comparison to grassland sites. The observed reduction of fungal richness on arable sites can be partially attributed to a negative effect of tillage on fungi which was shown when tillage and no-till cropping systems were compared. Fungal community is thought to be susceptible to agricultural disturbance due to tillage physical disruption of hyphal

network, disturbance of the plant root colonization by arbuscular mycorrhizal fungi, or lack of substrate for specialized saprotrophic basidiomycetes (Plassart et al., 2008). Others found concurrent changes of fungal richness and diversity during secondary succession. Yang et al. (2017) found lower fungal Chao's richness (but not number of OTUs) and diversity (Shannon but not Simpson's) in arable soils than in regenerating grasslands in Loess Plateau in China and the indexes were higher in native vegetations (grasslands, forest) than in soils of regenerating sites. They further found that fungal diversity was related to plant diversity and SOC regardless of land use type. Zhang et al. (2017) did not observe a change of fungal OTU richness in successional grassland soils in Loess Plateau, while fungal diversity was higher in older grassland soils (20 and 30 years old) than in arable soils.

Overall, results of these studies suggest tendency for higher bacterial richness and diversity in soils with intensive agricultural management, although variation may be present at some location, which was the case of the present study that determined only changes in diversity but not in richness. On the other hand, former studies suggested an opposite pattern for soil fungal diversity indices in relation to effect of intensive agriculture which was also confirmed by the present study in the case of fungal richness. Higher diversity might be important for continuation of soil functioning under a variation of environmental conditions (i.e. insurance hypothesis, Yachi and Loreau, 1999). Indeed, Tardy et al. (2014) demonstrated reduction of functional stability with reduced microbial diversity and Louis et al. (2016) showed diversity indices can be used to explain differences native OC mineralization. As such, changes in microbial diversity may have consequences for soil function.

Bacterial community composition in the soil

Phylum *Proteobacteria* did not change its relative abundance with age of grasslands and in comparison between grasslands and arable sites. This is in contrary with Trivedi et al. (2017) who showed an increase of relative abundance of *Proteobacteria* in natural ecosystems in comparison with agricultural land. Nevertheless, class *Alphaproteobacteria* increased its relative abundance in grasslands comparing to arable sites and also towards Older grasslands. This is similar to Kuramae et al. (2010). *Alphaproteobacteria* is known to be associated with plant roots when for instance Thompson et al. (2013) showed that removing plants decreased relative abundance of *Alphaproteobacteria* in comparison to control grassland plots. Its increase might reflect greater root biomass which would be expected for a permanent grassland compared to annual plant cropping system (e.g. DuPont *et al.*, 2014).

Alphaproteobacteria is generally considered copiotrophic species and was found to increase in sites with N and P addition in grasslands globally (Leff et al., 2015). In the present study, it increased its relative abundance on extensively managed grasslands (incl. sites with over 100 years without significant agricultural influence) compared to arable sites. It can be anticipated that the arable sites would have more available mineral N and P than these extensive grasslands. Thus, the increase of *Alphaproteobacteria* with grassland age may point to another driver of its abundance in these soils. Relative abundances of orders *Rhizobiales* and *Rhodospirillales* (the most abundant *Alphaprotebacterial* orders, not tested statistically) increased for older grasslands (not significant for *Rhizobiales* however). Both orders include representatives capable of non-symbiotic N fixation (Anderson et al., 2011; Angel et al., 2016; Tsoy et al., 2016). Their dynamics in relation to land use history might be related to an effect of land use disturbance related to agricultural management as was suggested by Zhalnina et al. (2013) who found relative abundance of *Bradyrhizobium* (*Rhizobiales*) lower in agricultural soils than non-agricultural soils. Moreover, relative abundance of order *Sphingomonadales* (*Alphaprotebacteria*) which also possess N fixation capabilities (Angel et al., 2016) had higher relative abundance in arable soils than in grasslands in the present study. Members of this order were found in an extreme site such as soil biological crust in a desert (Angel et al.,

2016), which may suggest that this taxa may be more resilient/resistant to a disturbance or stress, as is also present in agricultural soils. This may explain higher relative abundance found in the present study. However, *Bradyrhizobium* members were also found in biological crust of deserts (Lester et al., 2007) which may suggest that the hypothesized stress tolerance may be phylogenetically conserved on lower than order taxonomic resolution.

Relative abundance of phylum *Acidobacteria* was affected by land use history when its relative abundance was lower for older grasslands when compared to arable sites and Recent grasslands. In contrary to the present research, relative abundance of *Acidobacteria* was higher in natural ecosystem than in agricultural sites in the global scale (Trivedi et al., 2017) and also was found to increase on restoring grasslands with age since beginning of restoration (Barber et al., 2017). Trivedi et al. (2017) argued that despite this phylum is generally thought as oligotrophic, their higher abundance in natural systems might be also related to decrease of soil pH towards natural ecosystem. Soil pH was found to be strongly associated with *Acidobacterial* relative abundance in a continental scale (Lauber et al., 2009). In the present research, *Acidobacteria* was dominated by subgroups 4 (class *Chloracidobacteria*) and 6 (class *Acidobacteria-6*). This composition reflected alkaline soil pH of the studied ecosystems because these two subgroups were found to increase their abundances with soil pH increase (Griffiths et al., 2011; Kielak et al., 2016). *Acidobacteria* is usually associated with oligotrophic lifestyle, potentially due to its frequent association with low soil pH and low soil pH is frequently associated with low nutrient sites. Increasing abundance of *Acidobacteria* with decreasing age since intensive management cessation towards arable sites in the present research, may suggest that subgroups 4 and 6 exhibit somewhat copiotrophic lifestyle. Indeed, Kielak *et al.*, (2016) reported that these subgroups appeared to be abundant in high nutrient-rich soils. Arable sites generally have high availability of N and P. This may suggest that the observed high relative abundance of *Acidobacteria* in arable sites in comparison to regenerating grasslands and decreasing relative abundance of *Acidobacteria* with the age of grasslands reflect the decreasing nutrient availability in the soil while high soil pH prevent immigration of other subgroups of *Acidobacteria*, more often found in acidic nutrient poor conditions.

Relative abundance of *Verrucomicrobia* was higher in soils of older grasslands than in arable soils and it was also higher in soils of Old grasslands than Recent and Medium grasslands. Kuramae *et al.* (2010) observed a similar response of relative abundance of *Verrucomicrobia* in cropland soils in comparison to regenerating grasslands. Szoboszlay *et al.* (2017) showed that *Verrucomicrobia* responded to land use change whereby its relative abundance increased when cropland was changed to grassland and vice versa. Bergmann *et al.* (2011) found that *Verrucomicrobia* had relatively high abundance in humid grasslands compared to other biomes including agricultural land, however their selection of biomes was not representative. They also showed that *Spartobacteria*, representing the most abundant class of *Verrucomicrobia* globally, had lower abundance in agricultural fields in comparison to grasslands, while subdivision 3 [Pedosphaerales] and *Opitutate* rose in agricultural fields, similar to Barber et al. (2017). This is the same trend as observed in this study. These studies together with the present data show that *Verrucomicrobia* is negatively impacted by intensive agricultural management. Barber et al. (2017) showed relatively slow recovery of *Spartobacteria* during ecosystem regeneration (27 year lag phase until its noticeable abundance) which was also shown in the current study. After 6 years of restoration management (Recent grasslands), this phylum was still showing lower relative abundance when compared to Old regenerating grasslands. Similarly, (Hirsch et al., 2017) found no recovery of *Verrucomicrobia* during a period of 3 years after bare fallow or cropland was converted to grassland, however land use change in the opposite directions showed decrease of its relative abundance.

These studies are in contrary to a global study where relative abundance of *Verrucomicrobia* was higher on agricultural land in comparison to natural ecosystem in temperate continental and also

tropical regions (Trivedi et al., 2017). This would suggest that the most abundant *Verrucomicrobial* classes are those ones associated with agricultural soils such as subdivision 3 [Pedosphaerales] and Opitutate (e.g. Bergmann *et al.*, 2011; Barber et al., 2017), however it was suggested that *Spartobacteria* is the most abundant *Verrucomicrobia* class globally. *Spartobacteria* was also showed to be the most abundant *Verrucomicrobia* class in the present research (not tested statistically). Navarrete *et al.* (2015) speculated that *Verrucomicrobial* taxa associated with low nutrient soils are oligotrophic species and those in soils of optimal fertility are copiotrophic species based on their study of tropical soils of different fertilities. Cultured representative *Chthoniobacter flavus* (class *[Chthoniobacterales]*, phylum *[Spartobacteria])* was suggested to respond rapidly to changes in substrate availability and can grow on sugars, sugar polymers and pyruvate (Hirsch et al., 2017). This can be characterized as a copiotrophic life-style (Fierer et al., 2007). Nevertheless, (Brewer et al., 2016) characterized this taxa as slow-growing instead. They also argued, that other *[Spartobacteria]* member, phylotype DA101, belongs to the most dominant phylotypes worldwide. Hirsch et al. (2017) showed a decrease of its relative abundance after grassland was changed to bare fallow, which may be attributed to lack of root exudates in bare fallow, and as it prefers soils receiving elevated amounts of labile C inputs (Brewer et al., 2016). Detailed characterisation of *Verrucomicrobia* phylotypes may shed more light into its response to land use change and ecosystem recovery.

Relative abundance of phyla *Chloroflexi* and *Bacteroidetes* were higher in arable soils than in grassland soils, a trend which was also seen for classes and orders belonging to these phyla. In accordance to these results, Trivedi et al. (2017) found generally higher relative abundance of phylum *Chloroflexi* in agricultural soils than in natural ecosystem soils over all biomes tested. Similarly, high representation of *Bacteroidetes* in agricultural and early transitional grasslands sites was observed on two chalk soil sites in Netherlands (Kuramae et al., 2010).

Fungal community in the soil

Ascomycetes represented the most abundant fungal phylum and it decreased on grasslands in comparison to arable sites. Leff et al. (2015) observed higher relative abundance of phylum *Ascomycetes* in N and P fertilized grassland soils worldwide. Thus the observed increase of relative abundance of some classes of *Ascomycota* on arable sites compared to grassland sites may be attributed to a positive response of these classes to anticipated N and P additions as fertilizers on arable sites (as it is a common practise).

Basidiomycetes increased its relative abundance on grassland sites in comparison to arable sites. Overall relative abundance of *Basidiomycetes* showed strong underrepresentation in comparison to its mean global relative abundance (Tedersoo et al., 2014), which was due to low relative abundance of class *Agaricomycetes.* Relative abundance of order *Agaricales*, the most abundant order in class *Agaricomycetes*, increased in grasslands compared to arable sites in the present research. This can be seen as a similar trend to Lauber *et al.* (2008) who found its increase on pastures and towards forest when compared to cropland.

Both most abundant fungal phyla showed opposite trends whereby relative abundance of *Ascomycota* was higher and relative abundance of *Basidiomycetes* was lower in arable soils in comparison to regenerating grassland soils. Others have observed similar trend in reaction of these fungal phyla to ecosystem regeneration or a change of land use to intensive agriculture. Relative abundance of *Ascomycota* and *Basidiomycetes* in the soil decreased and increased respectively for native forest with grassland understorey when compared to intensive grasslands (Yan et al., 2018) or when native tropical forest was transformed to intensive plantations (Brinkmann et al., 2019). Yan et al. (2018) suggested (based on their results and those of others) that representation of *Ascomycota*

and *Basidiomycetes* within the fungal community can represent an indicator of ecosystem degradation, but further tests are needed to confirm this. On the other hand, Yang et al. (2017) observed higher relative representation of *Basidiocycetes* than *Ascomycota* in arable soils in Loess Plateau, and *Ascomycota* showed higher relative abundance for restored and native grasslands or forest when compared to the arable sites. Similarly, *Ascomycota* increased relative abundance within restoring grassland chronosequence in Loess Plateau and *Basidiomycetes* showed opposite trend, however arable sites showed relative abundances in between those of grasslands of 40 and 50 years old (Zhong et al., 2020). In a natural succession chronosequence of 30 years on Loess Plateau, *Ascomycota* accounted for 50 % of OTUs and did not change in relation to successional development (Zhang et al., 2017). These studies suggest that trends of relative abundances of *Ascomycota* and *Basidiomycota* to land use and ecosystem restoration are complex might not represent a degree of ecosystem restoration as suggested by (Yan et al., 2018).

In conclusion, comparison of intensive agricultural land use with regenerating grasslands of different periods under extensive management showed a negative impact of agriculture on soil structure and SOC as well as an impact on microbial community structure. Alteration of aggregate structure was marked at higher aggregation level (macro- and micro- aggregation) but also at the very fine level such as silt. Mass of silt-size fractions of different sizes (coarse and fine) within microaggregates (both free- and intra-macroaggregate- microaggregates) showed opposite trends when croplands and grasslands were compared. Differential dynamics of OC pools associated with the two silt size fractions separated (coarse and fine silt) was suggested by the data, and however a more research is needed in order to prove some of these trends significant, it may show that if the overall soil OC stock is approaching equilibrium, its small part may be still gaining C such as OC associated with coarse silt fraction. These results are lending suggestion that mass of organic C pool of these silt sub-fractions (or their ratio) can be used as a tool for monitoring of soil structure/SOC recovery during ecosystem regeneration after land use change. Soil microbial community is a key driver of transformations of organic matter in the soil, and as such largely responsible for fate of C derived from primary production. Its composition can be strongly affected by land management, as found here and elsewhere, however course and time of its recovery is not fully understood. The present research showed increase of relative abundance of phylum *Verrucomicrobia* during grassland regeneration suggesting this phylum as a key indicator of grassland recovery from disturbance by intensive land use. Its relatively slow recovery during the regeneration as observed here by also by Barber et al. (2017) may point towards its greater dependence on overall ecosystem recovery such as overall microbial community or soil structure and/or associated organic C pools. Future research thus may address microbial community diversity at the microscale as well as its relationship to regeneration of soil aggregation from intensive land use to further understanding of drivers of soil aggregation and organic C stock recovery.

Chapter 4: **Response of grassland soil biotic and abiotic characteristics and soil and ecosystem processes related to C, N and P cycles to manipulation of soil pH**

4.1 Introduction

Grassland management is traditionally aimed at enhancing a single ecosystem service such as yield (Carolana and Fornara, 2016). Recent interest in provision of multiple ecosystem services from agroecosystems brings new challenges for land managers in achieving these multiple goals. For instance, apart from food delivery, grasslands are frequently gaining interest for their potential of C sequestration (Conant et al., 2001). Increasing soil pH through liming is a typical practice for improving grassland yield quantity and quality on acid soils (Holland et al., 2018). Although liming was shown to promote SOC sequestration and thus it can be an important element in the effort to reduce atmospheric CO_2 (Fornara et al., 2011), it has also been associated with no effect on SOC or even its decrease (Paradelo et al., 2015). SOC stock is a result of a variety of processes and interactions within ecosystem components including plants and microbes. Microbial processing of plant inputs and OC already stored in the soil is the key driver of SOC (Schimel and Schaefer, 2012). Mechanistic understanding of a relationship of soil pH with soil microbes and processes they drive should be thus beneficial for improvements of grassland management practices aiming at multiple ecosystem services delivery and especially promotion of SOC.

This chapter aims at studying soil nutrient cycles in grassland as affected by soil pH modification where liming is the key treatment apart from soil acidification.

4.1.1 The effect of soil pH on microbes

Soil abiotic factors have been shown to strongly affect soil microbial community structure (e.g. Lauber et al., 2008) (Figure 4.1). Considering microbial size, soil represents immediate environment for soil microbial community within which different microbial populations thrive based on their ecophysiological traits. It has been widely documented that soil pH is a strong factor structuring microbial communities as observed across different spatial scales and taxonomic resolutions within a community (Lauber et al., 2009; Rousk et al., 2010; Griffiths et al., 2011; Tedersoo et al., 2014). Bacterial diversity was shown to respond to soil pH whereby it peaked at near neutral pH (Lauber et al., 2009) or increased linearly with soil pH (Griffiths et al., 2011). Fungal community was suggested to be less responsive to soil pH and fungi were suggested to have wider soil pH optima (Rousk et al., 2010). Bacterial phylum *Acidobacteria* is frequently highlighted as the most responsive phylum in relation to pH gradient from acidic to alkaline levels (e.g. Griffiths et al. 2011), however at lower taxonomic resolutions, orders of this phyla react to soil pH differently. Lauber et al. (2009) showed that also other phyla responded to soil pH including *Bacteroidetes* and *Actinobacteria*. Bacteria respond to soil pH also at a level of functional types when bacterial GP:GN ratio increased with soil pH (Frostegård et al., 1993; Fornara et al., 2011).

Soil pH effect on microbes has been linked to microbial ecophysiology whereby microbial communities at distinct pH levels resulted in different abundancies of particular genes (Malik et al., 2018). Specifically, low pH microbial community was enriched in energy processing and membrane proton transporter genes, while high pH community was enriched in genes coding metabolism and nitrate transformations. This results in distinct microbial functional attributes in relation to soil pH such as that in lower pH soils microbial investment preference is into strategies to survive stressful

acidic conditions represented by maintenance respiration, while at higher soil pH, microbes can invest more resources to growth (Malik et al., 2018).

4.1.2 Effect of soil pH on SOC dissolution

Soil pH have been shown to affect solubility of soil OC whereby the solubility was enhanced after raising the pH (Bolan et al., 1996, but see Bolan et al., 2003; Curtin et al., 1998; see Kemmit et al., (2006) for summary of other studies) (Figure 4.1). Solubilisation of SOC counterbalances changes in dissolved cations after application of a base and it maintains soil equilibrium (Curtin et al., 2016). On the other hand, decrease of soil pH can reduce solubility of SOC by affecting clay minerals by increasing complexation of SOM with metal ions released with pH decrease.

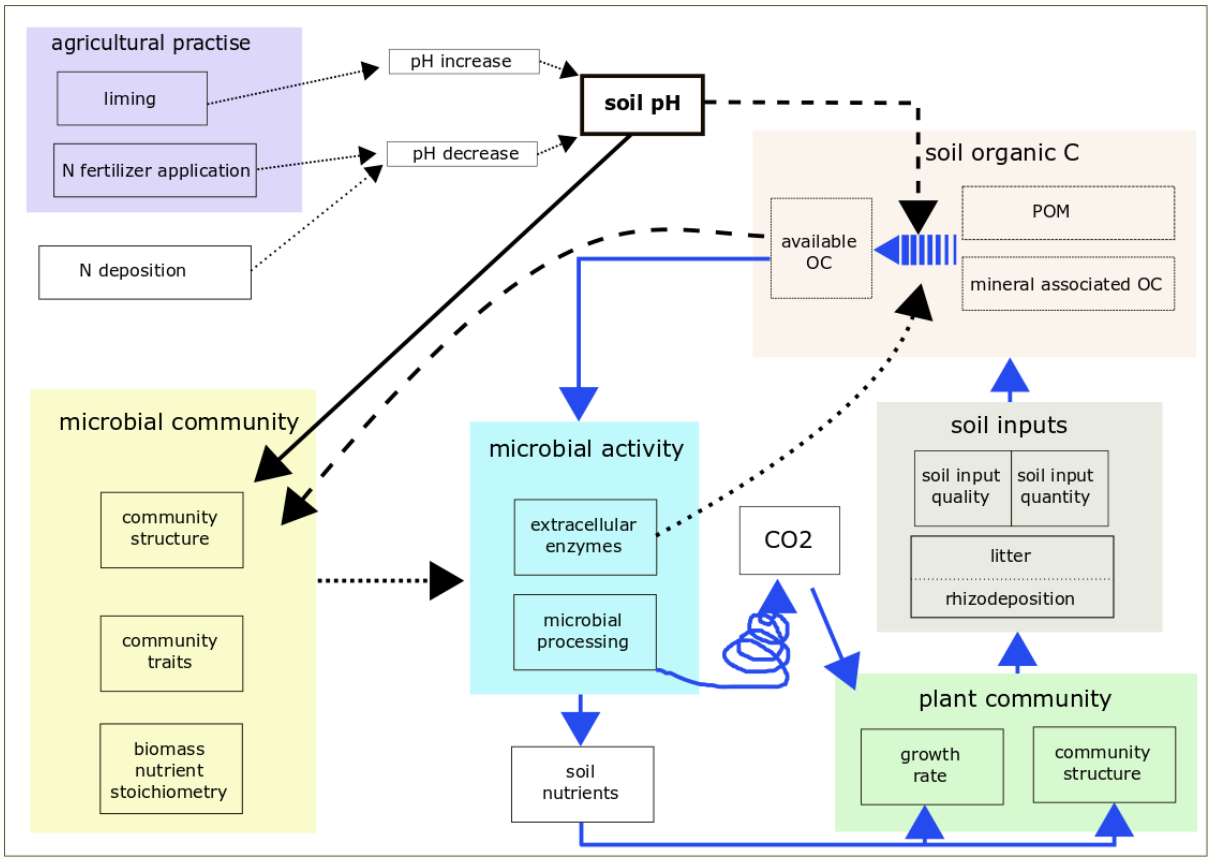


Figure 4.1 Direct and indirect effects of soil pH change on microbial community and its functioning. Solid line denotes direct effect of soil pH on microbial community. Dashed lines denote indirect effect of soil pH on microbial community through an effect on dissolution of SOC (blue dashed line) resulting in modification of available substrates for microbial processing. Blue lines denote simplified ecosystem nutrient cycle: microbes process substrates which become available through an effect of physical or chemical (e.g. dissolution of SOC under high pH) or biological (e.g. extracellular enzyme action) factors while they release excessive nutrients into the soil (based on substrate stoichiometry and microbial requirements) for plant uptake driving plant primary production (and potentially also community structure such as effect of excess of mineral N); plant primary production impacts inputs of organic matter into the soil (quality and quantity) for microbial processing and stabilization within soil structure.

4.1.3 Effect of soil pH on microbial community functioning

Microbes are key for nutrient transformations in the soil (Schimel and Schaefer, 2012) (Figure 4.1). At a community level, microbes can respond to alteration in environmental conditions by changes in their community structure driven by an increase of traits which are better suited to new conditions (Wallenstein & Hall, 2012). These changes in community traits can affect microbial function through functional trade-offs because these trade-offs can affect recourse use efficiency (such as C use efficiency (CUE)) (Malik et al., 2019). CUE represents an amount of energy and substrates invested in progeny vs. transformed and exuded as various compounds or mineralized and released into the atmosphere (Geyer et al., 2016). As such, an environmental stress (such as low soil pH or lack of available substrates) would result in greater channelling of resources into stress response than to growth. Therefore, microbial metabolism as affected by changes in environmental conditions can directly control amount and form of OC compounds available for stabilization within the soil matrix.

Soil pH can also affect microbial functioning indirectly through changes of availability of substrates for processing. At the individual level, a set of microbial traits emerges as a life strategy. Microbial life strategies can be characterized according to microbial response to different levels of nutrients. Copiotrophic microorganisms are selected in more resource-rich environments whereas oligotrophic microorganisms are adapted to growth under resource-poor environments (Koch, 2001; Fierer et al., 2007; Roller and Schmidt, 2015). Oligotrophic microbes are expected to have higher CUE than copiotrophic microbes (Roller and Schmidt, 2015).

Additionally, quality of available substrates such as chemical composition and stoichiometry is an important determinant of the direction of microbial processing with consequences for CUE and availability of soil nutrients (Mooshammer et al., 2014; Roller and Schmidt, 2015). Whilst microbial biomass stoichiometry has been shown to be relatively strongly constrained (Cleveland and Liptzin, 2007), imbalances between stoichiometry of microbial biomass and available substrates would most likely result in an adjustment in substrate use efficiency releasing element in excess (Mooshammer et al., 2014). Excess of C would thus be released through microbial respiration of C compounds while excess of N within substrates brought into cells will be shown as its excessive mineralization and release into the soil.

4.1.4 Soil extracellular enzymes

Extracellular enzymes (EEs) are principal agents of initial processing of complex soil substrates before simpler compounds can be brought inside of cells and utilized (Allison and Vitousek, 2005). Microbes allocate resources and energy to EEs synthesis and their release in order to enhance availability of limiting nutrients based on their growth requirements (Allison et al., 2011). Shifts in activities of EEs (such as changes in their ratios) can thus indicate changes in availabilities of nutrients in the soil due to environmental conditions and/or land management alterations (Ekenler and Tabatabai, 2003). Studying enzymatic ratios along a gradient or following a change can help to understand soil processes and elucidate their drivers (Cenini et al., 2015; Nottingham et al., 2015).

Soil pH have been found to affect EEs activities as determined by EE assays (EEAs) (Acosta-Martínez and Tabatabai, 2000), however relationship of EEs with soil pH is complex (Hendriksen et al., 2016). Glycosidases, enzymes involved in soil C cycle, have pH optimum at 5 ± 1 (Sinsabaugh et al., 2008). They have been found to respond positively to soil pH increase from 5 to 7 as induced by liming whereby β-glucosidase (BG) was the most pH sensitive and α-glucosidase (AG) was the least pH sensitive enzyme. BG is typically showing the greatest activity of glucosidases studied in the soil (e.g. Hendriksen et al., 2016) which together with its role in cellulose degradation can help in understanding dynamics of plant derived organic matter in the soil. Cenini et al. (2015) showed that

liming decreased BG activity due to potential changes in relative availability of C and N from decomposing organic substrates (i.e. higher C availability resulting from increased root C:N ratio on limed plots and under N-only fertilization treatments only). Employment of EEAs can thus help in understanding drivers of soil nutrient cycles.

4.1.5 Effect of soil pH on SOC and other ecosystem parameters

It will be important to know what the change of soil pH means for soil C storage. Studies related to an effect of liming of grasslands on SOC did not bring conclusive results as reviewed by Paradelo et al. (2015). For instance, Fornara et al. (2011) studied grasslands with over 100 years of history of liming (Park Grass experiment, UK) and found that SOC increased on limed plots whereby the increase occurred primarily in organo-mineral associated C fraction. The increase could not be attributed to increased plant productivity as hay yield was similar regardless of liming. Limed soils showed lower soil C:N ratios suggesting greater microbial processing of plant residues. Bacterial community has changed as demonstrated by a decrease of GP:GN ratio which was correlated with increase of organo-mineral associated C and soil respiration among other parameters. Research performed at another long term experimental field (Nash's field, UK, over 19 years of liming treatment) showed no effect of liming on total SOC stock or C concentration in soil fractions but it resulted in greater C pool of small macroaggregates (250 – 2000 μm diameter), microaggregates and silt+clay fraction due to mass redistribution from large macroaggregates (Egan et al., 2018). The liming also increased soil mineral N (NO_3-N) (Heyburn et al., 2017b).

Increased soil N availability due to impacts of liming has a potential to affect plant community. N is typically a limiting factor of NPP and its increase can positively impact on NPP (Suding et al., 2005; LeBauer and Treseder, 2008). Furthermore, soil N increase can affect interactions among plant species as those species which are more positively responsive to N availability might supress growth of less or negatively responsive species and this can shift plant species composition towards more 'nitrophilous' community (Bobbink et al., 2010). It can be also assumed that plants growing in soil with higher N levels will respond by changes in their biomass nutrient stoichiometry as was shown by Fornara and Tilman (2012). They also demonstrated that plants can modify their biomass allocation between aboveground and belowground plant compartments after soil N increase. Heyburn et al. (2017b) observed an increase of aboveground biomass yield and C:N ratio and decrease of root biomass yield without a change in root C:N ratio on limed grasslands. Lochon et al. (2019) observed no response of plant above- and below- ground biomass growth and decrease of aboveground biomass C:N ratio on limed plots in a short term (2-year) study.

These changes in plant traits in response to liming can have impacts on soil nutrient cycling and C sequestration through alteration of quality and quantity of soil inputs (De Deyn et al., 2008). Heyburn et al., (2017) showed increased root decomposability and N mineralization in decomposing roots sampled from limed grasslands. Soil respiration would also respond to liming as a result of microbial activity change in response to substrate quantity and quality alterations. Plants on limed plots had higher photosynthetic rates as deduced from increased ^{13}C label in the shoot biomass (Rangel-Castro et al., 2004). This label was found to decrease faster in limed plots suggesting faster allocation of recently photosynthesized C to roots or faster shoot respiration. They argued that limed plants lost the label from the roots faster than those on un-limed plots if the label was preferentially allocated to the roots and not respired by aboveground plant part. Greater microbial utilization of recent plant photosynthates may promote SOC storage through microbial biomass production and its stabilization in the soil (Liang et al., 2017) as well as it can promote SOC decomposition resulting from priming effect (Kuzyakov, 2010). Johnson et al. (2005) showed higher root colonization by AMF on limed plots which might suggest that loss of recent photosynthate C from roots on limed plots as

observed by Rangel-Castro et al. (2004) is related to its greater allocation to microbial food web through fungal network connected with roots.

Measurements of C flux after pH manipulations are rare. Lochon et al. (2018) did not find effect of liming on *in situ* soil respiration in a 2-year grassland study. Egan et al. (2018) found lower in situ soil respiration in May but not in Jun and Jul on 22-year limed grasslands. Greater insight into soil C cycle can be obtained from partitioning soil respiration into its components such as bulk soil and soil connected to roots through mycorrhiza (Johnson et al., 2001).

Understanding the effects of land management on agricultural ecosystems and in particular on SOC is key for improvements on the management practises. Despite recent excellent study of land use intensification effect on SOC as it depends on soil pH (Malik et al., 2018) we still do not fully understand effect of liming on SOC in grasslands.

4.2 Hypothesis

In order to address uncertainties on overall plant-soil ecosystem responses to altered soil pH, a field experiment was initiated measuring response of multiple plan-soil biodiversity and functional properties to two modifications of soil pH of acid grasslands. Specifically, responses of soil nutrients, microbial community and soil and ecosystem scale processes were studied together with those of plant biomass characteristics and plant community structure during the second season after the start of the pH manipulation.

As it has been previously shown that increasing soil pH through liming affected soil substrate availability and microbial community structure resulting in increased N mineralization, it was hypothesized that (1) liming application, increasing soil pH to near neutral level, will change bacterial community structure and promote microbial taxa known as copiotrophic, because these taxa are understood to be associated with high nutrient availability. Bacterial phyla considered as copiotrophic include *α-Proteobacteria*, *β-Proteobacteria*, *Actinobacteria* and *Firmicutes* (Fierer et al., 2007; Leff et al., 2015). At the same time, relative abundance of phylum *Acidobacteria* will be reduced after pH increase due to its preference of acidic soils (Griffiths et al., 2011). Lowering of soil pH would promote opposite changes.

Soil pH can affect substrate availability through dissolution of SOC, alteration of soil aggregation (calcium ions can promote aggregation through cation bridges between organic and mineral component (von Lutzow et al., 2006) while liming was also shown to reduce macro-aggregation (Edgar et al., 2018) potentially making large POM unprotected). Soil pH is also a factor affecting extracellular enzyme activity (Sinsabaugh et al., 2008) potentially also affecting substrate availability. Changes in soil microbial community and availability of substrates after the liming will result in the changes of soil processes which will be detected by extracellular enzymes assays and by soil and ecosystem C flux measurements. Liming typically increases soil N availability and (2) related increase of N mineralization will be detected by an increase of activity of extracellular enzymes involved in N cycle.

The changes in soil N availability as expected on high pH plots will affect plant growth when (3) plants on limed plots will increase investment into aboveground growth resulting in greater biomass yield while decrease investment into root growth in comparison to acidic soils of the other treatments.
(4) Soil respiration will increase on limed plots because liming is expected to increase soil C cycle due to higher allocation of recently photosynthesized C compounds to soil microbial food-web increasing

microbial growth and also due to increased microbial activity resulting from alleviation of low soil pH constraints (such as stress response at low pH increasing C allocation to maintenance respiration and thus reducing C use efficiency).

4.3 Methodology

4.3.1 Site description

The experiment was established at the Lancaster University Field Station, UK (54°01'37.2"N 2°77'44.0"W), on a gentle south facing slope. The overall experimental site consisted of five separately fenced blocks, each split into 25 plots of 3 x 3 m. Sheep were introduced during winter and early spring. Vegetation was cut and removed at the peak of season. Species-poor plant community on the site was dominated by grasses including *Agrostis capillaris*, *Anthoxanthum odoratum* and *Holcus lanatus*. Soil was nutrient poor silt loam with 5.58 ± 0.04 pH (mean ± standard deviation), 4.51 ± 0.16% C and 0.19 ± 0% N.

4.3.2 Experiment establishment and maintenance

The experiment was set up in August 2015 and maintained for two consecutive seasons. The experiment described in this chapter was part of a larger experiment (see Chapter 4 for details). Soil pH treatments were established in separate plots and repeated within each of the 5 blocks. The plots where the experimental treatments were imposed were selected from the plots located in the middle of each row of plots alongside of the sides of the blocks (Figure 4.1). The exact position for each pH treatment within these middle side plots was randomly assigned. Low soil pH (range of 4.5 – 5.0 pH) and high soil pH (range of 6.5 – 7.5 pH) treatments were established and maintained by addition of diluted hydrochloric acid [HCl] and hydrated lime [$Ca(OH)_2$] respectively. HCl was selected as it does not contain soil nutrients, such as S-based additives. For each chemical, a single application dose was established based on field and laboratory testing. Field testing ensured that the application dose of the acid solution did not cause a negative effect on plants (assessed by visual examination of plants following application of acidic solutions of increasing strength). Laboratory testing determined buffering capacity of the soil after application of lime solutions of different strength. The dose for HCl and $Ca(OH)_2$ which was used for imposing of experimental treatments was 11.6 ml $m^{-2}$and 34.5 g m^{-2} of the chemical respectively. The dose was applied diluted in 5 L of tap water. The application of chemicals was followed by application of 5 L of tap water per m^2 to each treatment level. Control treatment plots received tap water only. Potential introduction of exogenous microbial community in the water would thus be similar for all treatments. Nine weeks after the start, soil pH on both high and low pH plots was at the required levels and total application of chemicals was 141 ml m^{-2} of HCl and 613 g m^{-2} of $Ca(OH)_2$ applied to low and high pH plots respectively. Nine months after the start when it was found that soil pH at low pH plots was not stable and required further adjustments, additional 64 $ml.m^{-2}$ of HCl was applied during the two growing seasons when the experiment was run.

Each plot was divided into two subplots (3 x 1.5 m) (Figure 4.1), and only one subplot was used for installation of the rings, root ingrowth mesocosms and measurements described in this chapter. Plastic rings of 30 cm diameter and 10 cm height were installed to each plot in the beginning of the first season (beginning of May). The rings were pushed into the soil leaving approximately 5 cm of the ring height above the ground. At the same time, root ingrowth mesocosms (Nottingham et al., 2005) were installed. They were made of PVC pipe (6 cm diameter, 25 cm length) with mesh covering the bottom and two windows cut into the pipe leaving 2.5 cm rim at both ends of the pipe and 2.5 cm stripes on opposite sides of the pipe. The treatments according to mesh size were: root

and mycelia ingrowth (1 mm mesh), mycelia ingrowth (35 µm mesh) and soil-only control (1 µm mesh). For each soil pH treatment level in each block, three holes were made into the soil, the soil was homogenised passing through a 4 mm mesh, large roots were removed, mesocosms with each mesh treatments were fitted into the holes leaving 2 cm of the rim aboveground and filled with calculated amount of homogenized soil to achieve same soil bulk density across mesocosms.

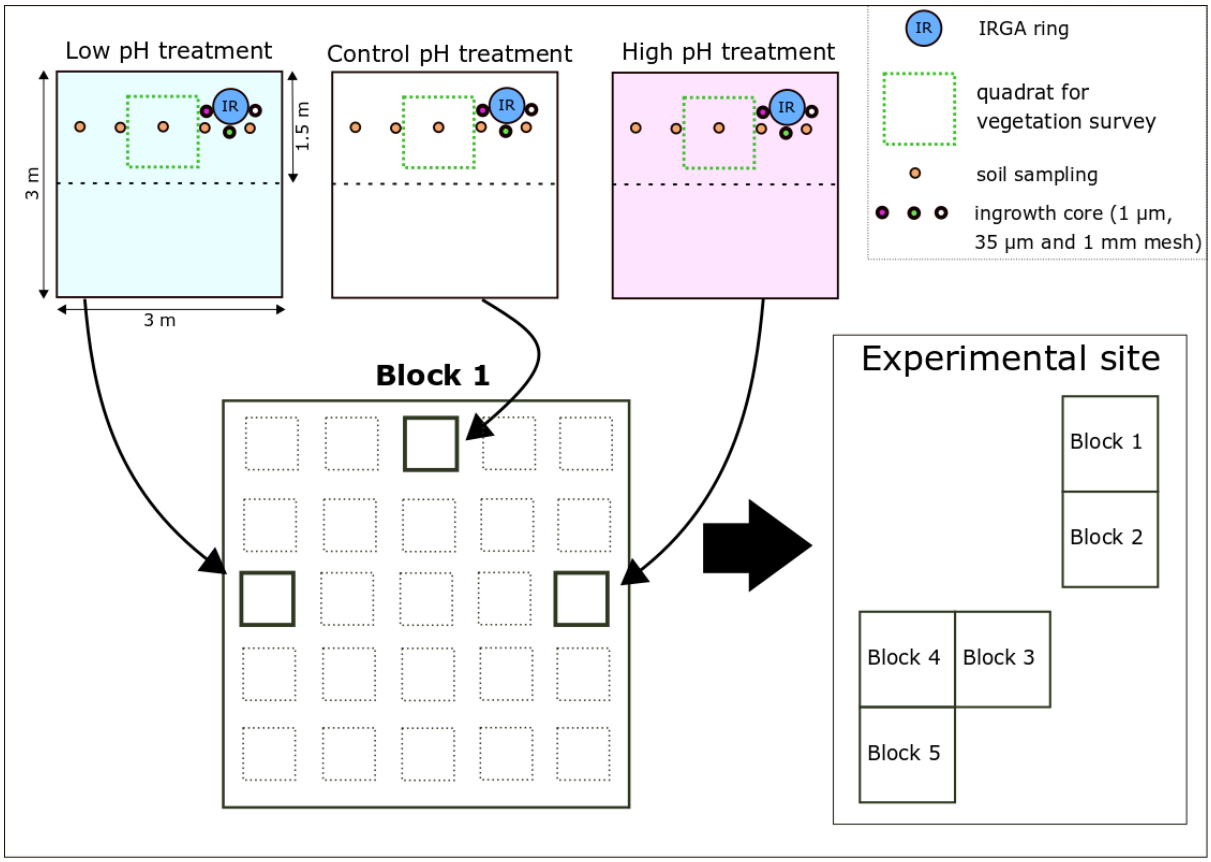


Figure 4.2 Experimental site design of soil pH manipulation experiment in grassland.

4.3.4 Measurements

Measurements and their timing are summarized in Figure 4.3. Common laboratory procedures are listed in the Methods chapter (Chapter 2).

Soil sampling and processing

Soil was sampled on the 13th of July, 2017. For each treatment, soil cores (5 cm diameter from 5 cm depth) were taken from 5 random locations within the plot along a transect in the middle of the plot (Figure 4.2), transported to the lab and stored at 4 ˚C. It was then homogenized by passing through a 4 mm mesh within 48 hours of sampling. At the same time, a subsample was taken for soil DNA analysis and enzyme assays and stored at -20 ˚C. A metal ring (6 cm diameter, 5 cm high) was pushed to the ground in the middle of each subplot, after a top layer of roots and undecomposed material was removed, dug out and the soil from inside the ring was used for bulk density determination. Methods of soil analysis (abiotic and biotic characteristics) are detailed in methods chapter (Chapter 2).

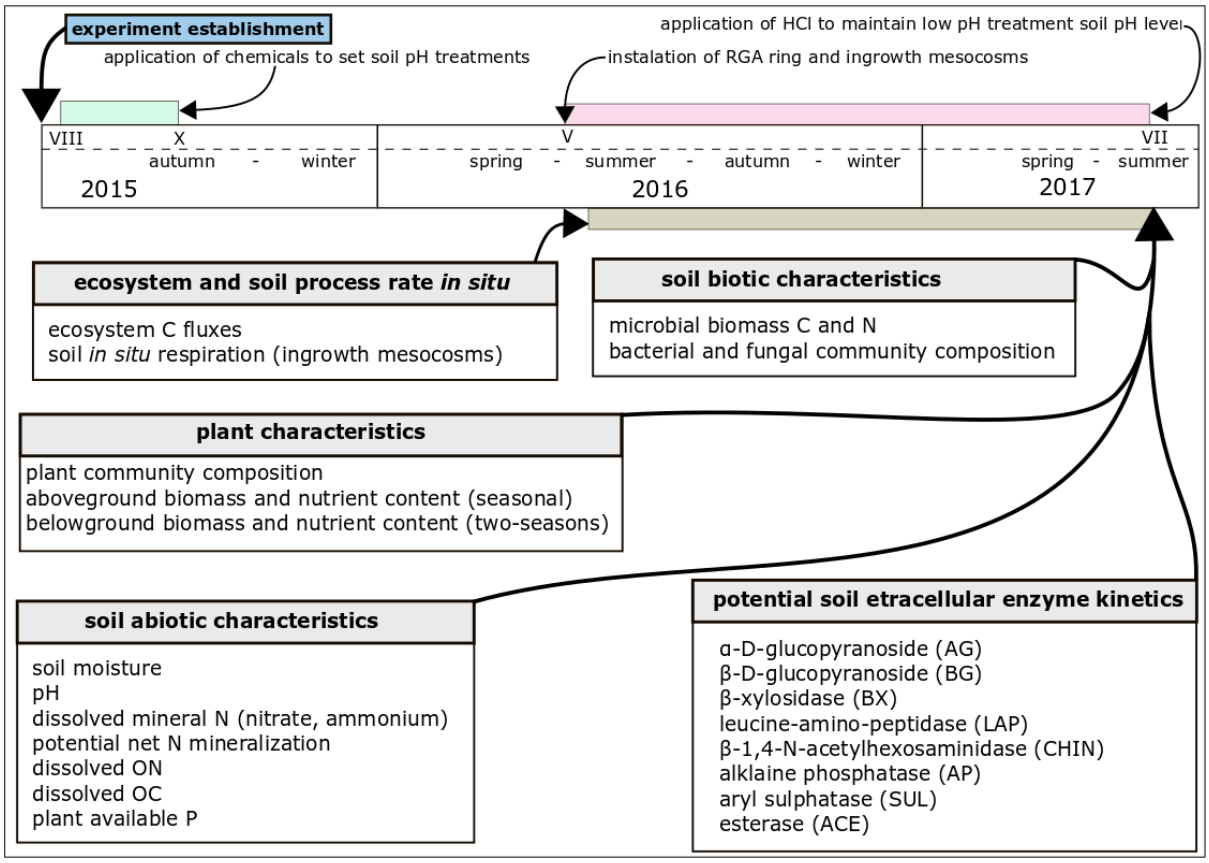


Figure 4.3 Experiment establishment, maintenance and measurements timing.

Plant community, growth rate and traits

Plant community determination was performed on the 11th of July, 2017. A 1 x 1 m quadrat was used to survey vegetation with all plants identified to a species level and cover estimated.
Plant biomass and soil cores were harvested on the 13th of July, 2017. Plant aboveground biomass was harvested from within the area of the installed rings (707 cm^2), oven dried at 65 ˚C for 3 days and stored in a dry place. Soil cores from the ingrowth mesocosms were cut lengthwise and half was used for root traits. Soil was washed off and root material was collected on 1 mm sieve and stored in 50 % ethanol solution. Half of the roots were then scanned and analysed in WinRhizo (Reagent Instruments Inc., Ville de Québec, Canada) to determine root lengths and diameters. Specific root length (SRL) and other specific root characteristics (root area, root volume, root surface) were calculated for the total root and for root diameter classes by the software. Both, scanned and un-scanned portion of roots were then oven dried at 65 ˚C until constant weight was achieved, biomass determined, and root characteristics determined by scanning were recalculated to total root mass. Methods of plant traits analysis are detailed in methods chapter (Chapter 2).

Ecosystem and soil process rates

Field measurements of C flux were performed monthly during growing season and bimonthly in winter using EGM-4 Environmental Gas Monitor (IRGA; PP systems, Amesbury, USA). Measurements of gross ecosystem soil respiration and net ecosystem exchange (NEE) were made using light chamber and dark chamber respectively, placed over the installed plastic rings (Ward et al., 2007). At the same time as C flux measurements, soil temperature and soil moisture were determined. The

field soil moisture was measured on three places in each plot using portable moisture probe (Delta-T, Cambridge, UK) and averaged.

In situ soil respiration measurements were partitioned into three components according to the size of the ingrowth into constructed soil mesocosms: roots, AMF mycelia and free living microorganisms (Nottingham et al., 2010). The field measurements were performed monthly during the season and bimonthly in winter using IRGA (as above) and a custom made dark-chamber (0.325 l).

Details of method for determining extracellular enzyme activity analysis are described in methods chapter (Chapter 2).

Soil microbial community

Microbial biomass C and N was performed as specified in Methods chapter (Chapter 2). Bacterial and fungal community composition was determined employing 16S and ITS amplicon sequencing respectively. For bacteria, the V4 hypervariable region of 16S rRNA gene was amplified using 515f-806r primers (Walters et al., 2015) and for fungi, the ITS2 region was amplified using fITS7-ITS4r primer sequences (Ihrmark et al., 2012). Extraction of DNA, amplification of target gene sequences, sequencing and sequences processing and taxonomy assignment was performed as specified in methods chapter (Chapter 2).

4.3.5 Data analysis

Effect of soil pH manipulation (categorical variable) or soil pH (continuous variable) on soil and plant characteristics, plant species cover and soil and ecosystem processes was determined using one-way ANOVA or Spearman's correlation analyses respectively. Differences between levels of the factor were tested using Tukey HSD post hoc comparison with Bonferroni correction for multiple comparisons. Models that violated assumptions of normality and homoscedasticity received ln(y) transformation. Non-parametric Kruskal-Wallis test and Dunn's pairwise comparison with Benjamini-Hochberg correction for multiple comparisons were used if ln(y) transformation did not improve model fit.

Response of microbial community to soil pH treatments was assessed by multivariate generalised linear models (M-GLMs) using GLM framework from MVABUND 3.10.4 package in R (Y. (Y.Wang et al., 2012). Details of the analysis are listed in methods chapter (Chapter 2).

To identify individual phylotypes significantly associated with soil pH treatments, indicator species analysis (R package indicspecies) was used. Details of the analysis are listed in method chapter (Chapter 2). Abundance of selected indicator species (with $P < 0.05$) for each soil pH treatment level was grouped at class taxonomic resolution.

All analyses were conducted in R of version 3.5.0 (R Core Team, 2018).

4.4 Results

4.4.1 Effect of soil pH treatment on soil characteristics

Soil pH manipulations created three distinct pH levels of low (4.9 ± 0.1 [mean ± s.e.]), control (5.5 ± 0) and high (7.3 ± 0.2) pH ($F_{2,11}$ = 95.4, P < 0.001; posthoc comparisons P < 0.05).

Soil pH treatment affected concentrations of dissolved soil nutrients including DOC ($F_{2,11}$ = 20.8, P < 0.001), DON ($F_{2,11}$ = 22.3, P<0.001), dissolved inorganic N (DIN) ($F_{2,12}$ = 14.1, P < 0.01) and Olsen P ($F_{2,12}$ = 8, P < 0.01) (Table 4.1). These effects were predominantly due to an impact of high soil pH treatment when DOC, DON and DIN were higher and Olsen P was lower on high pH treatment plots when compared to the other two pH treatments (posthoc comparison P < 0.05). Ammonium was the main component of DIN while its mean value was over 5 times higher than nitrate mean value and thus showed the same trend as DIN ($F_{2,11}$ = 22.9, P<0.001, posthoc comparison P < 0.05) (Table 4.1). No ammonium or nitrate were found in low and control pH plots. The same trends were observed for the stocks of these nutrients in the soil recalculated from BD data for 5 cm soil depth, apart from Olsen P stock when difference was only between low and high pH treatments (Table 4.1).

The ratio between concentrations of soil dissolved C and N pools including DOC:DON and DOC: dissolved total N (DN) was not affected by soil pH treatments ($F_{2,11}$ = 1.0, P = 0.41 and $F_{2,11}$ = 2.2, P = 0.16 respectively) but ratio between these pools and Olsen P changed in response to pH change when DOC:Olsen P was higher and DN:Olsen P was lower in high pH treatment than control and low pH treatment soils ($F_{2,11}$ = 15.2, P < 0.01 and $F_{2,11}$ = 25.2, P < 0.001 respectively, posthoc comparison P < 0.05).

In contrast to the dissolved soil nutrients, SOC concentration and stock (as determined by LOI) were not affected by soil pH treatments ($F_{2,12}$ = 1.2, P = 0.75 and $F_{2,12}$ = 1.2, P = 0.35 respectively). Soil moisture and bulk density showed no effect of soil pH manipulation ($F_{2,12}$ = 0.4, P = 0.43 and $F_{2,12}$ = 0.4, P = 0.69 respectively). Similarly, soil microbial community biomass C and N concentration did not respond to pH changes ($F_{2,12}$ = 0.4, P = 0.69 and $F_{2,12}$ = 0.2, P = 0.82 respectively) nor their ratio ($F_{2,12}$ = 0.3, P = 0.74).

Table 4.1 Response of soil properties to soil pH manipulation and soil pH.

	F[1)]	p[1)]	soil pH treatment			p[3)]	cor[3)]
			low	control	high		
soil characteristics - concentration							
LOI	1.2	0.35	0.11 ± 0	0.16 ± 0	0.11 ± 0	0.75	-0.09
DOC (ug OC g^{-1} dry soil)	20.8	***	29.25 ± 1.3[a]	35.68 ± 2.1[a]	93 ± 12.2[b]	***	0.95
DON (ug ON g^{-1} dry soil)	22.3	***	2.88 ± 0.4[a]	4.41 ± 0.3[a]	8.48 ± 0.9[b]	***	0.90
DIN (ug N g^{-1} dry soil)	14.1	**	0 ± 0[a]	0 ± 0[a]	6.25 ± 1.7[b]	***	0.85
ammonium (ug NH_4^--N g^{-1} dry soil)	22.9	***	0 ± 0[a]	0 ± 0[a]	5.5 ± 1.1[b]	***	0.88
nitrate (ug NO_3^--N g^{-1} dry soil)	1.4	0.28	0 ± 0	0 ± 0	0.76 ± 0.6	0.06	0.49
Olsen P (ug PO_4^{3-}-P g^{-1} dry soil)	8.0	**	42.44 ± 2.1[a]	36.8 ± 2.8[a]	24.74 ± 4.3[b]	**	-0.75
DOC:DON ratio	2.6	0.12	8.45 ± 0.2	8.13 ± 0.3	11.25 ± 1.7	*	0.64
DOC:DN ratio	2.2	0.157	11.65 ± 2.7	8.13 ± 0.3	6.71 ± 1.2	0.14	-0.42
DOC:Olsen P ratio	15.2	**	0.72 ± 0.035[a]	0.99 ± 0.091[a]	4.2 ± 0.808[b]	***	0.89
DN:Olsen P ratio	25.5	***	0.07 ± 0.01[a]	0.12 ± 0.01[a]	0.65 ± 0.1[b]	***	0.9
soil characteristics - stock							
OC (ug OC m^{-2})	0.9	0.43	27.11 ± 1.6	39.99 ± 13.6	26.87 ± 1.1	0.80	-0.07
DOC (ug OC m^{-2})	15.4	**	1.24 ± 0.1[a]	1.45 ± 0.1[a]	4.03 ± 0.6[b]	***	0.93
DON (ug ON m^{-2})	17.7	***	0.12 ± 0[a]	0.18 ± 0[a]	0.36 ± 0[b]	***	0.91
DN (ug N m^{-2})	14.2	***	0.12 ± 0[a]	0.18 ± 0[a]	0.64 ± 0.1[b]	***	0.88
DIN (ug N m^{-2})	12.1	**	0 ± 0[a]	0 ± 0[a]	0.28 ± 0.1[b]	***	0.83
Olsen P (ug PO_4^{3-}-P m^{-2})	9.2	**	1.8 ± 0.1[a]	1.5 ± 0.1[ab]	1.03 ± 0.1[b]	**	-0.74
microbial characteristics							
microbial biomass C (ug biomass C g^{-1} dry soil)	0.4	0.69	210 ± 21	197 ± 11	219 ± 20	0.70	0.11
microbial biomass N (ug biomass N g^{-1} dry soil)	0.2	0.82	31.2 ± 3.6	30.5 ± 2.3	33.4 ± 4.0	0.72	0.10
microbial biomass C:N ratio	0.3	0.74	6.8 ± 0.2	6.5 ± 0.2	6.7 ± 0.3	0.92	0.03

+ *P<0.1,* * *P<0.05,* ** *P<0.01,* *** *P<0.001;* *correlation:* -1 0 1

[1)] *Differences in mean values (±standard errors) for soil properties on low, control and high treatment level plots; letters indicate significant differences at P < 0.05 (Tukey HSD post hoc tests).*

4.4.2 Effect of soil pH treatment on soil microbial community

The sequencing yielded total of 518,929 and 699,370 reads for bacterial and fungal community respectively. Non-identified sequences at phylum level accounted for <0.05 % and <2 % of the total reads for bacteria and fungi respectively. Richness and diversity indices (Simpson's and Shannon) were not affected by soil pH manipulation for both, bacterial and fungal communities in the soil (Kruskal-Wallis test, all P > 0.05). Bacterial community composed primarily of *Proteobacteria* (35 % of all bacterial sequences, on average), *Firmicutes* (18 %), *Verrucomicrobia* 15 %) and *Acidobacteria* (13 %) and the fungal community composed primarily of Ascomycota (63 %) and Basidiomycota (30 %). The distribution of bacterial and fungalphyla and classes of relative abundance greater than 1 % for each repeated treatment level are presented in Figures 4.4, 4.5, 4.6 and 4.7.

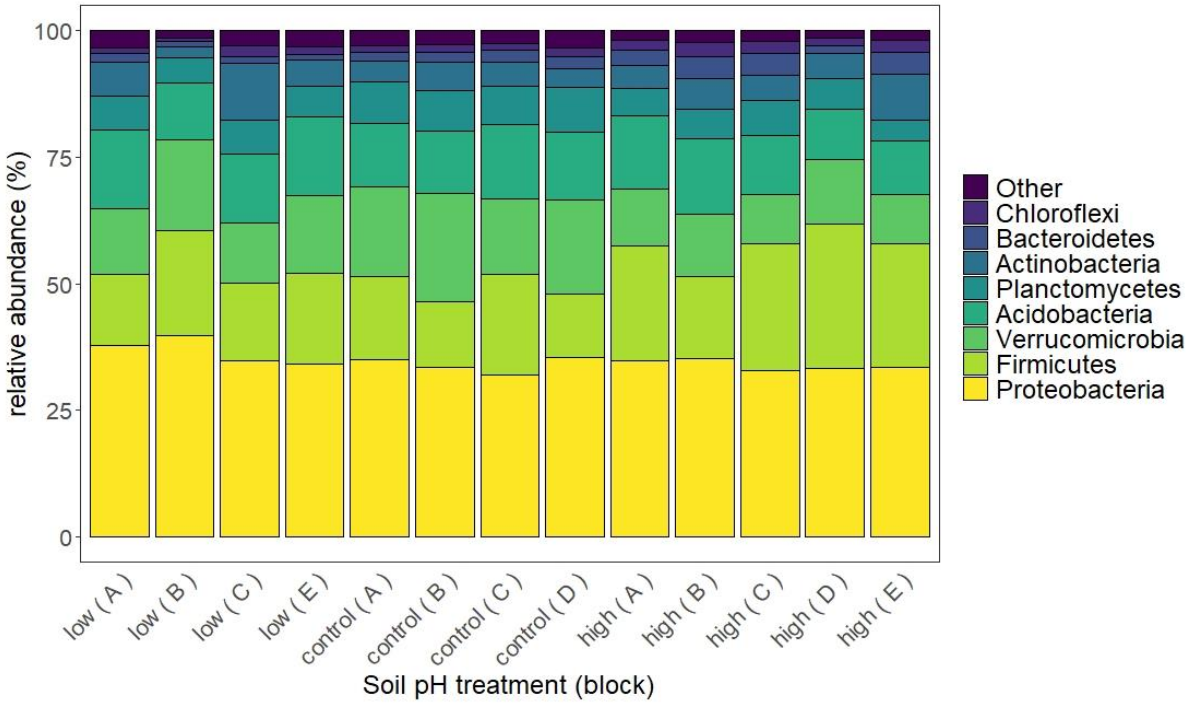


Figure 4.4 Relative abundance of bacterial phyla in the soil of different soil pH treatment plots. Phyla of relative abundance lower than 1 % are grouped as 'Other' together with unassigned phyla.Soil pH treatment levels are low, control and high pH treatments. Letter in brackets denotes experimental block.

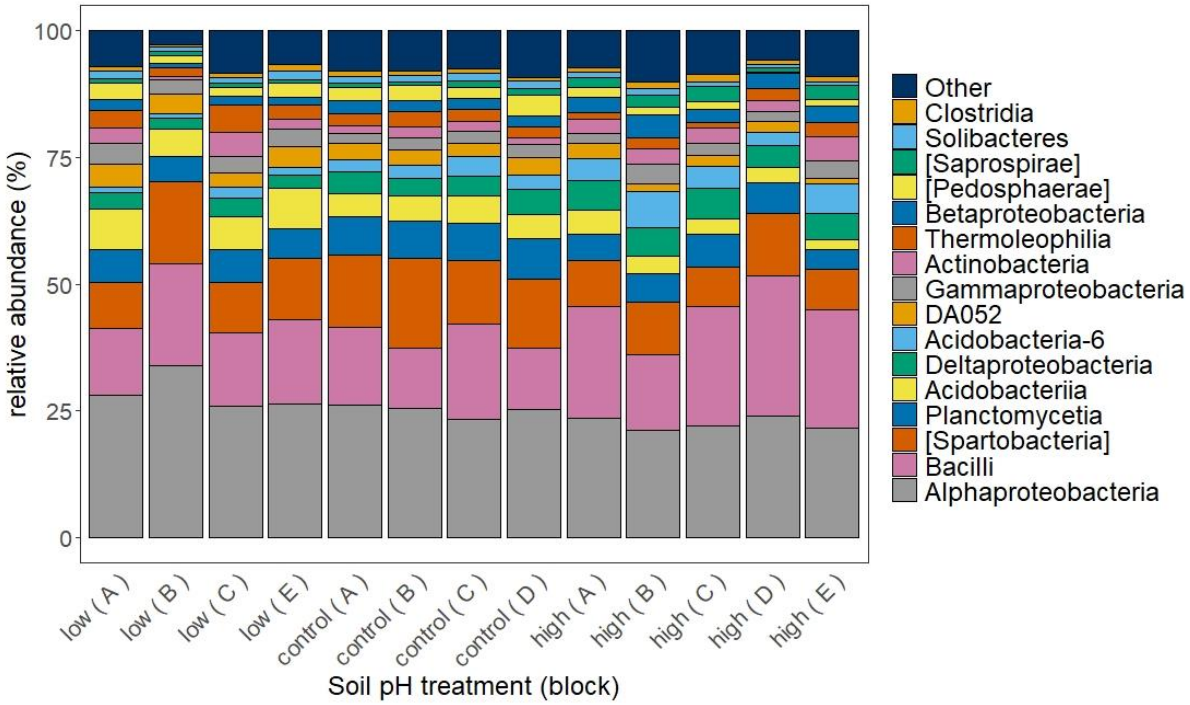


Figure 4.5 Relative abundance of bacterial classes in the soil of different soil pH treatment plots. Classes of relative abundance lower than 1 % are grouped as 'Other' together with unassigned classes. Identified classes are ordered according to mean relative abundance.

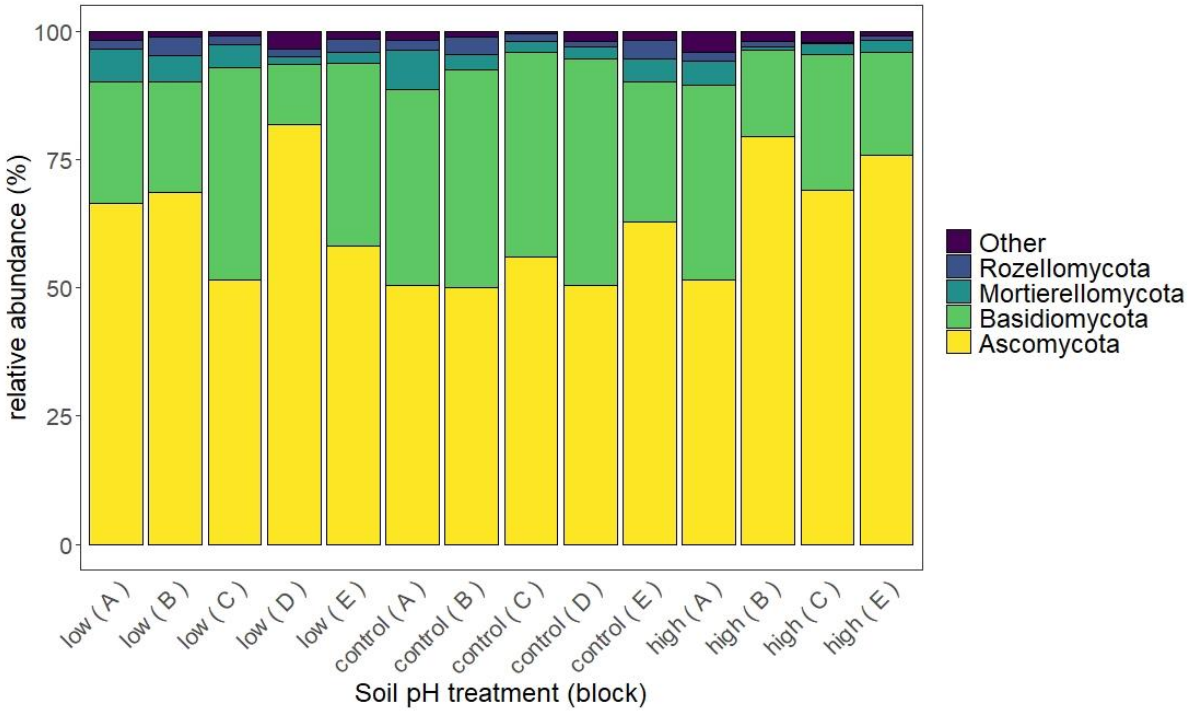


Figure 4.6 Relative abundance of fungal phyla in the soil of different soil pH treatment plots. Phyla of relative abundance lower than 0.1 % are grouped as 'Other' together with unassigned phyla.
Soil pH treatment levels are low, control and high pH treatments. Letter in brackets denotes experimental block.

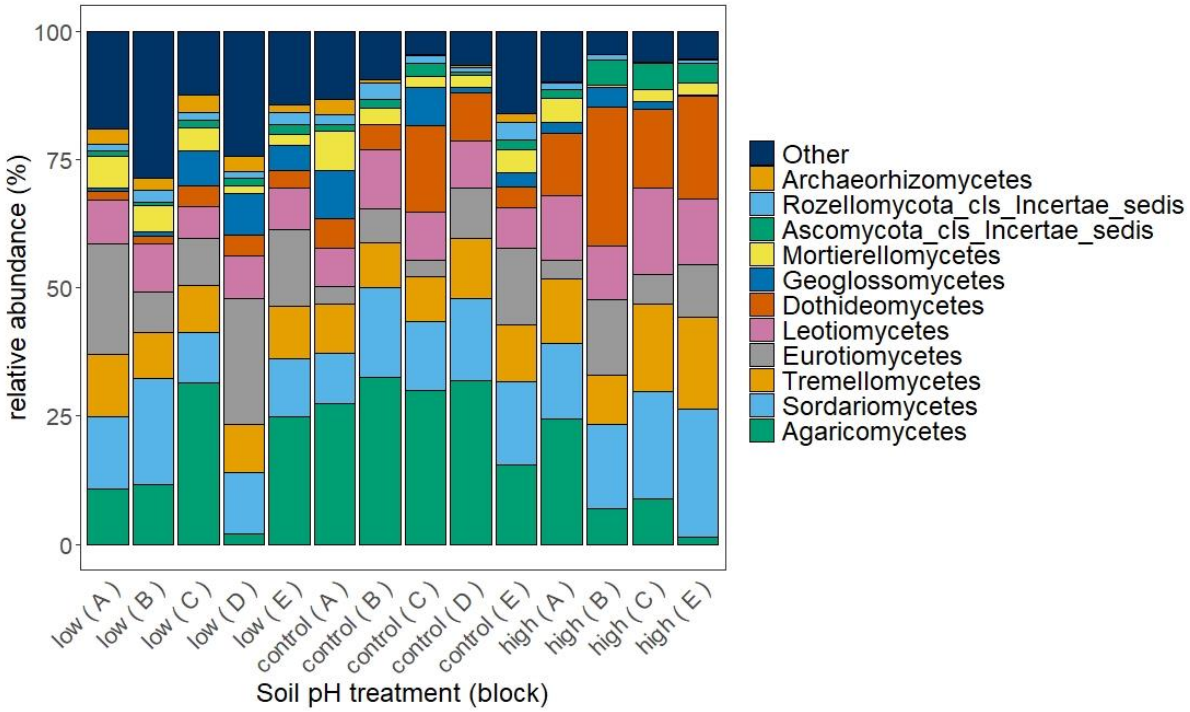


Figure 4.7 Relative abundance of fungal classes in the soil of different soil pH treatment plots. Classes of relative abundance lower than 1 % are grouped as 'Other' together with unassigned classes. Identified classes are ordered according to mean relative abundance.

Soil pH manipulation affected structure of bacterial and fungal communities at phylum, class, order and species taxonomic resolutions (Table .2). Pairwise comparison between soil pH treatment levels showed differences only for fungal community at order and species taxonomic resolutions whereby fungal community on high pH level treatment were different from the other communities (i.e. on low and control pH levels) at order resolution while it was different only from low pH treatment community at species resolution (Table .2). NMDS of bacterial and fungal community composition is displayed in Figure 4.8.

Table 4.2 Results of GLM analysis of effect of soil pH manipulation on microbial community structure at different taxonomic resolutions.

Microbial community	Taxonomic resolution levels [1]	Effect on overall community structure		Pairwise comparison between soil pH treatment levels					
				Test statistic [2]			P [3]		
		Test statistic	P	low - control	control -high	low - high	low - control	control -high	low - high
Bacteria	Phylum	20.1	**	8.1	21.7	16.3	0.50	0.38	0.40
	Class	51.6	**	136	193	168	0.20	0.18	0.18
	Order	40.1	*	86	137	128	0.28	0.22	0.22
	Species	7786	**	2775	4160	4016	0.13	+	+
Fungi	Phylum	6.9	*	10.3	21.0	17.6	0.18	0.10	0.10
	Class	18.4	***	27.5	82.4	79.5	0.37	0.17	0.17
	Order	23.6	**	40.6	181.8	135.0	0.44	*	*
	Species	5277	***	1983	2655	3080	0.12	+	*

+ P<0.1, * P<0.05, ** P<0.01, *** P<0.001;

[1] Selected taxonomic units (total abundance > 0.1 % only) within each taxonomic resolution level were grouped from raw sequencing data with abundance of individual sequences higher than 2 reads (species data were not grouped). [2] Scores statistics used for Species and Wald test statistic used the other data. [3] P adjusted within Taxonomic resolution level using Holm's step down procedure.

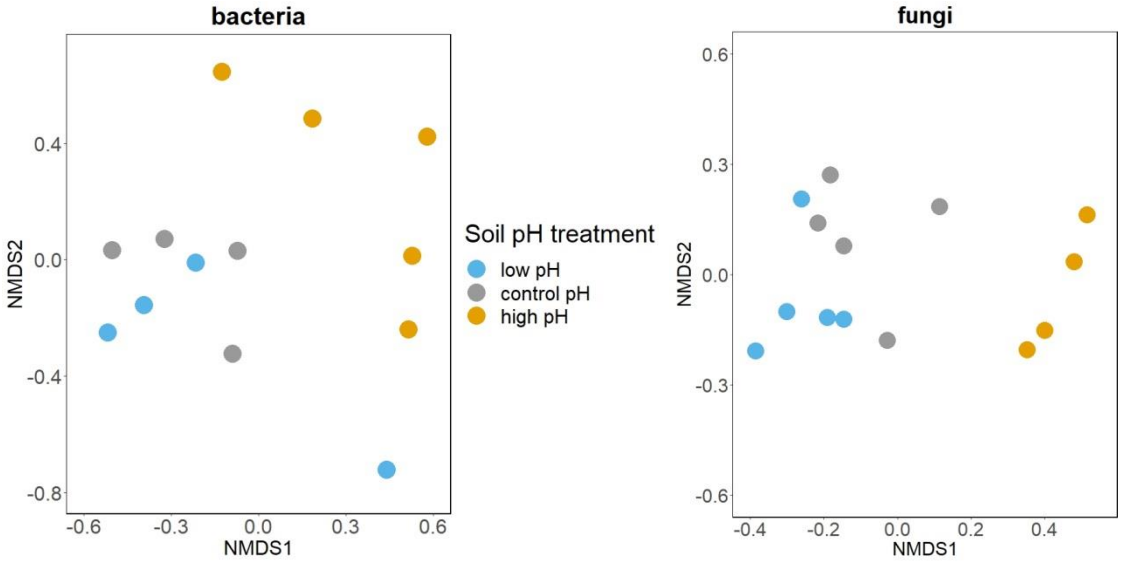


Figure 4.8 NMDS of bacterial and fungal community composition.

Only bacterial phyla with lower relative abundances were correlated with changes of soil pH and from those *Bacteroidetes*, *Chloroflexi* and *Nitrospirae* showed positive correlation with soil pH (rho = 0.76, P < 0.05; rho = 0.69, P < 0.05 and rho = 0.64, P < 0.05 respectively) and *WPS-2* showed negative correlated with soil pH (rho = - 0.72, P < 0.05) (Table 4.).

Classes of overall relative abundance greater than 0.1 % that responded to soil pH belonged to phyla including *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Nitrospirae* and *WPS-2* (Table 4.3). For the most abundant phyla with multiclass responses such as *Proteobacteria* and *Acidobacteria*, the responding classes within each phylum did not respond to the soil pH in the same direction. Soil pH was positively correlated with relative abundances of *Deltaproteobacteria* (rho = 0.83, P < 0.001), *BetaproteobacteriaI* (rho = 0.86, P < 0.001), *Acidobacteria-6* (rho = 0.9, P < 0.001), *iii1-8* (rho = 0.66, P < 0.05), *Acidobacteria-5* (rho = 0.62, P < 0.05), [*Chloracidobacteria*] (rho = 0.67, P < 0.05), *Acidimicrobiia* (rho = 0.68, P < 0.05), *Flavobacteriia* (rho = 0.69, P < 0.05), *Ellin6529* (rho = 0.6, P < 0.05), *Anaerolineae* (rho = 0.83, P < 0.001), and *Nitrospira* (rho = 0.64, P < 0.05) and negatively correlated with *Alphaproteobacteria* (rho = -0.82, P < 0.001), *Acidobacteriia* (rho = -0.77, P < 0.01) and *DA052* (rho = -0.74, P < 0.01) (Table 4.4).

Indicator species analysis showed that for the most abundant bacterial classes, the classes with relatively high (< 10 %) cumulative abundance of indicator species for high soil pH treatment level included *Acidobacteria-6* (52 % of total class are indicator species), *Deltaproteobacteria* (25 %) and *Betaproteobacteria* (21 %) and for control pH level included *Solibacteres* (20 %) (Table 4.4). Low pH treatment soils did not show indicator species which were higher than 10 % of class abundance (Table 4.4).

Table 4.3 Microbial community phyla correlation with soil pH.

Kingdom	Phylum[1)]	Overall relative abundance (%)	Soil pH treatment mean[2)]			Spearman's correlation analysis	
			low	control	high	P[3)]	rho
Bacteria	*Proteobacteria*	35	4671 ± 230	4217 ± 102	4173 ± 62	0.23	-0.47
	Firmicutes	19	2181 ± 170	1976 ± 182	2860 ± 238	0.27	0.43
	Verrucomicrobia	16	2006 ± 256	2411 ± 210	1353 ± 78	0.14	-0.54
	Acidobacteria	13	1513 ± 231	1544 ± 105	1543 ± 125	0.83	-0.1
	Planctomycetes	6.1	664 ± 110	912 ± 97	685 ± 62	1	0
	Actinobacteria	5.2	678 ± 207	509 ± 80	741 ± 113	0.71	0.15
	Bacteroidetes	2.2	154 ± 13	246 ± 27	418 ± 73	*	0.76
	Chloroflexi	1.5	134 ± 44	154 ± 25	277 ± 26	*	0.69
	Gemmatimonadetes	0.4	55 ± 23	34 ± 11	48 ± 8	0.89	-0.06
	WS3	0.4	28 ± 15	66 ± 8	49 ± 9	0.30	0.39
	Chlamydiae	0.3	40 ± 11	29 ± 8	31 ± 7	0.39	-0.32
	TM6	0.3	37 ± 8	38 ± 6	22 ± 6	0.30	-0.38
	Nitrospirae	0.2	7 ± 3	25 ± 10	33 ± 11	*	0.64
	WPS-2	0.2	37 ± 10	28 ± 3	3 ± 2	*	-0.72
	Cyanobacteria	0.1	16 ± 5	19 ± 2	8 ± 2	0.39	-0.32
	Elusimicrobia	0.1	15 ± 8	31 ± 10	8 ± 5	0.70	-0.17
Fungi	*Ascomycota*	62.8	23235 ± 1828	19203 ± 892	24533 ± 1707	0.61	0.19
	Basidiomycota	30.1	9489 ± 1889	13589 ± 1066	8976 ± 1284	0.75	-0.09
	Mortierellomycota	3.4	1379 ± 316	1405 ± 378	868 ± 232	0.39	-0.31
	Rozellomycota	1.8	801 ± 145	815 ± 173	337 ± 81	*	-0.65
	Glomeromycota	0.1	71 ± 22	58 ± 17	4 ± 1	***	-0.82

\+ P<0.1, * *P<0.05,* ** P<0.01, *** P<0.001; correlation: -1 0 1

[1)] *Microbial phyla of relative abundance lower than 0.1% not shown.* [2)] *Differences in mean values (±standard errors) for soil properties on plots of low, control and high pH treatment levels.* [3)] *Spearman correlation, Benjamini-Hochberg corrected P.* Rarefied sequencing data used for mean calculation and analysis.

For the fungal community, only two phyla correlated with soil pH such as *Rozellomycota* and *Glomeromycota* when both reacted negatively to soil pH increase (rho = -0.65, $P < 0.05$; rho = -0.82, $P < 0.001$ respectively) however they were of marginal presence (Table 4.3).

Only classes of phylum *Ascomycota* correlated with soil pH. Soil pH was positively correlated with relative abundances of *Leotiomycetes* (rho = 0.64, $P < 0.05$), *Dothideomycetes* (rho = 0.93, $P < 0.001$), *Ascomycota_cls_Incertae_sedis* (rho = 0.72, $P < 0.05$) and negatively correlated with relative abundances of *Archaeorhizomycetes* (rho = - 0.77, $P < 0.01$) and *Lecanoromycetes* (rho = - 0.80, $P < 0.001$) (Figure 4.5). Interestingly, phylum *Ascomycota* contained 14 % of species unassigned at a class level which mostly belonged to low and control pH plots.

For the indicator species analysis classes with relatively high (< 10 %) cumulative abundance of indicator species for high pH treatment plots included *Ascomycota_cls_Incertae_sedis* (99.8 % of species are indicator species), *Dothideomycetes* (57 %), Sordariomycetes (21 %) and *Eurotiomycetes* (17 %), for control plots included *Rozellomycota_cls_Incertae_sedis* (58 %) and for low pH plots included *Archaeorhizomycetes* (57 %), *Eurotiomycetes* (19 %), *Sordariomycetes* (14 %) and *Rozellomycota_cls_Incertae_sedis* (12 %) (Table 4.5).

Table 4.4 Bacterial class relationship with soil pH.

Phyla	Class	Relative abundance (%)	soil pH treatment mean			correlation		indicator species			
			low	control	high	P	rho	none	low	control	high
Proteobacteria	*Alphaproteobacteria*	26.68	3785 ± 331	3277 ± 214	2768 ± 78	***	-0.82	88.1	8.1	1.8	2.0
	Deltaproteobacteria	3.87	326 ± 46	434 ± 75	667 ± 39	***	0.83	74.6		0.6	24.8
	Gammaproteobacteria	2.56	372 ± 57	238 ± 41	334 ± 42	0.58	-0.2	90.9	6.0		3.0
	Betaproteobacteria	2.26	169 ± 35	263 ± 30	400 ± 42	***	0.86	76.7		2.7	20.6
Firmicutes	*Bacilli*	18.06	2072 ± 180	1861 ± 175	2720 ± 245	0.23	0.41	91.6			8.4
	Clostridia	0.98	109 ± 20	113 ± 16	140 ± 16	0.52	0.25	94.7		5.3	
Verrucomicrobia	*[Spartobacteria]*	13.39	1748 ± 322	2029 ± 240	1155 ± 100	0.21	-0.43	92.6	6.6	0.6	0.2
	[Pedosphaerae]	1.9	220 ± 73	324 ± 50	157 ± 33	0.56	-0.22	89.2		10.8	
	Opitutae	0.18	23 ± 7	40 ± 10	5 ± 2	0.13	-0.49	63.7		36.3	
Acidobacteria	*Acidobacteriia*	4.86	770 ± 107	608 ± 19	413 ± 55	**	-0.77	89.4	7.2	3.4	
	DA052	2.87	441 ± 56	363 ± 33	254 ± 35	**	-0.74	98.8		1.2	
	Acidobacteria-6	2.82	130 ± 41	320 ± 46	589 ± 87	***	0.9	46.8	1.4		51.8
	Solibacteres	1.07	126 ± 35	157 ± 22	110 ± 11	0.54	-0.23	69.9	3.7	20.4	5.9
	iii1-8	0.31	21 ± 8	27 ± 6	67 ± 12	*	0.66	100.0			
	Acidobacteria-5	0.24	12 ± 5	36 ± 11	41 ± 6	*	0.62	100.0			
	[Chloracidobacteria]	0.11	2 ± 1	18 ± 6	21 ± 5	*	0.67	100.0			
Planctomycetes	*Planctomycetia*	5.83	642 ± 104	844 ± 85	660 ± 61	0.94	0.03	95.0		3.8	1.2
	Phycisphaerae	0.15	13 ± 5	37 ± 7	6 ± 2	0.58	-0.19	72.3		27.7	
Actinobacteria	*Actinobacteria*	2.46	305 ± 83	207 ± 21	393 ± 66	0.24	0.4	94.7			5.3
	Thermoleophilia	2.28	323 ± 108	263 ± 53	254 ± 34	0.58	-0.17	97.4			2.6
	Acidimicrobiia	0.48	48 ± 29	38 ± 8	90 ± 19	*	0.68	72.2			27.8
Bacteroidetes	*[Saprospirae]*	1.41	95 ± 6	136 ± 21	290 ± 49	*	0.71	83.3		5.3	11.4
	Cytophagia	0.3	19 ± 9	47 ± 12	43 ± 11	0.07	0.55	50.4		31.1	18.6
	Sphingobacteriia	0.28	34 ± 5	30 ± 8	37 ± 8	0.94	0.04	91.1		8.9	
	Flavobacteriia	0.19	1 ± 1	30 ± 8	40 ± 14	*	0.69	84.2		15.8	
Chloroflexi	*Ktedonobacteria*	0.52	59 ± 21	46 ± 12	86 ± 12	0.49	0.26	100.0			
	Ellin6529	0.5	32 ± 15	53 ± 11	98 ± 31	*	0.6	62.2			37.8
	Anaerolineae	0.17	7 ± 4	14 ± 5	43 ± 11	***	0.83	84.2			15.8
	TK10	0.15	15 ± 7	25 ± 9	16 ± 4	0.98	-0.01	100.0			
Gemmatimonadetes	*Gemmatimonadetes*	0.3	46 ± 19	30 ± 9	33 ± 8	0.58	-0.19	94.3		5.7	
WS3	*PRR-12*	0.39	28 ± 15	66 ± 8	49 ± 9	0.24	0.39	70.9		29.1	
Chlamydiae	*Chlamydiia*	0.27	40 ± 11	29 ± 8	31 ± 7	0.36	-0.32	100.0			
TM6	*SJA-4*	0.24	35 ± 7	32 ± 8	21 ± 6	0.31	-0.35	100.0			
Nitrospirae	*Nitrospira*	0.18	7 ± 3	25 ± 10	33 ± 11	*	0.64	100.0			
WPS-2	*other WPS-2*	0.19	37 ± 10	28 ± 3	3 ± 2	**	-0.72	63.8		36.2	
Elusimicrobia	*Elusimicrobia*	0.15	15 ± 8	31 ± 10	8 ± 5	0.58	-0.17	100.0			

+ P<0.1, * *P<0.05,* ** P<0.01, *** P<0.001; correlation (rho): -1 0 1 cumulative abundance (%): 100 50 0

[1)] *Fungal classes of relative abundance lower than 0.1% not shown.* [2)] *Differences in mean values (±standard errors) for soil properties on plots of low, control and high pH treatment levels.* [3)] *Benjamini-Hochberg corrected P.*

Table 4.5 Fungal classes relationship with soil pH.

Phyla	Class[1)]	Relative abundance (%)	soil pH treatment mean[2)]			Correlation[3)]		indicator species			
			low	control	high	P	rho	none	low	control	high
Ascomycota	*Sordariomycetes*	15.77	4787 ± 692	5230 ± 486	6796 ± 631	0.21	0.45	64.7	13.5		21.8
	Leotiomycetes	10.13	2913 ± 180	3237 ± 258	4652 ± 368	*	0.64	87.9	3.1	0.5	8.5
	Eurotiomycetes	10.62	5563 ± 1190	2707 ± 774	3056 ± 684	0.21	-0.43	64.1	18.9		17.0
	Dothideomycetes	9.93	1029 ± 188	2887 ± 827	6676 ± 890	***	0.93	39.4		3.3	57.3
	Geoglossomycetes	3.47	1511 ± 553	1483 ± 640	702 ± 191	1.00	0	98.8		1.2	
	Ascomycota_cls_Incertae_sedis	2.27	457 ± 71	582 ± 114	1377 ± 222	*	0.72	0.2			99.8
	Archaeorhizomycetes	1.33	948 ± 114	427 ± 192	40 ± 18	**	-0.77	43.0	57.0		
	Pezizomycetes	0.44	116 ± 28	112 ± 19	246 ± 73	0.40	0.26	100.0			
	Orbiliomycetes	0.21	32 ± 28	112 ± 42	82 ± 18	0.21	0.41	82.6			17.4
	Lecanoromycetes	0.1	69 ± 15	39 ± 13	0 ± 0	***	-0.8	100.0			
	other Ascomycota	8.47	5797 ± 953	2335 ± 669	896 ± 76	***	-0.86	60.2	35.0		4.7
Basidiomycota	*Agaricomycetes*	17.97	5748 ± 1903	9727 ± 1139	3681 ± 1366	0.33	-0.31	97.4	2.0	0.5	0.05
	Tremellomycetes	11.4	3543 ± 212	3524 ± 226	5088 ± 523	0.21	0.41	93.0			7.0
	Microbotryomycetes	0.3	90 ± 24	126 ± 30	104 ± 19	1.00	0.02	94.8	5.2		
	other Basidiomycota	0.25	50 ± 12	149 ± 46	73 ± 17	1.00	0.01	88.0		12.0	
Mortierellomycota	*Mortierellomycetes*	3.42	1379 ± 316	1403 ± 379	868 ± 232	0.33	-0.31	99.4	0.6		
Rozellomycota	*Rozellomycota_cls_Incertae_sedis*	1.58	628 ± 83	761 ± 172	291 ± 64	+	-0.54	37.1	12.0	50.8	
	other Rozellomycota	0.26	173 ± 78	54 ± 14	47 ± 18	*	-0.66	75.3	21.4		3.3

+ P<0.1, * P<0.05, ** P<0.01, *** P<0.001; correlation (rho): -1 0; cumulative abundance (%): 100 0

[1)] Marginal fungal classes of relative abundance lower than 0.1% not shown. [2)] Differences in mean values (±standard errors) for soil properties on plots of low, control and high pH treatment levels. [3)] Benjamini-Hochberg corrected P.

4.4.3 Effect of soil pH treatment on plant community and plant traits

All the dominant grass species reacted strongly to high pH treatment whereby *H. lanatus* increased its cover area and *A. capillaris* and *A. odoratum* decreased their cover areas in the high pH plots ($F_{2,12}$ = 21.4, P < 0.001, $F_{2,12}$ = 15.8, P < 0.001 and $F_{2,12}$ = 110.4, P < 0.001 respectively) (Table 4.6).

Table 4.6 Effect of soil pH manipulation on plant species cover.

Plant species	One-way ANOVA analysis		Soil pH treatment [2]			Spearman's correlation analysis	
	F	P [1]	low	control	high	P [1]	rho
Agrostis capillaris	15.8	***	42 ± 2[a]	40 ± 4.5[a]	12.5 ± 5.2[b]	**	-0.70
Anthoxanthum odoratum	110.4	***	25 ± 1.6[a]	22 ± 1.2[a]	2 ± 0.5[b]	***	-0.81
Holcus lanatus	21.4	***	18 ± 3.4[a]	20 ± 0[a]	59.2 ± 8.0[b]	**	0.78
Ranunculus spp.	4.1	+	0.9 ± 0.4	4.5 ± 1.8	0.4 ± 0.2	0.69	-0.11
Festuca rubra	3.9	+	2.1 ± 0.8	2.72 ± 1.5	16.5 ± 7.0	0.10	0.48
Rumex acetosa	0.5	0.59	1.6 ± 0.4	1.6 ± 0.4	1.1 ± 0.4	0.40	-0.26
Alopecurus pratensis	10.1	**	0 ± 0[a]	0.04 ± 0.02[a]	3 ± 0.9[b]	**	0.78

P symbols and colours: + *P<0.1,* * *P<0.05,* ** *P<0.01,* *** *P<0.001;* *correlation:* -1 0 1

[1] Benjamini-Hochberg adjusted P.[2] Differences in mean values (±standard errors) for soil properties on low, control and high treatment level plots; letters indicate significant differences at p < 0.05 (Tukey HSD post hoc tests).

Plant aboveground biomass yield during one season and belowground biomass yield over two seasons were not affected by soil pH manipulation treatments ($F_{2,12}$ = 1.7, P = 0.223 and $F_{2,12}$ = 0.38, P = 0.693 respectively). Allocation of biomass, calculated by the ratio of aboveground biomass and belowground biomass, was positively correlated with soil pH (r^2 = 0.57, P < 0.05) (Table 4.7).

Plant aboveground biomass C concentration was affected by soil pH treatment while it was lower on high pH treatment plots than on low pH treatment plots ($F_{2,12}$ = 5.33, P < 0.05, posthoc comparisons P < 0.05). Plant root biomass C concentration was not affected by the soil pH treatment ($F_{2,12}$ = 1.32, P = 0.3). Root N concentration was greater on high soil pH than on control pH plots while low pH plots values were similar to both of them ($F_{2,12}$ = 5.36, P < 0.05, posthoc comparisons P < 0.05). Aboveground P concentration was lower on high soil pH than on control pH plots while low pH plots values were similar to both of them ($F_{2,12}$ = 4.21, P < 0.05). For the plant nutrient stocks, only aboveground N stock was affected by soil pH treatment ($F_{2,12}$ = 5.83, P < 0.05) and showed relatively strong positive correlation with soil pH (r^2 = 0.7, P < 0.01) Table 4.7). Plant biomass C:N ratios, aboveground and root, did not respond to soil pH manipulations ($F_{2,12}$ = 1.85, P = 0.2 and $F_{2,12}$ = 3.2, P = 0.07 respectively) while both showed relatively modest negative correlations with soil pH (r^2 = -0.54, P < 0.05 and r^2 = -0.53, P < 0.05 respectively) (Table 4.7). Plant aboveground biomass N:P ratio showed a strong positive correlation with soil pH (r^2 = 0.79, P < 0.001) and also an effect of soil pH treatments ($F_{2,12}$ = 9.06, P < 0.01) (Table 4.7). Root biomass N:P ratio was also affected by soil pH treatment when it was greater on high pH plots than on control plots but low pH plots were indifferent from them ($F_{2,12}$ = 4.88, P < 0.05, posthoc comparisons P < 0.05). Allocation of C, N and P stock to aboveground plant compartment over belowground compartment showed a modest positive correlation with soil pH P (r^2 = 0.57, P < 0.05; r^2 = 0.53, P < 0.05; r^2 = 0.57, P < 0.05 respectively). Only the finest root class (root class of diameter smaller than 0.1 mm) reacted to soil pH changes when it decreased at high pH plots when compared to both, control and low pH plots ($F_{2,12}$ = 7.33, P < 0.01, posthoc comparisons P < 0.05). pH effect was also shown by a relatively strong negative correlation with soil pH (r^2 = -0.54, P < 0.05). Such strong response of the finest root class was not observed on the total root length response ($F_{2,12}$ = 2.05, P < 0.17), potentially due to relatively high variation of the total root length data (Table 4.7).

Table 4.7 Response of plant characteristics to soil pH manipulation.

	One-way ANOVA analysis		Soil pH treatment [1)]			Pearson's correlation analysis	
	F	P	low	control	high	P	cor
plant biomass							
aboveground (g m^{-2})	1.70	0.22	386 ± 58	453 ± 27	545 ± 84	0.11	0.43
root (g m^{-2})	0.38	0.69	221 ± 13	236 ± 24	203 ± 38	0.48	-0.2
aboveground/root ratio	3.10	+	1.7 ± 0.2	2.1 ± 0.4	2.8 ± 0.3	*	0.57
aboveground nutrient concentration							
C (mg C g^{-1} dry biomass)	5.33	*	451 ± 2^{a}	447 ± 1^{ab}	445 ± 1^{b}	**	-0.69
N (mg N g^{-1} dry biomass)	1.84	0.20	11 ± 1	11 ± 1	13 ± 1	*	0.54
P (mg P g^{-1} dry biomass)	4.21	*	3.0± 0.1^{a}	2.8 ± 0.1^{ab}	2.6 ± 0.1^{b}	+	-0.48
C/N ratio	1.85	0.20	41 ± 2	41 ± 2	35 ± 3	*	-0.54
C/P ratio	3.14	+	147 ± 5	165 ± 9	171 ± 7	0.11	0.43
N/P ratio	9.06	**	3.6 ± 0.1^{a}	4.1 ± 0.3^{ab}	4.9 ± 0.2^{b}	***	0.79
aboveground nutrient stock							
C (g C m^{-2})	1.55	0.25	174 ± 27	203 ± 12	242 ± 37	0.13	0.41
N (g N m^{-2})	5.83	*	4.2 ± 0.6^{a}	5.0 ± 0.1^{ab}	6.7 ± 0.7^{b}	**	0.7
P (g P m^{-2})	0.62	0.56	1.2 ± 0.1	1.2 ± 0.1	1.4 ± 0.2	0.30	0.28
root nutrients concentration							
C (mg C g^{-1} dry biomass)	1.32	0.30	427 ± 4.1	399 ± 23	426 ± 5.6	0.84	0.06
N (mg N g^{-1} dry biomass)	5.36	*	9.1 ± 0.4^{ab}	8.8 ± 0.3^{a}	11.4 ± 0.9^{b}	*	0.54
P (mg P g^{-1} dry biomass)	1.40	0.28	1.3 ± 0.1	1.4 ± 0.1	1.2 ± 0.1	0.34	-0.26
C/N ratio	3.20	+	47 ± 2.4	46 ± 2.7	38 ± 2.9	*	-0.53
C/P ratio	1.51	0.26	328 ± 25	290 ± 35	368 ± 35	0.34	0.27
N/P ratio	4.88	*	7.1 ± 0.9^{ab}	6.3 ± 0.5^{a}	9.8 ± 1^{b}	+	0.5
root nutrients stock							
C (g C m^{-2})	0.11	0.90	47.1 ± 2.9	46.6 ± 4.3	43.6 ± 8.6	0.55	-0.17
N (g N m^{-2})	0.19	0.83	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.2	0.71	0.1
P (g P m^{-2})	2.22	0.15	0.15 ± 0	0.17 ± 0	0.12 ± 0	0.19	-0.36
aboveground/belowground nutrient allocation							
C concentration	1.33	0.30	1.1 ± 0	1.1 ± 0.1	1.0 ± 0	0.68	-0.12
N concentration	0.59	0.57	1.2 ± 0.1	1.26 ± 0.1	1.14 ± 0.1	0.79	-0.08
P concentration	1.40	0.28	2.4 ± 0.2	2.0 ± 0.2	2.2 ± 0.1	0.87	-0.05
C stock	3.05	+	3.6 ± 0.4	4.6 ± 0.7	5.8 ± 0.7	*	0.57
N stock	1.67	0.23	4.2 ± 0.6	5.1 ± 0.8	6.5 ± 1.2	*	0.53
P stock	3.36	+	8.0 ± 0.6	8.4 ± 1.9	12.2 ± 0.9	*	0.57
root characteristics							
total root length (m)	2.05	0.17	16551 ± 1338	15137 ± 1147	12485 ± 1767	+	-0.51
SRL (m g^{-1})	1.38	0.29	415 ± 27	364 ± 35	350 ± 23	0.26	-0.31
root diameter class length							
[< 0.1 mm] class length (cm)	7.33	**	7770 ± 738^{a}	7433 ± 555^{a}	4648 ± 592^{b}	**	-0.7
[0.1 - 0.2 mm] class length (cm)	0.46	0.64	6792 ± 510	5766 ± 547	6159 ± 1097	0.60	-0.15
[0.2 - 0.5 mm] class length (cm)	0.87	0.44	1854 ± 122	1754 ± 176	1542 ± 203	0.15	-0.39
[0.5 - 1 mm] class length (cm)	1.00	0.40	121 ± 12	166 ± 39	120 ± 20	0.60	-0.15
[1 - 2 mm] class length (cm)	0.39	0.68	7.2 ± 2.3	11.2 ± 4.2	10.6 ± 3.5	0.70	0.11
[> 2 mm] class length (cm)	0.07	0.93	1.4 ± 0.9	1.04 ± 0.6	1.27 ± 0.6	0.99	0

\+ *P<0.1,* * *P<0.05,* ** *P<0.01,* *** *P<0.001;* *correlation:* -1 0 1

[1)] *Differences in mean values (±standard errors) for soil properties on low, control and high treatment level plots; letters indicate significant differences at p < 0.05 (Tukey HSD post hoc tests).*

4.4.5 Effect of soil pH treatment on processes

Soil basal respiration expressed per g of soil and also per microbial biomass C increased in high pH treatment sites compared to both control and low pH ($F_{2,12}$ = 14.3, P < 0.01 and $F_{2,12}$ = 7.1, P < 0.01 respectively; posthoc comparisons P < 0.05) (Table 4.8). From ecosystem C flux measurements, only ecosystem respiration was affected by soil pH treatment whereby it was greater on high pH treatment plots when compared to low pH plots ($F_{2,11}$ = 5.3, P < 0.05; posthoc comparisons P < 0.05) (Table 4.8). For ingrowth core C flux measurements, only those with 35 µm mesh were affected by the soil pH treatment and it was higher on high pH plots when compared to both, control and low pH plots ($F_{2,11}$ = 5.8, P < 0.05; posthoc comparisons P < 0.05).

Net N-mineralization rates were strongly affected by soil pH manipulation ($F_{2,12}$ = 19.1, P < 0.001) and both low and control pH treatment plots showed very low N-mineralization rate compared to the high pH (Table 4.8).

For potential activity of enzymes assayed (Table 4.8), only AG and LAP responded to the soil pH manipulation ($F_{2,12}$ = 9.9, P<0.01 and $F_{2,12}$ = 9.9, P < 0.01 respectively) and were positively correlated with soil pH (r^2 = 0.75, P < 0.01 and r^2 = 0.84, P < 0.001 respectively). Only enzyme ratios of C:N and N:P were affected by soil pH ($F_{2,12}$ = 14.6, P < 0.01 and $F_{2,12}$ = 24.2, P < 0.001 respectively) and negatively or positively correlated with soil pH (r^2 = -0.78, P < 0.01 or r^2 = 0.87, P < 0.001 respectively). Specific enzyme activities recalculated to microbial biomass C (and N in case of LEU) followed the same trend as potential enzyme activities (Table 4.8).

Table 4.8 Response of ecosystem processes to soil pH manipulation.

	One-way ANOVA analysis		Soil pH treatment [1)]			Pearson's correlation analysis	
	F	P	low	control	high	P	cor
basal respiration							
(µg CO_2-C g^{-1} dry soil h^{-1})	14.3	**	$1.52^{a} \pm 0.4$	$1.84^{a} \pm 0.4$	$6.9^{b} \pm 1.3$	***	0.85
(ng CO_2-C g^{-1} microbial-C h^{-1})	7.1	**	$8.0^{a} \pm 2.5$	$9.4^{a} \pm 2.2$	$34^{b} \pm 8.8$	**	0.77
ecosystem respiration							
(mg CO_2-C m^{-2} h^{-1})	5.3	*	$467^{a} \pm 45$	$672^{ab} \pm 31$	$854^{b} \pm 136$	**	0.72
(ng CO_2-C g^{-1} microbial-C h^{-1})	2.1	0.17	0.054 ± 0.007	0.085 ± 0.008	0.100 ± 0.026	*	0.52
ecosystem NEE							
(mg CO_2-C m^{-2} h^{-1})	0.3	0.75	-940 ± 127	-757 ± 186	-944 ± 267	0.72	-0.10
ecosystem photosynthesis							
(mg CO_2-C m^{-2} h^{-1})	0.8	0.45	1407 ± 136	1428 ± 213	1849 ± 403	0.13	0.43
(mg CO_2-C g^{-1} plant biomass-C h^{-1})	0.6	0.56	3.87 ± 0.5	3.2 ± 0.5	3.1 ± 0.6	0.58	-0.16
ingrowth core respiration							
1 µm mesh (mg CO_2-C m^{-2} h^{-1})	2.0	0.18	103 ± 2	141 ± 21	148± 17	0.25	0.33
35 µm mesh (mg CO_2-C m^{-2} h^{-1})	5.8	*	$130^{a} \pm 7$	$127^{a} \pm 8$	$180^{b} \pm 18$	*	0.64
1000 µm mesh (mg CO_2-C m^{-2} h^{-1})	1.6	0.25	151 ± 17	162 ± 12	187 ± 11	0.19	0.43
root associated (mg CO_2-C m^{-2} h^{-1})	2.1	0.17	21 ± 15	35 ± 7	-1 ± 11	0.18	-0.38
hyphal associated (mg CO_2-C m^{-2} h^{-1})	2.2	0.16	22 ± 6	-13 ± 14	31 ± 21	0.28	0.31
net N mineralization							
(µg N g^{-1} dry soil)	19.1	***	$0.8^{a} \pm 0.1$	$0.6^{a} \pm 0.1$	$27.2^{b} \pm 6.1$	***	0.92
(ng N g^{-1} microbial-C)	14.3	**	$4^{a} \pm 0.5$	$3^{a} \pm 0.3$	$128^{b} \pm 33.1$	***	0.90
(µg NH_4^+-N g^{-1} dry soil)	7.0	*	$0.04^{a} \pm 0$	$0.02^{a} \pm 0$	$18^{b} \pm 7$	***	0.81
(µg NO_3^--N g^{-1} dry soil)	5.4	*	$0.8^{a} \pm 0.1$	$0.6^{a} \pm 0.1$	$8.8^{b} \pm 3.5$	*	0.64
potential soil enzyme kinetics							
AG (nmol g^{-1} dry soil min^{-1})	9.9	**	$0.20^{a} \pm 0$	$0.22^{a} \pm 0$	$0.35^{b} \pm 0$	**	0.75
BG (nmol g^{-1} dry soil min^{-1})	1.4	0.29	1.9 ± 0.2	2.8 ± 0.4	2.5 ± 0.5	0.68	0.12
C enzymes (nmol g^{-1} dry soil min^{-1})	1.4	0.28	2.12 ± 0.22	3.06 ± 0.39	2.88 ± 0.58	0.53	0.17
CHIN (nmol g^{-1} dry soil min^{-1})	0.4	0.70	0.75 ± 0.04	0.88 ± 0.17	0.76 ± 0.12	0.72	-0.1
ACE (nmol g^{-1} dry soil min^{-1})	2.7	+	37 ± 3	44 ± 3	37 ± 2	0.30	-0.29
LEU (nmol g^{-1} dry soil min^{-1})	20.7	***	$2.3^{a} \pm 0.1$	$3.2^{a} \pm 0.3$	$6.3^{b} \pm 0.8$	***	0.84
PHO (nmol g^{-1} dry soil min^{-1})	3.4	+	$8.5^{a} \pm 0.5$	$11.9^{b} \pm 1.2$	$11.5^{ab} \pm 1.1$	0.27	0.30
SUL (nmol g^{-1} dry soil min^{-1})	7.5	**	$0.33^{a} \pm 0.02$	$0.52^{b} \pm 0.06$	$0.54^{b} \pm 0.04$	+	0.49
C:N enzyme kinetics ratio	14.6	**	$0.86^{a} \pm 0.10$	$0.89^{a} \pm 0.07$	$0.39^{b} \pm 0.05$	**	-0.78
C:P enzyme kinetics ratio	0.3	0.71	0.23 ± 0.03	0.24 ± 0.02	0.21 ± 0.03	0.48	-0.2
N:P enzyme kinetics ratio	24.2	***	$0.27^{a} \pm 0.02$	$0.27^{a} \pm 0.02$	$0.55^{b} \pm 0.05$	***	0.87
C:S enzyme kinetics ratio	1.3	0.32	5.77 ± 0.54	5.4 ± 0.42	4.52 ± 0.71	0.11	-0.43
specific soil enzyme kinetics							
AG (pmol g^{-1} microbial-C min^{-1})	4.0	*	1.0 ± 0.1	1.1 ± 0.1	1.7 ± 0.3	**	0.65
BG (pmol g^{-1} microbial-C min^{-1})	1.3	0.32	9.7 ± 1.7	14.4 ± 1.8	12 ± 2.6	0.81	0.07
CHIN (pmol g^{-1} microbial-C min^{-1})	0.4	0.65	3.8 ± 0.5	4.4 ± 0.8	3.6 ± 0.6	0.69	-0.11
ACE (pmol g^{-1} microbial-C min^{-1})	4.3	*	$182^{a} \pm 11$	$226^{b} \pm 10$	$174^{ab} \pm 19$	0.33	-0.27
LEU (pmol g^{-1} microbial-C min^{-1})	11.2	**	$11^{a} \pm 1$	$16^{a} \pm 1$	$30^{b} \pm 5$	***	0.8
LEU (pmol g^{-1} microbial-N min^{-1})	9.3	**	$76^{a} \pm 9$	$103^{a} \pm 4$	$204^{b} \pm 37$	***	0.79
PHO (pmol g^{-1} microbial-C min^{-1})	2.8	+	$42^{a} \pm 4$	$60^{bc} \pm 5$	$55^{ac} \pm 7$	0.41	0.23
SUL (pmol g^{-1} microbial-C min^{-1})	6.7	*	$1.65^{a} \pm 0.17$	$2.64^{b} \pm 0.18$	$2.57^{b} \pm 0.27$	0.10	0.44

+ *P<0.1,* * *P<0.05,* ** *P<0.01,* *** *P<0.001;* *correlation:* -1 0 1

[1)] *Differences in mean values (±standard errors) for soil properties on low, control and high treatment level plots; letters indicate significant differences at p < 0.05 (Tukey HSD post hoc tests).*

4.5 Discussion

Opposing soil pH modifications resulted in differential impacts on studied grassland: increasing soil pH initiated substantial changes of several important ecosystem parameters while lowering soil pH did not show such strong effects (Table 4.9). High pH treatment increased availability of soil substrates (DOC, DON) and mineral N which promoted plant investment into aboveground compartment vs. belowground compartment. Aboveground and belowground biomass yield was not changed, but very fine root length (< 0.1 mm) decreased on high pH plots. Induced soil N availability enhanced aboveground plant N stock when compared to low pH treatment plots and also raised root N concentration but not aboveground N concentration however positive correlation of biomass N of both plant compartments was observed. Plant community structure have changed whereby fast growing plant species were promoted on high pH treatment plots. Ecosystem respiration was enhanced on high pH plots, but this was not manifested by increased soil *in situ* respiration, when only respiration of 35 µm mesh ingrowth cores reacted positively to pH increase. Together with increases of soil N availability and net N mineralization activity of LAP was also enhanced, while higher ecosystem respiration was not accompanied by an increase of BG. Like many other measured variables, the largest impacts on microbial community were in the raised pH plots and greater response was observed at lower taxonomic resolution (i.e. class) than phylum and for fungal community composition when compared to bacterial composition. The nutrient rich environment on high pH plots did not affect microbial biomass C and N concentrations. In total, raising soil pH by liming to near neutral level (7.3 ± 0.2) resulted in more open soil N cycle (higher mineral N availability, faster N mineralization) and relatively rapid changes in plant community structure as well as bacterial and fungal composition changes.

Table 4.9 Summary of key findings.

	soil pH manipulation treatment		
	low pH	**control pH**	**high pH**
soil and microbial nutrients			
soil mineral N, DON	▬	▬	↑
DOC	▬	▬	↑
Olsen P	▬	▬	▬
microbial biomass C, N, C:N	▬	▬	▬
plant characteristics			
aboveground C concentration, P concentration	↑	▬	↓
aboveground N stock	↓	▬	↑
root N concentration	▬	▬	↑
< 0.1 mm diameter root length	▬	▬	↓
ecosystem process rates			
respiration (ecosystem, basal)	▬	▬	↑
soil respiration (*in situ*; 1 mm ingrowth mesocosm)	▬	▬	▬
35 µm ingrowth mesocosm respiration	▬	▬	↑
C cycle extracellular enzyme activity - AG	▬	▬	↑
C cycle extracellular enzyme activity - BG	▬	▬	▬
net N mineralization (ammonification, nitrification)	▬	▬	↑
N cycle extracellular enzyme activity - LEU	▬	▬	↑
P cycle extracellular enzyme activity – alkaline PHO	▬	▬	▬

↑ difference compared to other treatments; ↑ difference compared to treatment with opposite arrow of the same colour; ▬ no difference

4.5.1 Effect of soil pH on soil nutrients

The concentration of the dissolved fraction of OC in the soil was strongly positively correlated with soil pH and significant increases of DOC and DON concentrations were observed on high pH treatment plots. Similar to the present study, liming increased DOC and DON concentration in short term experiments with plants which were grown in limed soil in pots (Filep et al., 2003). Increase of DOC due to high pH is understood to be due to counterbalancing changes in dissolved cations after application of a base in order to maintain soil equilibrium (Curtin et al., 2016). Such increases in DOC as frequently observed in short term studies may not be sustained in the long term as shown by Kemmitt et al. (2006) studying sites with over 40 years of history of liming whereby DOC was lower on limed plots.

Liming treatment increased concentration of overall mineral N in the soil as driven by the increase of ammonium. This was expected as liming is a frequent agricultural management practise for increasing soil N cycling (Kemmit et al., 2006) helping to sustain and improve plant growth (Fornara et al., 2011). Similar to the present research, Zhalnina et al. (2014) found an association of mineral N (ammonia, nitrate-N) with soil pH in grassland with a pH gradient in a long term experiment. Heyburn et al. (2017a) also showed higher soil mineral N concentration in a 22-year limed experimental grassland plots but this was in the form of nitrate in contrast to the present study which showed only marginal nitrate concentrations on limed plots. Form of mineral N available in the soil reflects overall bioavailability of N in the soil (Schimel and Bennet, 2004). Ammonium would dominate the soil under moderate N bioavailability and combined plant and microbial uptake would limit overall ammonium supply to nitrifiers as they are poor competitors for ammonium, while increasing nitrate presence results from increased soil N bioavailability (Schimel and Bennet, 2004). The present results may thus suggest that although mineral N concentration increased on limed plots, the overall bioavailability of N is not high enough to sustain nitrification.

The observed lack of extractable N in the soil of control plots in the present study shows that the studied grassland is strongly N limited. It can thus be expected that observed increase of N availability would have a strong effect on the overall ecosystem.

4.5.2 Effect of soil pH on microbial community biomass

Microbial biomass C was not affected by soil pH, showing stability of microbial community size, even with increase of availability of soil nutrients such as DOC, DON and mineral N. Microbial community size is usually correlated with soil C stock. Overall soil C stock was not changed, thus similar can be expected for microbial biomass size. Others also did not find a change of microbial biomass after liming (Fornara et al., 2011; Lochon et al., 2019), however Fornara et al. (2011) showed an increase of SOC. Johnson et al. (2005) found no change of microbial biomass in A horizon after liming but its decrease in F and H horizons. On the other hand, Kemmitt et al. (2006) found increase of microbial biomass C despite no changes in SOC. Zhalnina et al. (2014) found positive correlation of microbial biomass C and soil pH.

Similar to microbial biomass C, microbial biomass N and C:N ratio did not change after soil pH manipulations. Lochon et al. (2018) did not find a change in microbial biomass N in Jun and Sep on limed grassland plots, which covers the period of the sampling in the present study, but they found a decrease of microbial biomass C:N ratio without biomass C change in April suggesting an increase of the biomass N. Kemmitt et al. (2006) did not find a change in microbial biomass N in response to increasing levels of lime application on grasslands but they found correlation of soil pH with the biomass N.

4.5.3 Effect of soil pH on microbial community composition

In the present study, soil pH affected both, bacterial and fungal community structure at different taxonomic resolutions including phylum, class, order and species as it was determined by M-GLM. However, pair-wise comparisons among sites showed differences only for the fungal community at the lower resolutions tested (i.e. order and species resolutions).

It was found that for bacterial phyla of abundance > 1 %, only *Bacteroidetes* and *Chloroflexi* correlated with soil pH. Others found positive correlation of soil pH with *Proteobacteria* at the local scale (Zhalnina et al., 2014), *Actinobacteria* and *Bacteroidetes* at the continental scale (Lauber et al., 2009) and negative correlation of *Acidobacteria* with soil pH (Lauber et al., 2009).

A greater responsiveness of bacterial taxa to soil pH was found at class resolution, the resolution that is often not reported in many studies (Lanzén et al., 2015). *Acidobacteria*, which is typically described as responding to soil pH, showed a greater response at class and order taxonomic resolutions than at the phylum resolution. *Acidobacteria's* group 6 was positively correlated with soil pH while *Acidobacteria*'s group 1 (*Acidobacteriia*) and group 2 (*DA052*) were negatively correlated with soil pH. This pattern of *Acidobacteria* classes response to soil pH was also found by Griffiths et al. (2011). Likewise, the phylum *Proteobacteria* did not show a response to soil pH but its classes of *Alpha-*, *Delta-* and *Gamma-Proteobacteria* showed a response to soil pH.

Interestingly, *Firmicutes* comprised somewhat high relative abundance than usually reported (e.g. (e.g. Kaiser et al., 2016) but similar to Zhalnina et al. (2014). They argued that presence of animal-associated microbiota (mainly belonging to phylum *Firmicutes*) which did not correlate with any examined soil parameters (e.g. soil pH) is related to a history of grazing on the site. This may suggest that the high relative abundance of *Firmicutes* in the present research can be due to winter sheep grazing on the plots.

The most abundant fungal phyla showed a similar relative abundance as compared to another study of grassland from the same region (Leff et al., 2018) including high relative abundance of *Ascomycetes*. Worldwide, phylum *Basidiomycetes* was found the most abundant phyla in grasslands and scrublands and also in general in soils (Tedersoo et al., 2014). It was found that class *Agaricomycetes*, which belongs to phylum *Basidiomycetes*, encompassed only 18% of the total sequences while it comprised over 50 % of the sequences for the similar ecosystem determined worldwide (Tedersoo et al., 2014). Moreover, it was found that ratio of *Ascomycetes* and *Basidiomycetes* OTU richness was higher by 1 unit than the same ratio calculated for grasslands and scrublands worldwide by Tedersoo et al. (2014). This all may suggest that *Basidiomycetes* sequences are underrepresented in the present study. Tedersoo et al. (2014) targeted a different region on fungal DNA to infer the phylogeny. Differences in the representation and structure of *Basidiomycetes* were observed using various primer sets (Kuramae et al., 2013). Phylum *Mortierellomycota* was found to comprise on average 6.3 % of the fungal sequences in a global study (Tedersoo et al., 2014) ranking the third most abundant fungal phylum which is similar to the present results. Phylum *Glomeromycota* was present at very low relative abundance compared to Leff et al. (2018).

The posed hypothesis that increase of soil pH of extensively managed grassland will promote copiotrophic over oligotrophic bacterial lifestyles cannot be confirmed using phylum and class level taxonomic resolution as was hypothesized. Bacterial taxa considered copiotrophic include *Actinobacteria*, *Betaproteobacteria*, and *Firmicutes* (Fierer et al., 2007; Ramirez et al., 2012). Only *Betaproteobacteria* showed copiotrophic lifestyle whereby it increased its relative abundance with soil pH increase, although the relative abundance of this class was lower than reported elsewhere

(Fierer et al., 2007a; Kaiser et al., 2016). *Actinobacteria* and *Firmicutes* did not respond to soil nutrient changes. Leff et al., (2015) determined *Alphaproteobacteria* as copiotrophic species and it has been found positively correlated with soil pH in an arable soil (Rousk et al., 2010b). On the contrary, the present research showed its negative relationship with soil pH suggesting it can be describe as oligotroph. Goldfarb et al. (2011) found that relative abundance of *Alphaproteobacteria* declined after addition of glycine and associated this class with oligotrophic lifestyle. Oligotrophic taxa include *Acidobacteria* and *Verrucomicrobia* (Fierer et al., 2007; Ramirez et al., 2012), however, in the present study, *Acidobacteria* and *Verrucomicrobia* showed no significant differences in relative abundance in relation to soil pH manipulation. This suggests that phylum and class based taxonomic resolution may not be appropriate for determination of microbial trophic strategy (Fierer et al., 2007; Morrissey et al., 2016) and specifically as a response to soil pH mediated changes of soil nutrients. Microbial responses examined at phylotype level might be more appropriate (Jones et al., 2019).

4.5.4 Effect of soil pH on plants

Plant aboveground and belowground biomass did not respond to soil pH treatments when analysed individually. This is in agreement with Hejcman et al. (2010) and Logon et al. (2019). However, the present plant biomass unresponsiveness to liming contrasts with others who showed positive plant aboveground biomass response to liming. Application of lime typically increases mineral N concentration in the soil (e.g. Kemmitt et al., 2006) and it was thus expected that aboveground biomass will respond positively to the higher N availability. Increase of aboveground biomass after liming was observed by Kemmitt et al. (2006), Galbally et al. (2010) and Egan et al. (2018) in a long-term crop rotation experiment (including grassland), short term grassland and long term grassland experiments respectively. In addition, increased N enrichment of ecosystems was shown to promote plant biomass as observed in a global study (Lebauer and Treseder, 2008; Xia and Wan, 2008).

On the other hand, the present research suggested higher biomass allocation to the aboveground rather than belowground compartment as aboveground:belowground biomass ratio positively correlated with soil pH. This could reflect the reduced role of roots for nutrient foraging under higher mineral N availability (Logon et al., 2019) because plants are expected to allocate more resources into root systems in low-nutrient environments to boost their uptake capacities (Müller et al., 2000). Root mass decreased after liming in a 19-year study (Cenini et al., 2015).

Overall root length did not show a statistically significant response to pH treatment because of high variability. Nevertheless, the finest root diameter class (of diameter less than 0.1 mm) decreased with soil pH increase. This fraction represented 37 % of total root length but was associated only with less than 20 % of the root mass (based on root volume calculation, data not shown) which might explain why overall mass of roots was not affected. The decrease of very fine roots might reflect a change of plant C allocation strategy which was initiated by high mineral N concentration (Eissenstat, 1992). Lower investment into very fine roots may be simply due to higher nutrient availability in the soil and thus either need for extensive root foraging or competition with microbes for N are lower under such conditions. It might also reflect a shift of plant strategy for nutrient acquisition whereby plants would rely less on acquisition function of own root system but may promote acquisition of nutrients through a fungal network associated with roots (i.e. AMF). Indeed, liming has been found to promote root colonization by AMF (Johnson et al., 2005).

Plant biomass N concentration was the most responsive biomass element when compared to concentrations of other main biomass elements (i.e. C and P) whereby its concentration was positively correlated with soil pH for both, belowground and aboveground plant compartments. This resulted in an enrichment of N relative to C in the plant biomass with soil pH increase as was shown

by negative correlation of biomass C:N concentration ratios with soil pH for both plant compartments. Logon et al. (2019) also showed plant biomass nutrients response to liming such as decrease of aboveground biomass C:N ratio. Observed plant biomass C:N ratio response to N availability induced by liming promoted soil N availability agrees with Fornara and Tillman (2012) who showed a decrease of root C:N ratio after N addition. However, Heyburn et al. (2018) showed an opposite trend whereby aboveground biomass C:N ratio increased after liming in a long term study.

Liming treatment strongly affected plant community composition. Biomass cover of *Agrostis capilaris* and *Anthoxanthum odoratum* correlated negatively, while the cover of *Holcus lanatus* positively with soil pH respectively. It can be anticipated that these shifts are associated with soil N availability due to strong correlation of soil pH with soil N availability. *H. lanatus* is a fast growing species (Baxendale et al., 2014) and strongly benefited from the increased mineral N availability on the high pH plots in this study. On the other hand, *A. odoratum* is a slow growing species (Baxendale et al., 2014) and decreased in the plots of high N availability. However, *A. capilaris* (fast growing; (Baxendale et al., 2014) reacted to the N availability in the opposite direction than would be predicted based on its growth rate characteristics.

4.5.6 Effect on soil processes

C cycle

Total potential extracellular enzyme hydrolytic activity associated with soil C cycle (sum of AG and BG potential enzyme activities) did not respond to soil pH manipulation, while its smaller component, the potential activity of AG enzyme, increased on liming plots and was positively correlated with soil pH. AG activity was on average more than 10 and 7 times lower than the BG activity on control and high pH plots respectively in the present research which is in the range found by others (Acosta-Martínez and Tabatabai, 2000; Hernández and Hobbie, 2010).

Soil organic C content and pH are strong factors shaping activities of extracellular enzymes in the soil (Turner et al., 2002; Sinsabaugh et al., 2008; Hendriksen et al., 2016). Glycosidase hydrolytic enzymes have pH optima at pH 5 (± 1) (Sinsabaugh et al., 2008) thus decrease in their activities may be expected in relation to increase of soil pH to above pH 7 such as for the high pH treatment in the present research. However, this was not the case. On the other hand, glycosidase activity was found to be positively related to soil pH increase initiated by liming in arable soils whereby BG activity was the most sensitive (Acosta-Martinez and Tabatabai, 2000). Turner et al. (2002) demonstrated that most of the variation of BG activity was related to SOC and microbial biomass C in acidic grasslands and similarly, Štursová and Baldrian (2011) showed that soil OC affected β-glucosidase activity in grasslands regardless of difference in soil pH. Ekenler and Tabatabai (2003) suggested that stimulation of microbial population and diversity is behind positive effect of soil pH on glycosidase activity in the soil. In the present study, both, SOC and microbial biomass C did not change with pH increase which might be behind non-responsiveness of BG activity to soil pH.

BG generally shows the greatest activity in the soil of all glycosidase enzymes studied (Ekenler and Tabatabai, 2003) and is involved in cellulose degradation producing ready available substrate for microbial use (i.e. glucose) (Eivazi and Tabatabai, 1988). Fornara et al. (2011) showed SOC increase after liming in the long term and argued that this increase was due to a greater microbial processing of plant inputs. Non-responsiveness of BG in the high pH treatment might thus suggest that decomposition of cellulose-containing compounds derived from plant primary production such as litter and POM was not enhanced in the present short-term study, however, it can be expected to increase in the future.

Interestingly, BG activity was correlated (Pearson's correlation, cor = 0.71, $P < 0.01$) with soil respiration related to soil without an influence of roots and mycorrhiza (1 µm mesh ingrowth core), This may suggest that in the soil not directly affected by rhizosphere, microbes are energy limited and use cellulose to satisfy their requirements. Nevertheless, respiration on 1 µm mesh ingrowth core did not respond to soil pH treatments.

The increased activity of AG on limed plots may point to higher starch decomposition in the soil. German et al. (2011) showed that AG activity was related to starch added to the soil, however, the threshold starch concentration for initiation AG response was relatively high compared to normal soil starch content. Nevertheless, increased soil starch decomposition was found after aggregate breakdown which exposes physically protected starch within soil aggregate pores (Adu and Oades, 1978). Such a greater aggregate turnover would also involve a greater decomposition of released POM (Six et al., 2004) and associated increase of BG activity, which has not been observed. On the other hand, AG can be also related to microbial biomass turnover as AG is able to degrade cell wall sugars (Gude et al., 2012). This could point towards a higher microbial biomass turnover on high pH plots.

N cycle

Strong positive correlation of LEU enzyme with soil pH and with total net N mineralization rate (Pearson's correlation, cor = 0.67, $P < 0.01$) was observed suggesting that LEU activity is responsible for the increase of soil mineral N availability. LEU is primary responsible for degradation of proteins in the soil releasing amino acids (mainly leucine) and it is understood to represent an overall peptidase activity in the soil (Sinsabaugh et al., 2008). Proteins and amino acids represent the largest input of organic N into agricultural systems and are consequently the major substrate for inorganic N production (Kemmitt et al., 2006). Proteins originates from plant inputs and microbial biomass turnover, however their majority is not freely availably but protected by association with soil matrix and must be solubilized prior mineralization (Lipson and Näsholm, 2001). Thus, proteolysis of soil proteins is generally considered to be the rate-limiting step in N mineralization (Weintraub and Schimel, 2005). LEU has pH optima at 7 – 9 and soil pH increase by high pH treatment in the present research may have been beneficial for its activity, however peptidase activity is not always strongly associated with soil pH and the relationship is complex (Hendriksen et al., 2016).

Other potential driver of LEU activity and the increase of mineral N in the soil of the high pH treatment might be a stoichiometric imbalance between available substrates and microbial requirements (Mooshammer et al., 2014). C:N ratio of available substrates (DOC:DON ratio) was on average 11.25 (± 1.7, s.e.) while microbial biomass C:N ratio was 6.7 (± 0.3, s.e.) on the high pH plots. Sinsabaugh et al. (2013) argued that mean terrestrial CUE approach 0.3. Microbial community thus needed to adjust its N use efficiency (Mooshammer et al., 2014b) on high pH plots by excreting the excess of N into the soil through its mineralization.

Interestingly, LEU activity strongly correlated with AG activity in the soil (Pearson's correlation, cor = 0.96, $P < 0.001$) suggesting that both enzymes might be associated with substrates which have the same turnover such as for instance microbial cells or fine roots degradation. In the case of the root degradation, BG activity should also increase as plant material contains cellulose, but such an increase was not determined.

4.5.7 Effect of soil pH on ecosystem nutrient pools

Raising soil pH resulted in an increase of labile C and N pools but a decrease of labile P pool within the ecosystem. Moreover, amount of C, N and P allocated to aboveground plant pool compared to the belowground pool positively correlated with soil pH. These nutrients allocated to the aboveground compartment were removed from the system by a hay cut at the peak of the season. This is important especially for P which originate in the soil and to some degree for N however it can also enter the system via N deposition. N fixation would have been minimal as legumes formed only a marginal part of plant community but presence of free living N fixers cannot be discounted.

Increase of ecosystem C cycling was represented by greater soil DOC pool and ecosystem respiration but it was not reflected in microbial biomass C or soil C related extracellular enzyme activity (such as BG activity). This raises the question whether the increased ecosystem C cycling was related to plant growth and increase of photosynthate assimilation and mineralization or originated from SOC stock.

Greater plant respiration and allocation of recent photosynthates to the soil for microbial utilization can be suggested by an increase of plant photosynthesis (Rangel-Castro et al., 2004). Plant photosynthesis measurements did not show a difference among treatments in the present study, however this might be due to a large within treatment variation, while treatment mean values indicated an increase of the photosynthesis with soil pH increase. Soil C fluxes measured employing ingrowth core microcosms showed an increase of C flux related to fungal ingrowth core (35 µm mesh) potentially representing root associated fungi. Altogether, this might imply a greater influx of recent plant photosynthates and their allocation to soil microbiota (Rangel-Castro et al., 2004). Plants on high pH plots reduced investment into the very fine roots. Nutrient acquisitive role of these very fine roots can be substituted by AMF network associated with plant roots (Schimmel and Bennet, 2004). Indeed, Johnson et al. (2005) found a higher AMF root infestation after liming. Greater relative abundance of AMF (i.e. phylum *Glomeromycota*) in the limed soil treatment was not confirmed by the present data. It can be thus only speculated to what extent is the increased soil respiration related to fungal growth related to greater plant AMF infestation and allocation of recent photosynthate into the microbial food web through fungal-root interactions. In addition, part of the ecosystem respiration will be attributable to plant respiration. Pausch and Kuzyakov (2018) estimated that up to a third of yearly gross primary production is diverted to shoot respiration and 6 % to root respiration in grasslands.

SOC was not affected by soil pH which can be expected in this very short-term study. Changes in SOC are known to be relatively slow over time, requiring many years for their detection (Heyburn et al., 2017a). Others found increase of OC associated with soil mineral matrix and decrease of aboveground litter layer (Fornara et al., 2011) or no change of SOC at all while OC was increasingly more associated with smaller soil aggregates due to soil mass transfer (Egan et al., 2018) after over 100 years and 22 years of liming respectively. Change of microbial biomass C:SOC ratio might be an early indicator of SOC response to a management change (Wiesmeier et al., 2019, but see Powlson et al., 1987). SOC and microbial biomass C did not change in the present research indicating equilibrium, however Morrien et al. (2017) showed that fungal uptake of organic C and its processing can be affected without changes in fungal biomass.

Overall, soil pH was found to affect fungal community structure and tended to affect bacterial community structure, however predictable changes in community composition related to bacterial life strategies were not fully achieved through taxonomic ranking at phyla and class resolution. Increased N availability could be linked to increase of activity of extracellular enzyme related to N cycle. Higher N availability in high pH treatment soil altered plant community composition whereby two of the three dominant plant species responded predictably according to their life strategy,

however overall plant growth was not affected. Plant biomass also increased its N concentration and overall N pool. Ecosystem C efflux as well as soil basal respiration increased as well after liming, however such increases cannot be made accountable to processing of plant derived organic matter but instead data suggested that they may be associated with recent plant labile compounds derived through mycorrhizal fungal network, as based on higher respiration of ingrowth microcosms for fungal ingrowth (35 µm mesh).

It will be interesting to determine which active microbial taxa are receiving plant derived photosynthates on high pH treatment vs. acidic pH plots (control pH/low pH treatments) and infer their traits and potential life strategy based on their overall genome. Furthermore, these data combined with overall soil metagenome might inform us about relative importance of individual microbial life strategies involved in soil nutrient cycling for its better understanding (e.g. Rangel-Castro et al., 2004; Hannula et al., 2018; Wood and Franks, 2018; Malik et al., 2018).

Chapter 5: **Effect of fungicide perturbation on soil biotic and abiotic characteristics and ecosystem cycle rates in grassland.**

5.1 Introduction

Soils represent an enormous reservoir of organism diversity which is equal or even surpasses the aboveground total diversity (Wall et al. 2010). Tens of thousands of different bacterial sequences (Roesch et al., 2007) and up to 3.5 km of fungal hyphae (Bardgett, 2005) can be found in 1 g of soil. With the recent development of high throughput sequencing technology and statistical methods, we are starting to determine such diversity more accurately and study its importance for ecosystem functioning (Prosser, 2012). Soil microbes have a key role in a variety of soil processes including cycling of C, N and P (Van Der Heijden et al. 2008) among others and as a result they affect plant community, and impact ecosystems and global climate (Bardgett et al. 2008; Bardgett and Van Der Putten, 2014).

Soil microbial communities are increasingly confronted with disturbances originating from intensification of human activities such as increasing land management intensity. With predicted population rise and growth of human consumption, the pressure on ecosystems is not likely to decrease in the near future. Climate change resulting in a less predictable weather patterns will further magnify the pressure on ecosystems and their services. At the same time, it is becoming evident that loss of soil biodiversity will likely reduce ecosystem multifunctionality (Wagg et al. 2014; Delgado-Baquerizo et al. 2016) resulting in a loss of ecosystem services (e.g. House and Bever 2018). Yachi and Loreau (1999) presented insurance hypothesis whereby they proposed that probability of finding taxa able adaptation to changing conditions and allowing ecosystem functioning is greater in a more diverse ecosystem. Moreover, it was reported that soil microbial community structure is not generally resistant to environmental disturbances (Allison and Martiny, 2008) and it was also confirmed for function as well as microbial composition (Shade et al., 2012) although it may be more difficult to publish studies resulting in no effect of disturbance on microbial communities (Shade et al., 2012). Overall, disturbance has thus a potential to affect ecosystem function through its effect on soil biodiversity. Studies related to disturbance effect on microbial community and its function can increase understanding of a role of microbial community composition in soil function, which is important for better representation of soil microbes in predictive models (Wieder et al., 2013).

Understanding mechanisms of microbial roles in soil responses to a disturbance is challenging due to high soil complexity and variability (Griffiths and Philippot (2013). Microbial community stability in response to a disturbance is a tendency of microbial community to return to pre-disturbance state through resistance and resilience. Biological characteristics contributing to microbial stability include individual, population and community properties (Shade et al., 2012). For instance, growth rate (population property) is an important characteristic for microbial community resilience from a pulse disturbance. Disturbances can result in death or inactivation of local resident taxa, which releases resources and create empty niches. Microbial community can regrow back from surviving individuals utilizing available resources (Shade et al., 2012; De Vries and Shade, 2013).

Disturbance effects on community structure will depends on specific traits (Wallenstein and Hall, 2012; Mouillot et al., 2013; Wood et al., 2018). These traits are related to direct response of individual cells to the disturbance in order to combat the impact of the disturbance (i.e. stress or response traits; Lavorel and Garnier, 2002). Trade-offs related to these traits will be important for community composition response such as for instance trade-offs between disturbance tolerance and growth rate (Shade et al., 2012). Moreover, soil function will be affected if these trade-offs are

related to microbial physiology (e.g. efficiency of C use, Malik et al., 2018c). One way to understand the consequences of a disturbance on soil processes is to determine disturbance response within microbial functional groups and relationship of these functional groups with phylogeny (Allison and Martiny, 2008). Then the prediction of process rate response to disturbance can be estimated from abundance of phylogenetic groups (Martiny et al., 2015). De Vries and Shade (2013) showed that characterizing microbial community life history strategy attributes can be used to explain differences in its responses to environmental disturbances. Specifically, copiotrophic taxa, characterized by fast growth and preference for nutrient rich sites, were suggested to be related to resilience of microbial community, while oligotrophic taxa, with opposite characteristics, were suggested to be related to community resistance. They also found that soil resource availability, among other factors, can impact microbial community response to environmental disturbances whereby more substrate rich sites showed greater microbial resilience.

Soils are subject to many stresses such as for instance drought, waterlogging, heat and freeze thaw. Additionally, anthropogenic additions of agricultural chemicals can also impact soils including fertilisers or pesticides. Fungicides are widely used in agriculture aiming to control fungus related crop diseases. They can act through a variety of modes including for instance affecting synthesis of membrane components (e.g. lipids and sterol). They can target non-specific binding sites and can thus potentially affect non-target organisms (Yang et al., 2011) with consequences for soil processes. Fungicide application to the soil was shown to affect soil fungal and also bacterial community compositions (Bending et al., 2007; Hussain et al., 2009; Imfeld and Vuilleumier, 2012; Feld et al., 2015) . They were also shown to affect soil N and C cycles (i.e. mineral N concentrations and N mineralization rates, and soil respiration) (Chen et al., 2001; Monkiedje et al., 2002; Muñoz-Leoz et al., 2013). For instance, Sukul (2006) showed that fungicide decreased total C and N contents in the soil during 30-day incubation. Their application can impact on potential nitrification activity as well as the microbial community involved (i.e. archaea and bacteria) and the function can be restored faster than community structural changes (Puglisi et al., 2012). Higher soil respiration after fungicide application can result from induced stress of soil microbial community which can last up to 90 days after application while overall microbial activity (i.e. dehydrogenase activity) is reduced (Munoz-Leoz et al., 2013). Fungicides may also affect microbial interactions and promote certain groups of microbes released from competition (Hussain et al., 2009). Results from above studies were derived from laboratory microcosms and soil system responses in natural settings might be different due to complex interactions within soil and between below- and above- ground.

Fungi can affect plant performance directly such as through organic compounds transformations releasing plant nutrients and through plant-associations (i.e. as mutualists or enemies) and indirectly through affecting for instance plant-plant competition. As such, use of fungicides controlling soil fungi is an important tool to study effect of disruption of the fungal community on soil processes and plant community (Smith et al., 2000; Helgason et al., 2007; Dostálek et al., 2013; Bennett and Cahill, 2016a). However, fungicide can have complex and often contradictory effects on different components of microbial communities and functionality. For instance, Smith et al. (2000) showed that fungicide application negatively affected AMF colonization resulting in a decrease of bacterial biomass while overall microbial biomass increased, but overall fungal contribution to total microbial activity decreased. Aboveground and root P concentrations of AMF-dependent plant species were reduced after fungicide application as well as specialist AMF while generalist AMF were promoted (Helgason et al., 2007). Reduction of AMF by fungicide was also shown to affect plant community structure after 3-year application into nutrient-poor acid grassland (Dostalek et al. 2013).

In order to increase the understanding of functional consequences of soil microbial community change, an experiment was designed to test the effects of disturbance of fungal community on soil

and plant properties and processes in soils of different pH levels. This experiment was part of a bigger study including (1) the effect of soil pH on soil (Chapter 4).

5.2 Hypothesis

It was hypothesised that effect of soil pH will be important for the response of soil microbial community and soil nutrient processing to application of a mixture of two different fungicide ('the biocide application'). Fungi are known to prefer acidic soils whereby bacteria are thought to be more active in higher pH soils, where fungal activity might be affected (i.e. reduced) by their competition with bacteria (Rousk et al., 2010b; Barcenas-Moreno et al., 2016). Therefore, the response of microbial community composition and functioning to biocide application targeting fungi might be more pronounced in acidic pH soils.

On the other hand, one of the biocides belongs to strobilurin fungicides, and other representative of this group was found to negatively impact mycorrhizal activity (Diedhiou et al., 2004). Disruption of mycorrhizal network can have consequences for distribution of plant derived organic compounds to the soil through the fungal network and interactions with bacteria and thus might cause a greater effect in high pH soils, where bacterial activity is expected to be higher than in acidic soils. Nevertheless, as it was shown that AMF relative abundance was relatively small and further decreased on high pH treatment plots (Chapter 4) the biocide application effect of mycorrhizal network might not have significant consequences for further soil function. Furthermore, the biocide application will release nutrients from dead microbial cells which can be capitalized by fast growing bacteria, thus affecting bacterial composition, however, in acidic soil where microbial activity is expected to be reduced by pH effect, such bacterial response might be less pronounced.

Moreover, oligotrophic microbial taxa (such as fungi or gram-positive bacteria) which are expected to be more abundant and or active in acidic soils might be more resistant to a disturbance than copiotrophic bacteria (De Vries and Shade, 2013), and thus the community change in low pH soil might be less pronounced. On the other hand, as Chapter 4 showed, association of taxa at high taxonomic resolution (i.e. phyla, class) did showed contradictory response, thus greater responses to the biocide application might be observed at lower taxonomic resolution (i.e. individual phylotypes).

Overall, it is hypothesized that the effect on fungal community will be more pronounced than effect on bacterial community because the biocide application primarily targets fungal community. At the same time, (1) an interactive effect of soil pH treatment and biocide application on soil bacterial and fungal communities will be observed whereby the two acidic soils (low pH and control pH treatment plots) communities will respond in a similar way and different to the communities in high pH treatment soils. This is expected because low pH and control pH treatment plots community compositions as well as soil nutrients and processes were relatively similar to each other (Chapter 4).

(2) Different effects on fungal diversity across the pH gradient will be apparent, due to altered physiological constraints and compositions. Fungal community diversity in low pH and control pH treatment soils will decrease because fungi are more active in acidic soils and thus a greater competition might be expected for substrates released from biocide-killed fungal (as well as bacterial) cells which might reduce fungal diversity. In contrast, fungal community on high pH plots will increase its diversity due to relatively slower fungal community activity in non-acidic soils resulting in a greater possibility for more fungal species to be able to coexist and share available resources.

(3) Plant growth and/or biomass N will respond to potential changes of N availability in the soil resulting from the biocide application in the beginning of the growing season. Potential decline of N availability will negatively affect plant growth and biomass N while increase of N will increase plant N uptake.

5.3 Methodology

5.3.1 Site description

Details of the experimental site are specified in Chapter 4 (Figure 5.1(A)).

5.3.2 Experimental Design

The experiment described was part of a larger experiment and was set up in two stages. The initial stage started in August 2015 and included soil pH manipulation establishment and maintenance for two consecutive seasons (Chapter 4). Control, low and high pH treatments were established in separate plots within each of the five blocks.

Each experimental plot was divided into 2 subplots, non-biocide and biocide subplot (only half of the biocide subplot was used for the experiment) (Figure 5.1(B)). Plastic rings of 30 cm diameter and 10 cm height were installed to each subplot in the opposite corners [not closer than 0.5 m from plot edge or middle line] in the beginning of the first season (spring, 2016). The rings were pushed into the soil leaving approximately 5 cm of the ring height above the ground. They were used for ecosystem C exchange measurements.

The second stage of the establishment included biocide application on the 3rd of May 2017 to half of the biocide sub-plots where the plastic rings were installed (area of 1.5 m x 1.5 m) (Figure 5.1 (B-C)). The biocide was a mixture of fungicides Horizon® 250 EW (Bayer Crop Science, Monheim, Germany; active ingredient: Tebuconazole) and Zato® 50 WG (Bayer Crop Science, Monheim, Germany; active ingredient: Trifloxystrobin) applied at a rate of 6.413 ml.m^{-2} and 4.328 g.m^{-2} of Tebuconazole and Trifloxystrobin respectively in 5 L of tap water. Non-biocide sub-plots received only tap water. Each sub-plot then received additional 5 L of tap water. The experiment comprised of 6 treatments in 5 replicates (Figure 5.1(B).

5.3.3 Measurements

Common laboratory procedures are listed in Methods chapter (Chapter 2).

Sampling

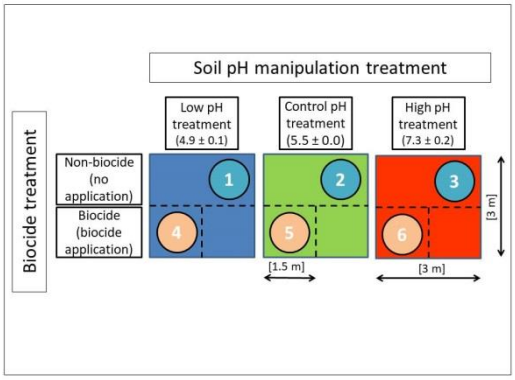
The treatments were sampled and field measurements were taken at time point of day 7 (10 May), 28 (31 May) and 70 (14 July) (all in year 2017) as specified in Figure 5.1(C).

For each treatment, soil cores (5 cm diameter from 5 cm depth) were taken from 5 locations along a transect run in the middle of the plot, transported to the lab, homogenized by passing through a 4 mm mesh and stored at 4 ˚C. A subsample was taken for soil DNA analysis and enzyme assays and stored at -20 ˚C within 48 hours of sampling.

Plant aboveground biomass was harvested from within the area of the installed rings (707 cm^2), pre-dried and stored in a dry place until analysis.

(A)

(B)



(C)

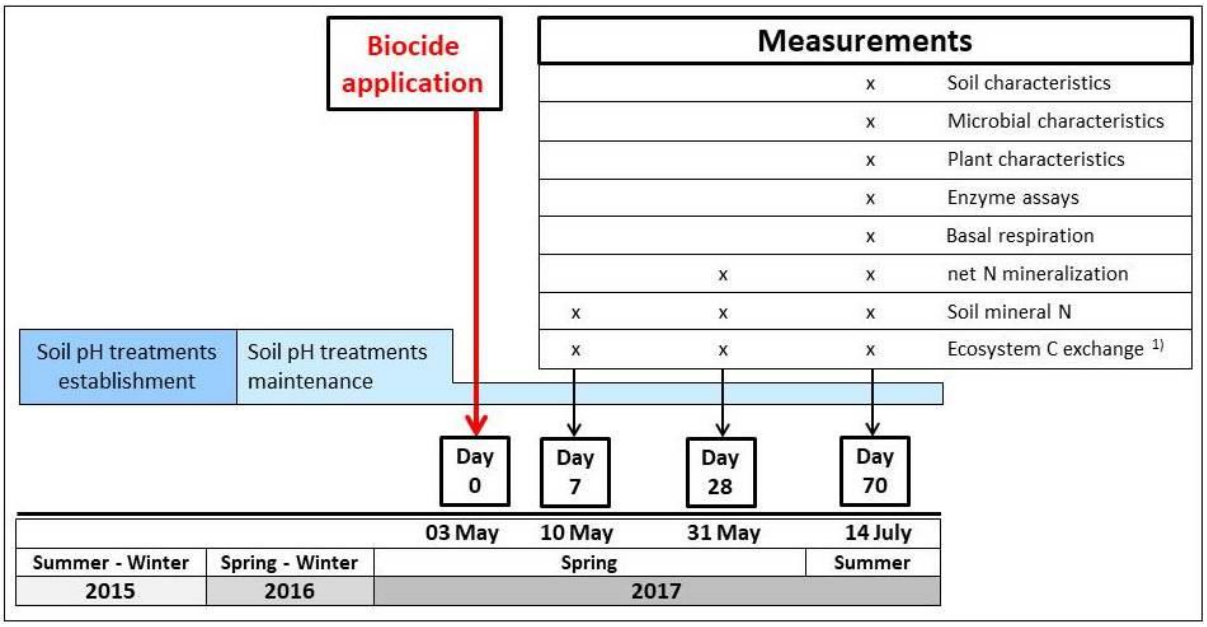


Figure 5.1 Experimental field at the time of biocide application (A), Scheme of experimental treatments (B) and Timeline of experiment establishment and measurements (C). 1(B): Coloured circles represent sides of each plot where the plastic rings for ecosystem C exchange measurements were installed. Soil pH manipulation treatment values (mean ± s.e.). 1(C): Soil pH treatments establishment and maintenance (coloured in blue) represent the first stage of the experiment, biocide application represents the second stage of the experiment. [1)] Ecosystem C exchange was measured on the 13th of July for the third time point.

Soil characteristics

Soil characteristics measured include gravimetric soil moisture, pH, dissolved mineral N ions (NO_3–N and NH_4–N), net N mineralization (ammonification and nitrification), DOC and DON, plant available P. Details of analytical method are described in Methods chapter (Chapter 2).

Soil microbial characteristics

Microbial biomass C and N was performed as specified in Methods chapter (Chapter 2). Bacterial and fungal community composition was determined employing 16S and ITS amplicon sequencing respectively. For bacteria, the V4 hypervariable region of 16S rRNA gene was amplified using 515f-806r primers (Walters et al., 2015) and for fungi, the ITS2 region was amplified using fITS7-ITS4r primer sequences (Ihrmark et al., 2012). Extraction of DNA, amplification of target gene sequences,

sequencing and sequences processing and taxonomy assignment was performed as specified in Methods chapter (Chapter 2).

Plant characteristics

Plant yield and aboveground biomass C, N and P concentration were determined. Details of analytical method described in Methods chapter (Chapter 2).

Ecosystem and soil process rates

Measurements of gross ecosystem soil respiration and net ecosystem exchange (NEE) were made using light chamber and dark chamber respectively, placed over the installed plastic rings (Ward et al., 2007). At the same time as C flux measurements, soil temperature and soil moisture were determined. The field soil moisture was determined using portable moisture probe (Delta-T, Cambridge, UK). Details of analytical method for potential extracellular enzyme activity are described in Methods chapter (Chapter 2).

5.3.4 Data analysis

Soil, plant and process measurements

Soil, plant and microbial characteristics measured only on day 70 after the biocide application (Table 5.1(C)) (including variables calculated from the measured variables such as for instance biomass nutrient ratios (full list in Table 5.12) were assessed for their response to soil pH manipulation and biocide application and their interactive effect using two-way ANOVAs. Factors of each ANOVA included soil pH treatment level (low pH, control pH or high pH), biocide treatment (control or treatment) and all interactions. Measurements of C and N cycle [soil ammonium, nitrate, net N mineralization for ammonium and nitrate and ecosystem respiration] taken at more time points after the biocide application (day 7, day 28 and day 70) (Table 5.1(C)) were assessed for their response to time, soil pH, biocide and their interactive effects using three-way ANOVA. The analysis was also performed at each time point using two-way ANOVAs. A model simplification procedure was applied to these ANOVA models and started with the full model with all the interactions. The model simplification involved subsequent removal of non-significant interaction or factor from the model followed by ANOVA of the simplified model. The final model included block factor and only significant factor(s) or interaction(s). All models violating assumptions of normality (tested by checking model parameters) were ln(y) or ln(y+1) transformed. Factors showing significant effects were tested for factor level differences effects using Tukey post hoc test. Analyses of effects on measurements on day 70 were corrected for multiple comparisons using Benjamini-Hochberg procedure.

Microbial diversity

Microbial diversity characteristics including total unique ASVs (richness) and Simpson's and Shannon diversity indexes were calculated using data with taxa of less than five reads removed and subsequently rarefied to the lowest sequencing depth to account for differences in the sequencing depth among samples. The effect of experimental treatments and their interactions on the diversity characteristics was determined using two-way ANOVA analysis including block factor. When heteroscedasticity was accounted, both ANOVA analysis of the dataset with outliers removed and Kruskal-Wallis test on the full data set were performed to support the results of the analysis. In order to fit the interactive effect into the Kruskal-Wallis test, the model was fit using a synthetic

factor created by combining levels of both factors (soil pH treatment and biocide application) for each sample. Spearman correlation was used to test relationship between soil pH measurements and diversity indices.

Multivariate analysis of microbial communities

Taxa with less than 5 reads were removed. Taxa with total relative abundance lower than 0.1 % for phylum and class taxa and lower than 0.01 % for class taxa were removed from the analysis. Response of microbial community composition at different taxonomic resolutions (phylum, class, order and species) to soil pH treatment and biocide application and their interactive effect was assessed by multivariate generalised linear models (M-GLMs) using GLM framework from MVABUND 3.10.4 package in R (Wang et al., 2012). Details of the analysis are listed in method chapter (Chapter 2). Two bacterial samples and one fungal sample with total number of reads below 20000 were removed from the subsequent analysis.

Indicator species

To identify individual phylotypes significantly associated with biocide treatment at different levels of soil pH, indicator species analysis (R package indicspecies) was used. Details of the analysis are listed in method chapter (Chapter 2). Abundance of selected indicator species (with $P < 0.05$) for each of the combination of factors and their levels (6 combinations) was grouped at order taxonomic resolution for each soil pH and biocide level separately. The results were expressed as relative proportion (%) of indicator species for biocide and non-biocide plots on biocide and non-biocide plots within a soil pH treatment to overall relative abundance of the order on specific soil pH treatment level.

Treatment effect on individual microbial taxa

The effect of experimental treatments on microbial taxa was assessed by univariate GLM with block factor and interactions of both treatment factors using function *manyglm* (R package MVABUND). Non-rarefied data are used and correction for variable sequencing depth among samples is achieved employing function argument '*offset'*. P values were corrected for multiple comparisons using Benjamini-Hochberg procedure. Pairwise comparisons between treatment levels was performed through function argument '*pairwise.comp*'.

All analyses were conducted in R of version 3.5.0 (R Core Team, 2018).

5.4 Results

5.1 Soil microbial community

Sequencing yielded total of 1,168,000 and 1,472,707 reads for the bacterial and fungal communities respectively. Unclassified sequences comprised of 0.07 % and 2.06 % of total bacterial and fungal reads respectively. Phylogenetic identification of sequences at taxonomic resolution of order and higher was > 97 % and > 80 % for bacteria and fungi (Table S5.1). The bacterial community composed primarily of orders of *Proteobacteria* (36% of all bacterial sequences, on average), *Firmicutes* (18%), *Verrucomicrobia* 15%) and *Acidobacteria* (13%) and the fungal community composed primarily of *Ascomycota* (63%) and *Basidiomycota* (28%) (Figure S5.1). Overall bacterial and fungal sequencing data at phyla and class taxonomic resolution are displayed in Figures 5.2, 5.3, 5.4 and 5.5. Two bacterial samples (low pH treatment without biocide and control pH treatment without biocide) and

one fungal sample (high pH treatment without biocide) with total number of reads below 20000 were removed from analysis.

Microbial diversity

In line with expectations, fungal diversity indices responded to the biocide application while bacteria did not respond (Table 5.1). Fungal Shannon diversity index responded to the treatments through an interactive effect of soil pH treatment and biocide application (ANOVA, $F_{2,21} = 4.6$, $P < 0.05$), however no differences between levels of the treatments were observed (posthoc comparisons $P > 0.05$). When an outlier (as visualized by boxplot; control pH treatment on non-biocide plot) was removed from analysis, interactive effect between soil pH and biocide application on Shannon index was confirmed (ANOVA, $F_{2,20} = 4.9$, $P < 0.05$) and pairwise comparisons showed increase of Shannon index after biocide application on control pH plots and higher Shannon index on high pH plots after biocide application when compared to non-biocide control pH plots (posthoc comparisons $P < 0.05$). Soil pH treatment affected fungal richness (number of unique sequences) (ANOVA, $F_{2,21} = 3.6$, $P < 0.05$) whereby low pH plots showed lower richness than high pH plots (posthoc comparisons $P < 0.05$) contrary to no effect of soil pH observed when half of the dataset was analysed (Chapter 4).

Bacterial community composition

Only soil pH manipulation showed an effect on bacterial community at phylum taxonomic resolution (M-GLMs, $Wald_{2,18} = 33.2$, $P < 0.001$) (Table 5.2, Table S5.2) while no response of bacterial phyla to biocide application was observed (ANOVA, all $P > 0.05$) (Table 5.3).

Bacterial community at class taxonomic resolution was affected by interactive effect of soil pH manipulation and biocide application (M-GLMs, $Wald_{2,18} = 35.1$, $P < 0.01$) (Table 5.2). Soil pH treatment showed stronger effect on relative abundances of bacterial classes when compared to biocide effect as only marginal effects of biocide on two classes were observed (ANOVAs, Table 5.4 and Table S5.3).

Soil pH manipulation and biocide application affected bacterial community at order taxonomic resolution (M-GLMs, $Wald_{2,18} = 85.4$, $P < 0.001$ and $Wald_{1,18} = 25.0$, $P < 0.05$ respectively) (Table 5.2). Biocide showed only marginal effect of relative abundance of bacterial classes compared to effect of soil pH treatment (Table 5.5). Soil pH manipulation affected (GLM, all $P < 0.05$) and tended to affect (GLM, all P from 0.05 to < 0.1) bacterial orders of total relative abundance of 31 % and 29 % respectively when unadjusted P values are considered, and it affected (GLM, $P < 0.05$) bacterial classes of total relative abundance of 3.8 % when adjusted P values are considered (Table 5.4). Biocide application affected (GLM, all $P < 0.05$) and tended to affect (GLM, all P range from 0.05 to < 0.1) bacterial classes of total relative abundance of 3 % and 29 % respectively when P values were not adjusted for multiple comparison, but only tended to affect relative abundance of order *Ellin6067* (*Betaproteobacteria*) (GLM, $Dev_{2,18} = 2.1$, $P < 0.1$) which is of total relative abundance of 0.5 % when P values were adjusted (Table 5.4).

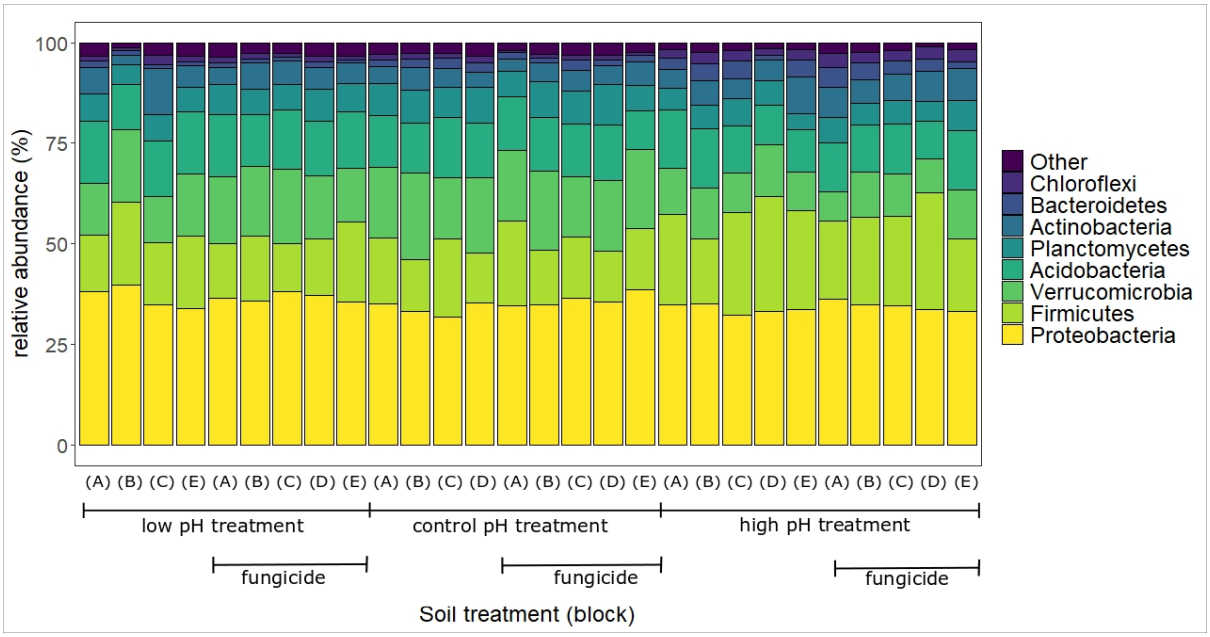


Figure 5.2

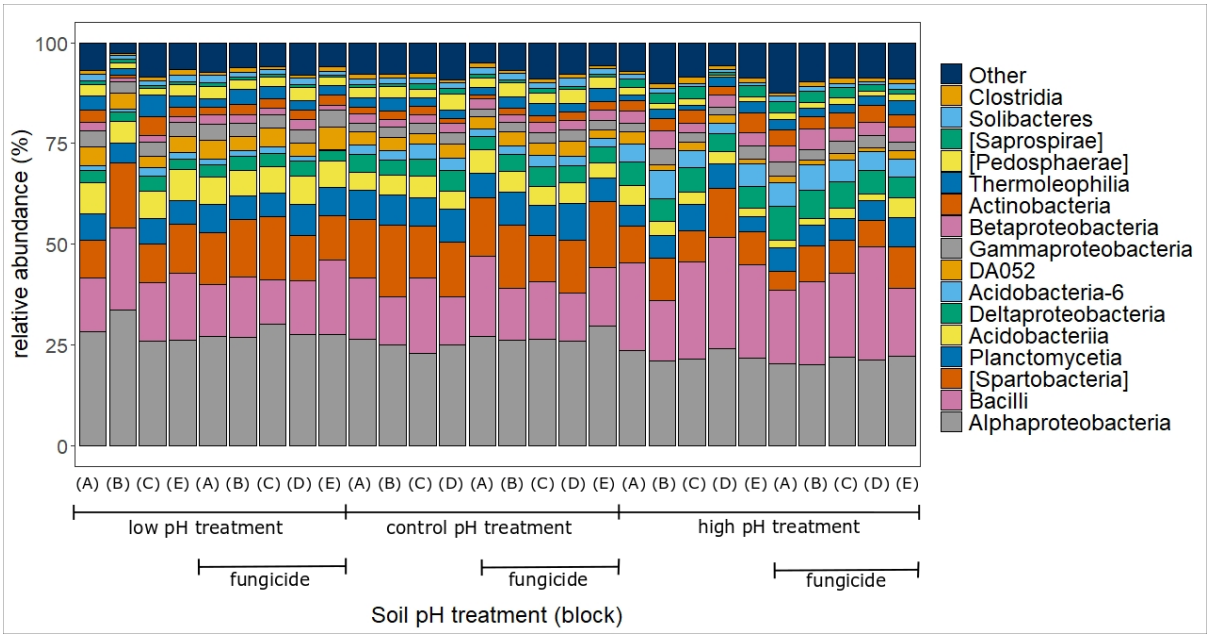


Figure 5.3 Bacterial class distribution on plots with different experimental treatments.

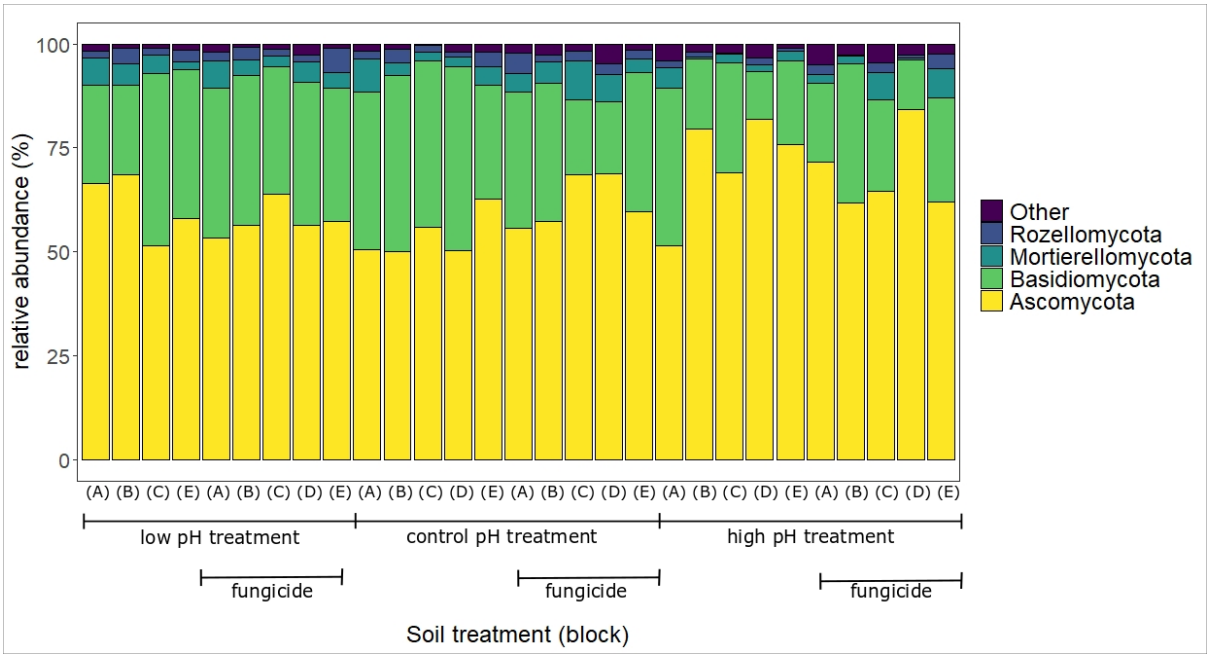


Figure 5.4 Fungal phyla distribution on plots with different experimental treatments.

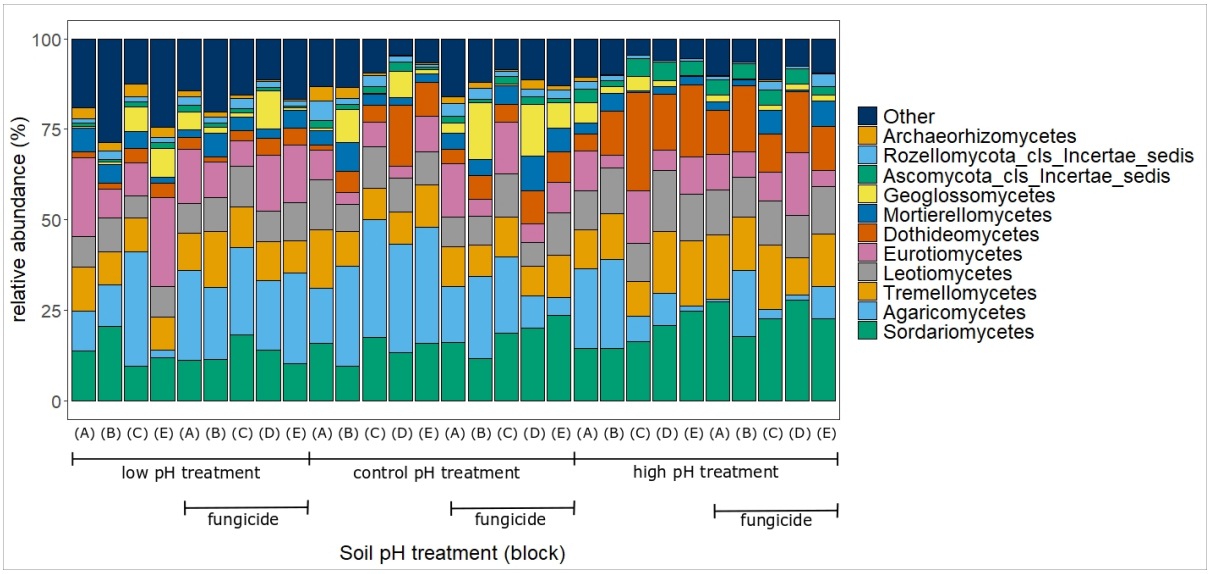


Figure 5.5 Fungal phyla distribution on plots with different experimental treatments.

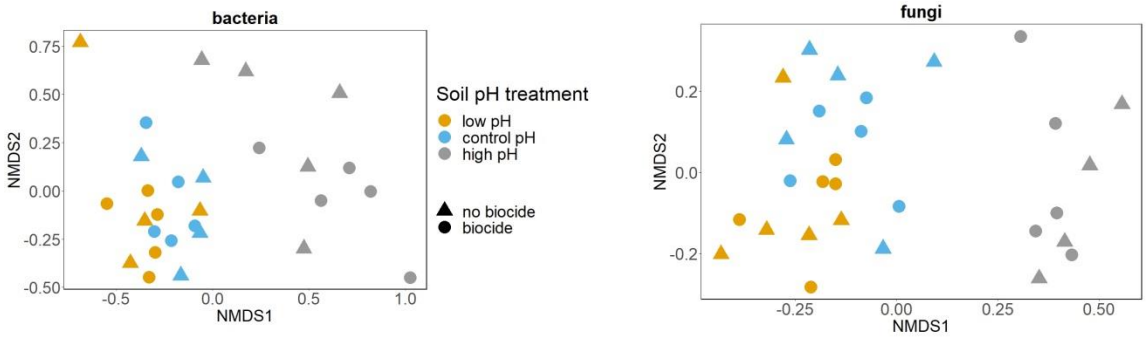


Figure 5.6 NMDS of bacterial and fungal community composition.

Table 5.1 Effect of experimental treatments on bacterial and fungal diversity indices.

Microbial community		F			P			Soil pH manipulation and biocide treatments (mean± s.e.)					
								Non-biocide			Biocide		
Diversity indices	df	pH	biocide	pH x biocide	pH	biocide	pH x biocide	low pH	control pH	high pH	low pH	control pH	high pH
Bacteria													
unique sequences	2, 1, 2	0.005	2.2	1.0	0.99	0.16	0.38	460 ± 96	529 ± 68	427 ± 76	545 ± 55	494 ± 54	616 ± 77
Simpson's diversity	2, 1, 2	0.003	1.2	0.6	1.00	0.28	0.55	0.987 ± 0.004	0.987 ± 0.001	0.985 ± 0.003	0.988 ± 0.001	0.987 ± 0.002	0.989 ± 0.001
Shannon diversity	2, 1, 2	0.054	1.8	0.9	0.95	0.19	0.44	5.21 ± 0.28	5.34 ± 0.10	5.16 ± 0.17	5.37 ± 0.09	5.28 ± 0.13	5.54 ± 0.11
Fungi													
unique sequences	2, 1, 2	3.6	1.7	3.1	*	0.21	+	337 ± 10	341 ± 16	343 ± 30	310 ± 6	372 ± 15	405 ± 23
Simpson's diversity	2, 1, 2	1.0	2.8	2.9	0.40	0.11	+	0.967 ± 0.004	0.95 ± 0.006	0.962 ± 0.008	0.96 ± 0.006	0.969 ± 0.003	0.973 ± 0.004
Shannon diversity	2, 1, 2	0.8	3.1	4.6	0.45	+	*	4.33 ± 0.05	4.17 ± 0.09	4.24 ± 0.04	4.17 ± 0.08	4.46 ± 0.05	4.45 ± 0.11

+ P<0.1, * P<0.05, ** P<0.01, *** P<0.001;

Data analysed using two-way ANOVA model. Diversity indices were calculated using sequencing data after rarefaction.

Table 5.2 Results of M-GLM analysis of effect of soil pH and biocide treatments on microbial community structure at different taxonomic resolutions.

Microbial community / Taxonomic resolution	df	Test statistics[3]			P		
		pH	biocide	pH * biocide	pH	biocide	pH * biocide
Bacteria							
Phylum [1]	2, 1, 2	33	13.8	12.2	***	0.18	0.12
Class [1]	2, 1, 2	128	20	35	***	*	**
Order [1]	2, 1, 2	85	25	35	***	*	+
Species [2]	2, 1, 2	14586	4782	2558	***	*	*
Fungi							
Phylum [1]	2, 1, 2	7.5	5.8	4.6	+	0.44	0.70
Class [1]	2, 1, 2	32	9.6	14	***	*	0.13
Order [1]	2, 1, 2	65	22	21	***	*	0.21
Species [2]	2, 1, 2	7562	2473	2216	***	0.12	*

+ P<0.1, * P<0.05, ** P<0.01, *** P<0.001;
[1] Block was included in the model. [2] Block was not included in the model. [3] Wald test statistics was used apart for Species analysis which used Score statistic. Only phyla, classes and orders of mean relative abundance greater than 0.1 % were selected for the analysis.

Table 5.3 Effect of soil pH manipulation, biocide application and their interaction on bacterial phyla.

Phylum	Relative abundance [mean ± s.e.] (%)	GLM [1] Deviance			GLM [1] P adjusted			Cor. [2]
		pH	biocide	pH x biocide	pH	biocide	pH x biocide	rho
Proteobacteria	35.4 ± 0.4	1.7	4.8	1.7	0.72	0.29	0.79	**-0.52**
Firmicutes	18.3 ± 0.9	13.8	1.5	2.3	*	0.53	0.72	**0.52**
Verrucomicrobia	14.6 ± 0.7	25.1	3.8	2.2	**	0.31	0.72	**-0.57**
Acidobacteria	13.1 ± 0.3	2.8	1.5	1.3	0.53	0.53	0.82	**-0.46**
Planctomycetes	6.78 ± 0.26	3.5	2.7	1.2	0.50	0.42	0.83	-0.31
Actinobacteria	5.71 ± 0.35	4.8	1.5	3.4	0.42	0.53	0.53	0.28
Bacteroidetes	2.21 ± 0.24	23.8	0.004	1.0	**	0.95	0.83	**0.83**
Chloroflexi	1.71 ± 0.15	17.4	0.01	6.8	**	0.95	0.31	**0.71**
Gemmatimonadetes	0.49 ± 0.04	2.7	1.5	0.5	0.50	0.42	0.83	-0.25
WS3	0.36 ± 0.05	6.9	0.1	1.2	0.20	0.83	0.82	**0.41**
Chlamydiae	0.29 ± 0.02	8.4	0.2	0.2	0.20	0.83	0.95	**-0.54**
WPS-2	0.27 ± 0.05	35.4	0.1	2.9	**	0.83	0.53	**-0.53**
TM6	0.26 ± 0.02	7.8	0.3	1.4	0.17	0.82	0.82	**-0.58**
Elusimicrobia	0.21 ± 0.03	15.7	2.9	0.5	**	0.31	0.83	**0.53**
Nitrospirae	0.17 ± 0.03	3.8	0.0	2.0	0.42	0.95	0.62	0.07
Cyanobacteria	0.12 ± 0.01	0.7	1.9	2.6	0.82	0.42	0.53	**-0.84**
FCPU426	0.10 ± 0.02	25.0	1.7	5.5	**	0.53	0.42	**-0.92**

* P<0.05, ** P<0.01,
Only phyla of mean relative abundance greater than 0.1 % were selected for the analysis. GLM univariate models included fixed block factor.[1] Univariate GLM model fitted using function *manyglm* (R package: MVABUND), P calculated using 1000 permutations and adjusted using Benjamini-Hochberg procedure. [2] Spearman correlation between phylum and soil pH: numbers in bold are significant (P < 0.05).

Table 5.4 Effect of soil pH manipulation, biocide application and their interaction on bacterial classes.

Phylum	Class	Relative abundance [mean ± s.e.]	GLM [1]						Cor. [2]
			Deviance			P adjusted			
			pH	biocide	pH x biocide	pH	biocide	pH x biocide	rho
Proteobacteria	*Alphaproteobacteria*	25.74 ± 0.63	10.5	4.8	0.99	+	0.20	0.89	**-1**
	Deltaproteobacteria	4.54 ± 0.28	15.0	3.6	2.6	*	0.26	0.65	**0.9**
	Gammaproteobacteria	2.94 ± 0.14	8.8	2.4	0.83	0.15	0.43	0.89	-0.4
	Betaproteobacteria	2.65 ± 0.19	14.3	3.9	1.8	*	0.24	0.79	**0.9**
Firmicutes	*Bacilli*	17.49 ± 0.9	14.3	1.5	2.4	+	0.58	0.73	**0.5**
	Clostridia	1.01 ± 0.05	2.7	0.98	0.71	0.58	0.65	0.89	0.32
Verrucomicrobia	*[Spartobacteria]*	11.9 ± 0.62	29.1	2.5	3.2	*	0.43	0.59	**-1**
	[Pedosphaerae]	2.29 ± 0.17	10.5	1.8	0.51	*	0.43	0.89	**-1**
	Opitutae	0.28 ± 0.04	16.4	6.2	3.1	*	+	0.52	**-1**
	Verrucomicrobiae	0.14 ± 0.04	22.0	0.42	2.5	*	0.79	0.63	**0.8**
Acidobacteria	*Acidobacteriia*	4.88 ± 0.36	29.4	0.07	0.06	*	0.91	0.98	**-1**
	Acidobacteria-6	3.1 ± 0.36	31.3	0.17	3.0	*	0.89	0.58	**1**
	iii1-8	0.38 ± 0.04	10.3	1.6	0.87	+	0.58	0.89	**0.6**
	Acidobacteria-5	0.29 ± 0.02	0.39	0.46	2.0	0.89	0.67	0.49	0.28
	[Chloracidobacteria]	0.15 ± 0.02	19.5	3.1	4.8	*	0.26	0.37	**0.8**
	DA052	3 ± 0.23	26.5	0.78	0.71	*	0.71	0.89	**-1**
	Solibacteres	1.3 ± 0.07	6.0	4.3	1.8	0.23	0.23	0.79	-0.4
Planctomycetes	*Planctomycetia*	6.48 ± 0.23	3.1	2.4	1.1	0.52	0.43	0.89	-0.3
	Phycisphaerae	0.19 ± 0.02	4.8	4.8	5.4	0.16	+	0.25	-0.3
Actinobacteria	*Actinobacteria*	2.62 ± 0.2	11.3	1.2	1.9	*	0.57	0.72	**0.5**
	Thermoleophilia	2.61 ± 0.16	4.5	1.9	5.6	0.43	0.47	0.37	-0.4
	Acidimicrobiia	0.54 ± 0.07	11.0	0.26	1.2	+	0.83	0.83	**0.8**
Bacteroidetes	*[Saprospirae]*	1.33 ± 0.16	30.5	0.01	1.1	*	0.97	0.89	**0.8**
	Cytophagia	0.33 ± 0.05	9.5	0.06	0.64	*	0.89	0.84	**0.7**
	Sphingobacteriia	0.3 ± 0.03	3.5	0.12	0.13	0.51	0.89	0.97	0.06
	Flavobacteriia	0.22 ± 0.04	26.8	0.04	0.25	*	0.92	0.96	**0.8**
Chloroflexi	*Ellin6529*	0.55 ± 0.09	21.3	0.48	6.9	*	0.79	0.25	**0.8**
	Ktedonobacteria	0.55 ± 0.04	8.2	0.01	0.81	0.16	0.97	0.89	-0
	Anaerolineae	0.26 ± 0.06	21.6	1.0	6.3	*	0.58	0.24	**0.8**
	TK10	0.18 ± 0.02	0.30	0.00	2.4	0.92	0.97	0.58	-0.1
Gemmatimonadetes	*Gemmatimonadetes*	0.43 ± 0.04	3.7	3.0	0.86	0.31	0.21	0.84	-0.4
WS3	*PRR-12*	0.37 ± 0.05	6.9	0.11	1.2	0.16	0.89	0.84	**0.4**
Chlamydiae	*Chlamydiia*	0.3 ± 0.02	8.4	0.17	0.18	0.20	0.89	0.97	**-1**
TM6	*SJA-4*	0.24 ± 0.02	5.9	0.20	1.4	0.21	0.89	0.84	**-0**
Elusimicrobia	*Elusimicrobia*	0.21 ± 0.03	15.7	2.9	0.48	*	0.23	0.89	**-1**
Nitrospirae	*Nitrospira*	0.17 ± 0.03	3.8	0.02	2.0	0.37	0.96	0.64	**0.5**

*+ P<0.1, * P<0.05,*

Only classes of mean relative abundance greater than 0.1 % were selected for the analysis. [1] Univariate GLM model fitted using function *manyglm* (R package: MVABUND), P calculated using 1000 permutations and adjusted using Benjamini-Hochberg procedure. [2] Spearman correlation analysis between class and soil pH: numbers in bold are significant (P < 0.05). GLM models included block factor.

Table 5.5 Effect of soil pH manipulation, biocide application and their interactive effect on selected bacterial classes.

Phylum	Class	Order	Relative abundance [mean ± s.e.]	GLM[1] Deviance			GLM[1] P unadjusted [2]			GLM[1] P adjusted[2][3]			Cor. [4]
				pH	biocide	pH x biocide	pH	biocide	pH x biocide	pH	biocide	pH x biocide	rho
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	19.37 ± 0.203	1.1	3.9	1.9	0.62	+	0.49	0.80	0.31	0.75	-0.29
		Rickettsiales	3.09 ± 0.496	51.6	3.7	2.5	***	+	0.42	*	0.31	0.71	**-0.81**
		BD7-3	0.01 ± 0.002	7.5	3.6	1.4	+	0.14	0.41	0.31	0.41	0.71	-0.28
	Deltaproteobacteria	*Myxococcales*	2.65 ± 0.175	12.8	4.0	2.3	**	+	0.43	+	0.31	0.71	**0.86**
	Betaproteobacteria	*Ellin6067*	0.51 ± 0.043	6.4	5.5	1.0	*	*	0.57	0.21	+	0.80	**0.65**
		MND1	0.14 ± 0.022	5.7	3.9	5.0	*	*	+	0.13	0.16	0.29	**0.52**
	Gammaproteobacteria	*Xanthomonadales*	2.04 ± 0.096	10.7	4.7	2.2	*	+	0.47	0.13	0.25	0.75	**-0.46**
		Legionellales	0.54 ± 0.048	4.3	1.5	4.8	0.14	0.25	0.14	0.41	0.57	0.42	-0.3
		Thiotrichales	0.04 ± 0.013	31.2	0.4	10.0	***	0.66	*	*	0.83	+	**0.8**
		Alteromonadales	0.02 ± 0.007	11.3	4.4	0.1	*	0.17	0.41	+	0.47	0.71	**0.53**
Verrucomicrobia	*Opitutae*	*Opitutales*	0.27 ± 0.035	15.8	6.2	3.2	***	*	0.26	*	0.13	0.59	**-0.48**
Acidobacteria	*Acidobacteria-6*	*CCU21*	0.11 ± 0.028	21.4	0.7	11.0	***	0.47	*	*	0.75	0.13	**0.85**
	Solibacteres	*Solibacterales*	1.32 ± 0.069	5.8	4.3	1.8	+	+	0.53	0.31	0.31	0.78	-0.37
	iii1-8	*DS-18*	0.04 ± 0.012	13.8	2.4	9.6	*	0.20	*	+	0.53	0.16	0.31
	Holophagae	*Holophagales*	0.03 ± 0.01	17.1	0.1	15.1	***	0.82	*	*	0.90	+	**0.68**
Planctomycetes	*Planctomycetia*	*Pirellulales*	1.65 ± 0.077	3.3	6.5	3.9	0.27	*	0.29	0.59	0.16	0.62	**-0.42**
		Planctomycetales	0.37 ± 0.017	12.6	6.9	4.8	**	*	0.21	+	0.15	0.53	**0.67**
	Phycisphaerae	*WD2101*	0.15 ± 0.024	9.3	2.7	2.9	**	+	0.23	*	0.31	0.56	-0.4
	vadinHA49	*DH61*	0.03 ± 0.007	4.4	0.8	9.1	+	0.40	+	0.33	0.71	0.23	-0.02
Chloroflexi	*Ktedonobacteria*	*Ktedonobacterales*	0.13 ± 0.021	5.8	3.8	1.2	+	+	0.67	0.31	0.31	0.83	0.18
		Thermogemmatisporales	0.09 ± 0.019	1.1	3.1	1.0	0.43	+	0.67	0.71	0.23	0.83	-0.19
	Anaerolineae	*SBR1031*	0.14 ± 0.042	14.7	2.8	2.2	**	+	0.40	+	0.33	0.71	**0.58**
		A31	0.04 ± 0.006	4.1	0.2	6.2	+	0.62	+	0.31	0.80	0.31	**0.61**
		H39	0.02 ± 0.005	1.4	2.1	9.1	0.48	0.22	+	0.75	0.55	0.26	0.32
	Chloroflexi	*[Roseiflexales]*	0.01 ± 0.008	11.1	3.3	0.0	*	0.32	0.54	0.16	0.64	0.79	**0.51**
Gemmatimonadetes	*Gemmatimonadetes*	*Ellin5290*	0.26 ± 0.024	1.1	2.1	0.8	0.42	+	0.61	0.71	0.31	0.80	-0.22
Elusimicrobia	*Elusimicrobia*	*Elusimicrobiales*	0.07 ± 0.015	14.2	5.3	2.4	***	*	0.35	*	0.19	0.67	**-0.58**
Cyanobacteria	*4C0d-2*	*MLE1-12*	0.06 ± 0.011	42.3	0.0	0.8	***	0.93	0.43	*	0.96	0.71	**-0.56**

*+ P<0.1, * P<0.05, ** P<0.01, *** P<0.001;*

[1] Univariate GLM model fitted using function *manyglm* (R package: MVABUND). [2] P calculated using 1000 permutations. [3] P values adjusted using Benjamini-Hochberg procedure. [4] Spearman correlation analysis between class and soil pH: numbers in bold are significant (P < 0.05). GLM models included block factor.

Bacterial structure at species taxonomic resolution was affected by interactive effect of soil pH manipulation and biocide application (M-GLMs, $Scores_{2,18} = 2558$, $P < 0.05$) (Table 5.2 5.2).

Bacterial indicator species

Indicator species aggregated at order taxonomic resolution showed that orders included indicator species for different combinations of the treatments (i.e. soil pH manipulation and biocide application). For instance, order *Xanthomonadales* included indicator species for biocide application on low and high pH plots as well as indicator species for non-biocide plots on low pH plots (Table 5.6).

Indicator species for effect of soil pH manipulation and biocide application (Table 5.6) was compared with indicator species selected for overall soil pH manipulation plots as performed by separate analysis including only soil pH treatments as factors (data not shown). Some orders included biocide application indicator species that were also marked soil pH manipulation indicator species (e.g. indicator species for biocide application determined for order *Rickettsiales* on high pH plots are also indicator species for high pH manipulation). Other orders included only part of biocide application indicator species that were also soil pH indicator species (e.g. *Rhizobiales* on high pH plots) and other orders included biocide indicator species that were not soil pH indicator species (e.g. *Burkholderiales* on high pH plots). This did not suggest that indicator species selected for soil pH manipulation are predominantly indicator species selected for biocide applied plots in the present analysis.

The phylum *Firmicutes* included relatively very low proportion of indicator species, however, it is the second most abundant phylum (Table 5.6).

Fungal community composition

Fungal community at phylum taxonomic resolution was not affected by soil pH treatment (M-GLMs, all $P > 0.05$, Table 5.2). Only phylum *Glomeromycota* showed an effect of experimental treatments whereby its abundance on high pH plots was lower compared to control pH and low pH plots (GLM, $Dev_{2,19} = 17.4$, $P < 0.05$, pairwise comparisons $P < 0.05$) (Table 5.6). Phylum *Glomeromycota* was also positively affected by biocide application (GLM, $Dev_{1,19} = 14.4$, $P < 0.05$, pairwise comparisons $P < 0.05$).

Table 5.6 Effect of soil pH manipulation, biocide application and interaction on fungal phyla.

Phylum	Relative abundance [mean ± s.e.]	GLM model [1)]						Cor. [2)]
		Deviance			P adjusted			
		pH	biocide	pH x biocide	pH	biocide	pH x biocide	rho
Ascomycota	63.8 ± 1.9	11.0	0.0	7.6	0.11	0.91	0.62	0.26
Basidiomycota	29.6 ± 1.8	6.1	0.3	6.8	0.14	0.73	0.23	-0.19
Mortierellomycota	4.12 ± 0.42	4.4	3.7	0.7	0.18	0.17	0.88	-0.27
Rozellomycota	2.26 ± 0.24	7.4	4.9	3.2	0.11	0.12	0.49	**-0.42**
Glomeromycota	0.24 ± 0.05	17.4	14.4	2.1	*	*	0.62	**-0.68**

* $P < 0.05$

Only phyla with mean relative abundance greater than 0.1 % selected for the analysis. [1)] Univariate GLM model fitted using function *manyglm* (R package: MVABUND), P calculated using 1000 permutations and adjusted using Benjamini-Hochberg procedure. [2)] Spearman correlation analysis between class and soil pH: numbers in bold are significant ($P < 0.05$). GLM models included fixed block factor.

Soil pH manipulation and biocide application affected fungal community structure at class taxonomic resolutions (M-GLMs, $Wald_{2,19} = 32.3$, $P < 0.001$ and $Wald_{1,19} = 9.6$, $P < 0.01$, respectively) (Table 5.2).

Fungal classes contributing the most to the biocide effect included *Glomeromycetes* and *Lecanoromycetes* comprising 15.3 % and 14.7 % of the overall model statistics respectively (data not shown). Soil pH manipulation affected (GLM, all $P < 0.05$) and tended to affect (GLM, all P from 0.05 to < 0.1) fungal classes of total relative abundance of 33 % and 58 % respectively when adjusted P values were considered (Table 5.7). While for the response to biocide application, only class *Lecanoromycetes* tended to be affected by the interactive effect of soil pH treatment and biocide application (GLM, $Dev_{2,19} = 14.8$, $P < 0.1$) (Table 5.7).

Table 5.7 Effect of soil pH manipulation, biocide application and their interaction on fungal classes.

Phylum	Class	Relative abundance [mean ± s.e.]	GLM[1)] Deviance pH	Deviance biocide	Deviance pH x biocide	P adjusted pH	P adjusted biocide	P adjusted pH x biocide	Cor.[2)] rho
Ascomycota	*Sordariomycetes*	19.07 ± 1.04	10.8	2.3	2.2	+	0.43	0.68	0.36
	Eurotiomycetes	11.6 ± 1.25	10.8	0.1	2.1	+	0.86	0.73	-0.35
	Leotiomycetes	11.61 ± 0.45	11.6	1.0	3.9	+	0.62	0.46	0.23
	Dothideomycetes	9.32 ± 1.25	53.6	2.3	1.3	*	0.44	0.79	**0.9**
	Geoglossomycetes	4.44 ± 0.89	4.2	0.4	4.9	0.44	0.76	0.44	-0.08
	Ascomycota_cls_Incertae_sedis	2.46 ± 0.27	30.4	0.1	2.8	*	0.86	0.61	**0.71**
	Archaeorhizomycetes	1.42 ± 0.26	30.2	0.0	9.0	*	0.88	0.13	**-0.77**
	Pezizomycetes	0.57 ± 0.11	16.9	0.3	2.9	*	0.79	0.62	0.37
	Lecanoromycetes	0.21 ± 0.05	12.7	7.4	14.8	*	+	+	**-0.67**
	Orbiliomycetes	0.21 ± 0.04	0.5	0.3	6.5	0.84	0.76	0.18	0.19
Basidiomycota	*Agaricomycetes*	18.33 ± 2.12	10.8	1.0	2.8	*	0.61	0.61	-0.34
	Tremellomycetes	13.32 ± 0.63	13.7	2.0	0.9	+	0.46	0.83	0.07
	Microbotryomycetes	0.43 ± 0.05	0.1	3.4	1.4	0.97	0.29	0.78	-0.24
	Spiculogloeomycetes	0.1 ± 0.03	7.9	0.3	7.7	+	0.76	0.15	0.16
Glomeromycota	*Glomeromycetes*	0.21 ± 0.05	23.3	7.4	0.7	*	0.13	0.86	**-0.68**
Mortierellomycota	*Mortierellomycetes*	4.58 ± 0.48	5.3	3.1	0.9	0.25	0.35	0.83	-0.37
Rozellomycota	*Rozellomycota_cls_Incertae_sedis*	2.13 ± 0.23	10.4	3.4	2.2	+	0.29	0.68	**-0.5**

+ P<0.1, * P<0.05

Only classes with mean relative abundance greater than 0.1 % selected for the analysis. GLM univariate models included fixed block factor. [1)] Univariate GLM model fitted using function manyglm, P calculated using 1000 permutations and adjusted using Benjamini-Hochberg procedure. [2)] Correlation between phylum and soil pH assessed by spearman correlation: numbers in bold are significant ($P < 0.05$).

Soil pH manipulation and biocide application affected fungal community composition at the order taxonomic resolution (M-GLMs, $Wald_{2,19} = 64.5$, $P < 0.001$, $Wald_{1,19} = 22.1$, $P < 0.01$ respectively) (Table 5.2). Soil pH manipulation affected (GLM, all $P < 0.05$) and tended to affect (GLM, all P from 0.05 to < 0.1) fungal orders of total relative abundance of 19 % and 31 % respectively when unadjusted P values were considered (data not shown). Biocide application affected fungal classes of total relative abundance of 4 % (GLM, all $P < 0.05$) when unadjusted P values were considered (Table 5.9) while it showed only a tendency for effect with adjusted P values on relative abundance of *Glomerellales* (*Sordariomycetes* (GLM, $Dev_{1,19} = 8.4$, $P < 0.1$).

Community composition at species taxonomic resolution was affected by interactive effect of soil pH treatment and biocide application (M-GLMs, $Scores_{2,19} = 2216$, $P < 0.01$) (Table 5.2) (Figure 5.8). Pairwise comparison showed a tendency for community differentiation between non-biocide low pH plots and biocide high pH plots (pairwise comparisons $P < 0.1$).

Table 5.8 Bacterial indicator species for biocide and non-biocide plots at each level of soil pH treatment aggregated at order taxonomic resolution.

Phylum		Overall relative abundance[1] (%)				Low pH				Control pH				High pH			
						Non-biocide indicator		Biocide indicator		Non-biocide indicator		Biocide indicator		Non-biocide indicator		Biocide indicator	
Class	Order	Total	Low pH	Control pH	High pH	non-bio[2]	bio[2]	non-bio	bio	non-bio	bio	non-bio	bio	non-bio	bio	non-bio	bio
Proteobacteria																	
Alphaproteobacteria	*Rhizobiales*	18.8			18.4	1								1	0	5	9
	Rickettsiales	3.0	5.5	3.4	0.44			0	2			0	1			12	30
	Rhodospirillales	2.7	2.8		2.4			0	1							1	3
Betaproteobacteria	*Ellin329*	0.54	0.83		0.27			13	19								
	Burkholderiales	0.80	0.65		1.15			18	24							5	14
	Ellin6067	0.49			0.63											12	38
	Methylophilales	0.004			0.011											0	197
	unassigned Order	0.26			0.38									19	13	0	28
Deltaproteobacteria	*Myxococcales*	2.6		2.6	3.4					9	3	4	17			11	22
	Syntrophobacterales	1.1			1.3											0	3
	Desulfuromonadales	0.57			1.00											4	24
	Bdellovibrionales	0.055			0.056											0	73
Gammaproteobacteria	*Xanthomonadales*	2.0	2.5		1.8	6	3	13	24							13	24
	Thiotrichales	0.042			0.114											48	88
	HTCC2188	0.032			0.085									122	78		
	Alteromonadales	0.018			0.046												
	[Marinicellales]	0.008			0.021											0	122
	unassigned Order	0.13	0.30					39	84								
Firmicutes																	
Bacilli	*Bacillales*	17.1			21.5											0	0
Clostridia	*Clostridiales*	0.99			1.1									4	0		
Verrucomicrobia																	
[Spartobacteria]	*[Chthoniobacterales]*	11.7	12.5		8.6			0	1							1	2
[Pedosphaerae]	*[Pedosphaerales]*	2.3	2.5	3.0	1.4			6	14	7	4					9	22
Opitutae	*Opitutales*	0.27	0.30	0.42	0.10			40	58	26	12	15	20			29	62
Verrucomicrobiae	*Verrucomicrobiales*	0.13		0.06	0.32					45	7					39	91
Acidobacteria																	
Acidobacteriia	*Acidobacteriales*	4.8	6.8			1	0	2	3								
Acidobacteria-6	*iii1-15*	2.9		2.5	4.9					8	3	0	3			8	18
	CCU21	0.11		0.08						114	0						
DA052	*Ellin6513*	2.9	4.1			3	1	2	2								
Solibacteres	*Solibacterales*	1.3	1.3	1.6	1.0	18	5	1	4			0	4			20	31
Acidobacteria-5	*unassigned Order*	0.28		0.27						33	13						
[Chloracidobacteria]	*RB41*	0.085			0.148											41	55
	PK29	0.053			0.089											57	131
Sva0725	*Sva0725*	0.049			0.109											12	35
Holophagae	*Holophagales*	0.032			0.080									93	59		
Planctomycetes																	
Planctomycetia	*Gemmatales*	4.4		5.5	3.7							0	0			2	5
	Pirellulales	1.6	1.8	1.6	1.4							6	16			1	22

	Planctomycetales	0.36			0.44											0	7
BD7-11	*unassigned Order*	0.053		0.126								0	27				
C6	*MVS-107*	0.019		0.059								65	129				
Pla4	*unassigned Order*	0.015		0.037								0	97				
Actinobacteria																	
Actinobacteria	*Actinomycetales*	2.6	2.5		3.4	7	3							7	3	23	35
Thermoleophilia	*Solirubrobacterales*	1.3	1.5		1.4			0	2					17	4	2	6
	Gaiellales	1.2		1.4	0.8					12	5	1	4			0	2
Acidimicrobiia	*Acidimicrobiales*	0.53			0.84											6	19
Bacteroidetes																	
[Saprospirae]	*[Saprospirales]*	1.3		0.93	2.2							5	11	8	6	13	28
Cytophagia	*Cytophagales*	0.33		0.41	0.47					75	41			23	12	22	48
Sphingobacteriia	*Sphingobacteriales*	0.30	0.31	0.24	0.34	39	11	6	19	40	20			45	11		
Flavobacteriia	*Flavobacteriales*	0.21			0.34									27	3	4	20
Chloroflexi																	
Ellin6529	*unassigned Order*	0.54			1.01											13	20
Anaerolineae	*SBR1031*	0.14	0.06		0.30			0	76							8	40
	Caldilineales	0.025			0.066									113	46		
Ktedonobacteria	*Thermogemmatisporales*	0.084	0.078					0	31								
WS3																	
PRR-12	*Sediment-1*	0.35		0.52	0.34					10	6					9	13
Gemmatimonadetes																	
Gemmatimonadetes	*Ellin5290*	0.25		0.26						17	4						
Gemm-1	*unassigned Order*	0.056	0.060			87	40										
Elusimicrobia																	
Elusimicrobia	*FAC88*	0.10		0.13						43	7						
	Elusimicrobiales	0.071		0.124						29	12						
Fibrobacteres																	
Fibrobacteria	*258ds10*	0.064		0.078						82	46						
Armatimonadetes																	
Armatimonadia	*FW68*	0.025	0.067					61	112								
Chlamydiae																	
Chlamydiia	*unassigned Order*	0.023	0.028					0	72								
Chlorobi																	
OPB56	*unassigned Order*	0.006			0.017											0	175
unassigned Class	*unassigned Order*	0.007			0.004											0	208
Cyanobacteria																	
4C0d-2	*SM1D11*	0.009			0.023											44	155

Relative contribution of sum of indicator species at order level to the mean relative abundance of particular order for each pH treatment level (%): 100 0

[1] 'Total' represents relative abundance for whole order while 'Low pH', 'Control pH' and 'High pH' represent relative abundance of orders for each soil pH treatment (displayed are only relative abundances of orders with indicator species). [2] Split of indicator species to the plots where they are present (i.e. 'non-bio'means plots without pesticide application and 'bio' means plots with biocide application).

Fungal indicator species

Indicator species analysis, combining indicator species into their orders, showed that biocide application indicator species associated with low and high pH plots belonged to orders with considerably high relative abundance (Table 5.10). On the other hand, indicator species associated with control pH plots belonged to orders with considerably low relative abundance or belonged to orders with considerably high relative abundance but covered only very low fraction of total mean abundance of the order (Table 5.10). Orders with higher relative abundance (> 5 %) did not include high proportion (i.e. < 10 %) of indicator species apart from orders of *Chaetothyriales* (*Sordariomycetes*) and *Pleosporales* (*Dothideomycetes*) (both *Ascomycota*). Orders tended not to contain indicator species for biocide application at multiple soil pH treatment levels apart from order *Geoglossales*. Interestingly, order *Chaetothyriales* included non-biocide indicator species on high pH plots and biocide indicator species on low pH plots. Also, *Sordariales* (*Sordariomycetes*), *Helotiales* (*Leotiomycetes*), *Pleosporales* (*Eurotiomycetes*) (all *Ascomycota*) and *Mortierellales* (*Mortierellomycetes*, *Mortierellomycota*) included indicator species for both biocide and non-biocide plots (Table 5.10). Orders of lower relative abundances (< 1 %) tended to contain relatively larger proportion of indicator species for the biocide effect than orders of higher relative abundances such as orders *Glomerellales*, *Spiculogloeales* and *GS07*.

Indicator species for effect of soil pH manipulation and biocide application (Table 5.10) was compared with indicator species selected for overall soil pH manipulation plots as performed by separate analysis including only soil pH treatments as factors (data not shown). This showed that most of indicator species for soil pH treatment are also indicator species for biocide application.

Table 5.9 Effect of soil pH manipulation, biocide application and their interaction on selected fungal orders.

Phylum	Class	Order	Relative abundance [mean ± s.e.]	GLM[1)] Deviance			GLM[1)] P unadjusted[2)]			GLM[1)] P adjusted[2)3)]			Cor.[4)]
				pH	biocide	pH x biocide	pH	biocide	pH x biocide	pH	biocide	pH x biocide	rho
Ascomycota	*Sordariomycetes*	*Glomerellales*	0.52 ± 0.14	39.1	8.4	3.4	***	*	0.29	*	+	0.66	**0.71**
		Sordariales	1.57 ± 0.1	11.6	3.8	1.5	*	+	0.60	+	0.33	0.89	**0.47**
	Dothideomycetes	*Capnodiales*	1.49 ± 0.29	18.6	6.3	1.8	**	*	0.52	*	0.13	0.87	**0.84**
	Archaeorhizomycetes	*Archaeorhizomycetales*	1.52 ± 0.28	26.4	0.1	8.3	***	0.81	*	*	0.96	0.21	**-0.78**
	Orbiliomycetes	*Orbiliales*	0.01 ± 0	10.2	18.2	0.0	*	*	0.60	0.17	0.13	0.89	**0.5**
Basidiomycota	*Agaricomycetes*	*Sebacinales*	0.22 ± 0.07	9.3	0.0	6.4	*	0.98	0.12	0.16	0.99	0.37	0.27
		Boletales	0.03 ± 0.01	1.7	2.8	1.5	0.36	+	0.56	0.73	0.35	0.89	-0.16
	Tremellomycetes	*Trichosporonales*	1.82 ± 0.53	32.7	7.3	0.3	***	*	0.90	*	0.12	0.98	**0.52**
	Microbotryomycetes	*Leucosporidiales*	0.25 ± 0.04	1.3	5.4	0.6	0.63	+	0.80	0.89	0.23	0.96	-0.15
		Sporidiobolales	0.03 ± 0.01	0.9	0.1	5.7	0.59	0.73	0.11	0.89	0.94	0.37	-0.27
	Spiculogloeomycetes	*Spiculogloeales*	0.11 ± 0.03	8.1	0.2	7.9	*	0.65	+	0.13	0.89	0.22	0.15
Glomeromycota	*Glomeromycetes*	*Glomerales*	0.22 ± 0.05	23.3	7.5	0.7	***	*	0.83	*	0.16	0.96	**-0.69**
	Archaeosporomycetes	*Archaeosporales*	0.02 ± 0.01	4.2	6.6	5.0	0.18	*	0.20	0.51	0.19	0.55	-0.34

*+ P<0.1, * P<0.05, ** P<0.01, *** P<0.001;*

[1)] Univariate GLM model fitted using function *manyglm* (R package: MVABUND). [2)] P calculated using 1000 permutations. [3)] P values adjusted using Benjamini-Hochberg procedure. [4)] Spearman correlation analysis between class and soil pH: numbers in bold are significant (P < 0.05). GLM models included block factor.

Table 5.10 Outcome of indicator species analysis showing combined relative abundance of species determined as indicator species of biocide and non-biocide plots at each level of soil pH treatment for bacterial community.

Phylum			Relative abundance[1] (%)				Low pH				Control pH				High pH			
							Non-biocide indicator		Biocide indicator		Non-biocide indicator		Biocide indicator		Non-biocide indicator		Biocide indicator	
Class	Order	Total	Low pH plots	Control pH plots	High pH plots	non-bio[2]	bio[2]	non-bio	bio	non-bio	bio	non-bio	bio	non-bio	bio	non-bio	bio
Ascomycota																		
Sordariomycetes	*Hypocreales*	8.0			10.1									0	0	1	3
	Coniochaetales	1.9			3.1									49	15		
	Sordariales	1.3			1.5									16	5	4	9
	Chaetosphaeriales	0.49	0.42		0.56			12	31					13	3		
	Glomerellales	0.45			1.3											69	101
	unassigned Order	4.4		5.4	4.0							0	0			0	0
Eurotiomycetes	*Chaetothyriales*	8.9	12.1		7.5	57	11									37	67
	Eurotiales	0.75															
	Onygenales	0.38			0.60											2	6
	unassigned Order	0.16															
Dothideomycetes	*Pleosporales*	6.7		5.8	12.9					0	0	0	1	26	14	2	5
	Tubeufiales	0.042															
	Dothideales	0.002															
	unassigned Order	0.56															
Leotiomycetes	*Helotiales*	4.7	4.5	4.2	5.5	2	0					0	1	3	1	1	3
	Thelebolales	0.006															
Geoglossomycetes	*Geoglossales*	3.9	3.6	6.4				0	14			0	9				
Ascomycota_cls_Incertae_sedis	*Ascomycota_ord_Incertae_sedis*	2.2		1.8								0	1				
Archaeorhizomycetes	*Archaeorhizomycetales*	1.2	2.0			17	3										
	unassigned Order	0.006															
Pezizomycetes	*Pezizales*	0.51															
Xylonomycetes	*GS34*	0.052															
Lecanoromycetes	*Pertusariales*	0.006															
	unassigned Order	0.18	0.29					32	110								
Saccharomycetes	*Saccharomycetales*	0.014															
unassigned Class	*unassigned Order*	7.9	14.2	6.4	2.6	19	9					0	0	42	30	0	10
Basidiomycota																		
Agaricomycetes	*Agaricales*	15.2	17.0	20.5		3	1			0	0						
	Atheliales	0.012															
	Auriculariales	0.002															
	unassigned Order	0.60	0.85	0.51	0.41	65	18					7	28			6	36
Tremellomycetes	*Tremellales*	6.8			7.6									1	0		
	Trichosporonales	1.6			3.8											50	118

Spiculogloeomycetes	*Spiculogloeales*	0.096		0.185										28	95				
Cystobasidiomycetes	*Cystobasidiales*	0.018	0.044			149	52												
	Erythrobasidiales	0.004																	
Microbotryomycetes	*unassigned Order*	0.011		0.014										14	101				
unassigned Class	*unassigned Order*	0.22		0.31	0.23							28	12					1	24
Mortierellomycota																			
Mortierellomycetes	*Mortierellales*	4.0	4.1	4.8		1	0							1	2				
Rozellomycota																			
Rozellomycota_cls_Incertae_sedis	*GS11*	1.8	2.2			0	0												
	GS07	0.008			0.027													33	153
unassigned Class	*unassigned Order*	0.33			0.25											10	2		
Glomeromycota																			
Glomeromycetes	*Glomerales*	0.18	0.22					0	56										
Chytridiomycota																			
unassigned Class	*unassigned Order*	0.043		0.046	0.050									0	18	32	18		
Mucoromycota																			
unassigned Class	*unassigned Order*	0.004																	

Relative contribution of sum of indicator species at order level to the mean relative abundance of particular order for each pH treatment level (%): 100 0

[1] 'Total' represents relative abundance for whole order while 'Low pH', 'Control pH' and 'High pH' represent relative abundance of orders for each soil pH treatment (displayed are only relative abundances of orders with indicator species). [2] Split of indicator species to the plots where they are present (i.e. 'non-bio'means plots without pesticide application and 'bio' means plots with biocide application).

5.2 Soil nutrient and ecosystem process cycles

Ammonium. Biocide application affected soil ammonium concentrations at time points of day 7 and day 28 (ANOVA, $F_{2,22} = 13.4$, $P < 0.01$ and $F_{2,21} = 18.6$, $P < 0.001$ respectively) and its application increased soil ammonium concentration at each of these time points (pairwise comparisons $P < 0.05$) (Figure 5.7). Soil ammonium concentration was affected by soil pH treatment at all three time points (ANOVA; $F_{2,22} = 31.4$, $P < 0.001$, $F_{2,21} = 39.5$, $P < 0.001$ and $F_{2,21} = 42.4$, $P < 0.001$ respectively) whereby the concentrations were always higher on high pH plots than control pH and low pH plots (pairwise comparisons $P < 0.05$) (Table 5.11) (Figure 5.8) (half of the dataset also analysed in Chapter 4 with the same significant trend).

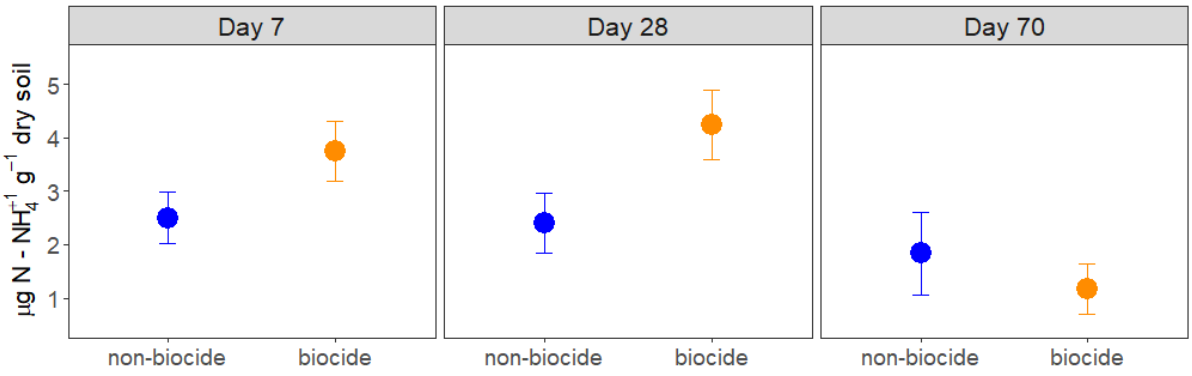


Figure 5.7 Effect of biocide application on soil ammonium concentration in grassland. Mean values of replicated measurements plotted. Error bar is ± standard error. Capital letters denote significant differences between means within each time point (pairwise comparisons $P < 0.05$).

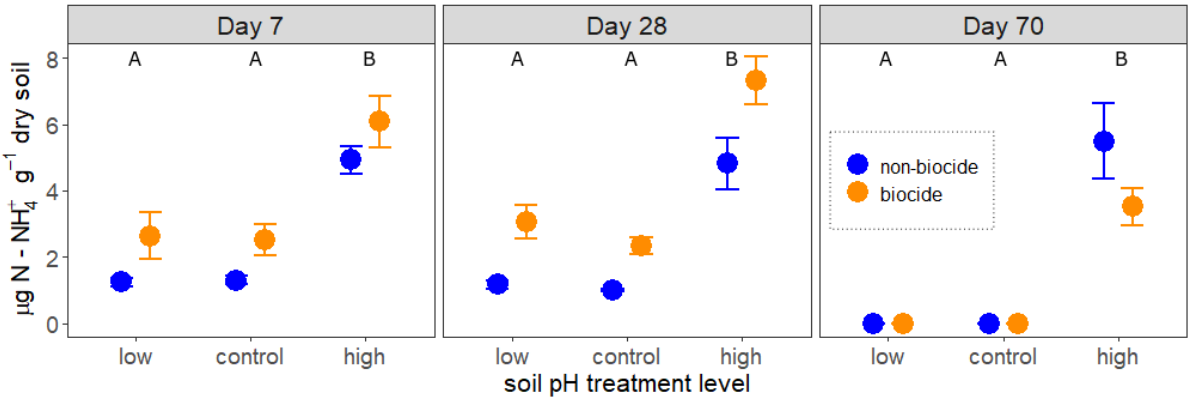


Figure 5.8 Effect of soil pH manipulation and biocide application on soil ammonium concentration in grassland. Mean values of replicated measurements plotted. Error bar is ± standard error. Capital letters denote significant differences between means of soil pH treatment levels within each time point (pairwise comparisons $P < 0.05$).

Nitrate. Soil pH treatment affected soil nitrate concentration at time points of day 7 and day 28 (ANOVA; $F_{2,23} = 9.7$, $P < 0.001$ and $F_{2,23} = 7.4$, $P < 0.01$ respectively) whereby high pH plots had always higher nitrate concentrations than control and low pH plots (pairwise comparisons $P < 0.05$) (Table 5.11) (Figure 5.9) (half of the dataset also analysed in Chapter 4 with the same significant trend).

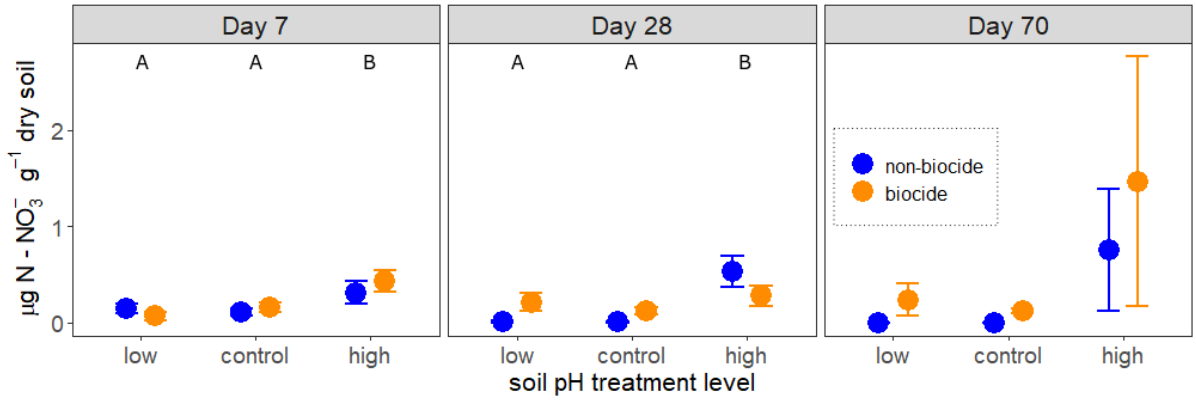


Figure 5.9 Effect of soil pH manipulation and biocide application on soil nitrate concentration in grassland. Mean values of replicated measurements plotted. Error bar is ± standard error. Capital letters denote significant differences between means of soil pH treatment levels within each time point (pairwise comparisons $P < 0.05$).

Net N mineralization. Overall net ammonification was higher at time point of day 70 than at time point of day 28 (ANOVA; $F_{1,52} = 21.2$, $P < 0.001$, pairwise comparisons $P < 0.05$) while net nitrification was lower at time point of day 70 than at time point of day 28 (ANOVA; $F_{1,50} = 4.8$, $P < 0.05$, pairwise comparisons $P < 0.05$) (Table 5.11) (Figure 5.10). Biocide application increased net ammonification only on day 70 (ANOVA; $F_{1,23} = 4.8$, $P < 0.05$, pairwise comparisons $P < 0.05$) (Figure 5.10). Net ammonification at earlier time point (day 28) responded differently to soil pH manipulation than at later time point (day 70; Chapter 4) (Figure 5.11). At time point of day 28, high pH plots showed lower net ammonification when compared to control and low pH plots (ANOVA; $F_{2,23} = 8.0$, $P < 0.01$, pairwise comparisons $P < 0.05$) In fact, values on high pH and control pH plots were negative. At time point of day 70, net ammonification was the highest on high pH plots (ANOVA; $F_{2,23} = 11.5$, $P < 0.001$, pairwise comparisons $P < 0.05$) (half of the dataset also analysed in Chapter 4 with the same significant trend).

Net nitrification was affected only by soil pH at both time points (day 28 and day 70) whereby high pH showed the highest level of nitrification at both time points (ANOVA; $F_{2,23} = 10.3$, $P < 0.001$, $F_{2,23} = 9.0$, $P < 0.01$, pairwise comparisons $P < 0.05$) (Figure 5.12) (half of the dataset at time point of day 70 also analysed in Chapter 4 with the same significant trend).

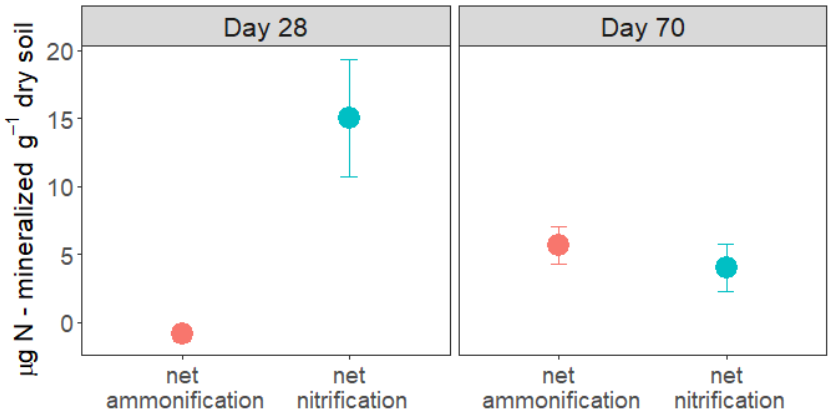


Figure 5.10 Effect of time on overall net N ammonification and nitrification in grassland. Overall mean values of replicated measurements (combined for pH and biocide treatments) plotted. Error bar is ± standard error.

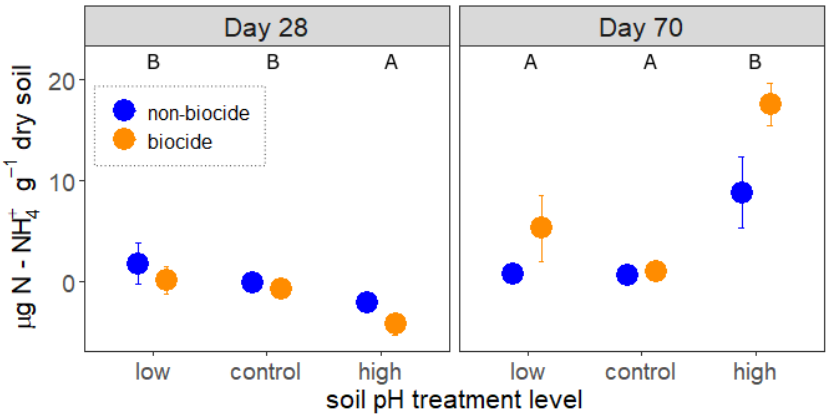


Figure 5.11 Effect of soil pH manipulation and biocide application on net ammonification in grassland. Mean values of replicated measurements plotted. Error bar is ± standard error. Capital letters denote significant differences between means of soil pH treatment levels within each time point (pairwise comparisons $P < 0.05$).

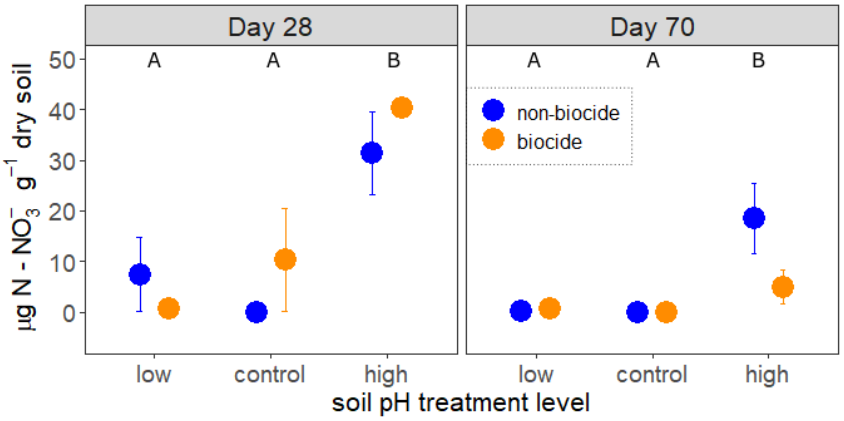


Figure 5.12 Effect of soil pH manipulation and biocide application on net nitrification in grassland. Mean values of replicated measurements plotted. Error bar is ± standard error. Capital letters denote significant differences between means of soil pH treatment levels within each time point (pairwise comparisons $P < 0.05$).

Ecosystem respiration. Interactive effects of soil pH manipulation and biocide application on ecosystem respiration rate were observed at time points of day 7 and day 28 (ANOVA, $F_{2,20} = 4.2$, $P < 0.05$ and $F_{2,19} = 6.9$, $P < 0.01$ respectively) (Table 5.11). At time point of day 7, ecosystem respiration rate was higher on high pH plots without biocide application than on control pH plots with biocide application and low pH plots without biocide application, while these differences were not seen for high pH plots with biocide application (posthoc comparisons $P < 0.05$) (Figure 5.13). This may suggest negative effect of biocide application on ecosystem respiration on high pH plots. At time point of day 28, biocide application reduced ecosystem respiration rate on control pH plots (posthoc comparisons $P < 0.05$) (Figure 5.13). Ecosystem respiration on low pH treatment plots did not respond to biocide application at any of these time points (posthoc comparisons $P > 0.05$). Only soil pH treatment showed an effect on ecosystem respiration at time point of day 70 (ANOVA, $F_{2,26} = 5.4$, $P < 0.05$) whereby it was higher on high pH plots than on low pH plots (posthoc comparisons $P > 0.05$) (Figure 5.13) (half of the dataset also analysed in Chapter 4 whereby high pH treatment showed higher respiration than both low pH and control pH treatments). When the data were analysed together for all three time points, interactive effect of soil pH treatment and biocide application was observed (ANOVA, $F_{2,76} = 9.6$, $P < 0.001$) apart from effects of time, soil pH

treatment and biocide application (ANOVA, $F_{2, 76} = 33.5$, $P < 0.001$, $F_{2, 76} = 17.4$, $P < 0.001$, and $F_{1, 76} = 12.8$, $P < 0.001$ respectively). This interactive effect showed a reduction of ecosystem respiration rate on control plots and high pH plots but not on low pH plots after biocide application (pairwise comparisons $P < 0.05$) (Figure 5.14).

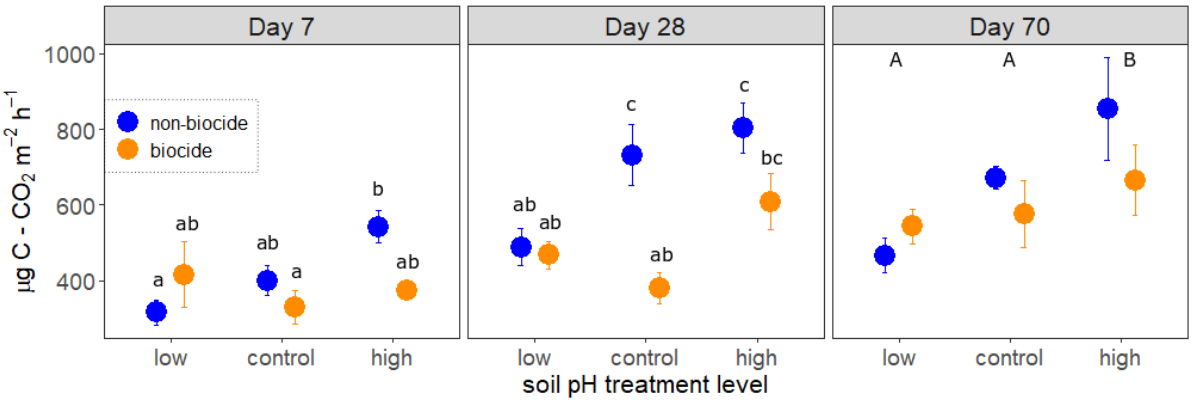


Figure 5.13 Interactive effect of soil pH manipulation and biocide application on ecosystem C flux in grassland. Mean values of replicated measurements plotted. Error bar is ± standard error. Capital letters denote significant differences between means of soil pH treatment levels within each time point (pairwise comparisons $P < 0.05$). Small letters denote significant differences between means of soil treatment levels within each time point (pairwise comparisons $P < 0.05$).

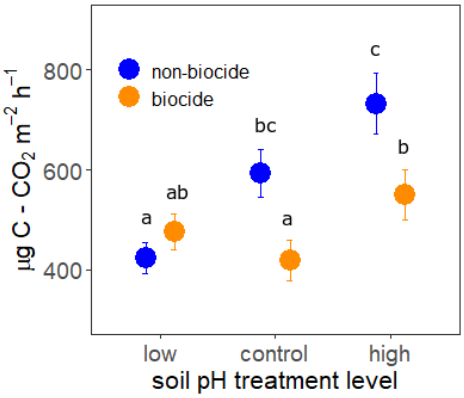


Figure 5.14 Effect of soil pH manipulation and biocide application on ecosystem C flux in grassland over all 3 time points studied. Mean values of replicated measurements plotted. Error bar is ± standard error. Letters denote significant differences between means of soil treatment levels (pairwise comparisons $P < 0.05$).

Table 5.11 Effect of soil pH manipulation and biocide application and their interaction on soil N and ecosystem C cycle in grassland.

Time of the analysis	Tested factors included in the model								
	pH manipulation			biocide application			pH manipulation x biocide application		
Response variable	df	F	P	df	F	P	df	F	P
Day 7									
ammonium	2	31.4	***	1	11.9	**			
nitrate	2	8.5	**						
ecosystem respiration [2)]	2	3.2	+				2	3.2	*
Day 28									
ammonium	2	70.8	***	1	53.5	***			
nitrate	2	9.5	***						
net N mineralization - ammonium	2	2.91	+						
net N mineralization - nitrate	2	10.3	***						
ecosystem respiration	2	13.3	***	1	21.6	***	2	6.9	**
Day 70									
ammonium	2	177	***						
nitrate									
net N mineralization - ammonium	2	17.7	***	1	6.39	*			
net N mineralization - nitrate	2	9	*						
ecosystem respiration	1	5.4	*						

+ P<0.1, * P<0.05, ** P<0.01, *** P<0.001;

ANOVA model for each response variable included all the factors and their interactions and non-significant terms were sequentially removed until all factors remaining were significant. [1)] *ln (y + 1) transformed,* [2)] *ln (y) transformed*

Plant available P. Interactive effect of soil pH manipulation and biocide application explained concentration of plant available P in the soil (ANOVA, $F_{2,24} = 7$, $P < 0.01$) (Table 5.12) whereby biocide application reduced the concentration only on high pH plots (pairwise comparisons $P < 0.05$ (Figure 5.15). On the plots without biocide application, a difference in the available P concentrations between low pH and high pH treatments was observed (pairwise comparisons $P < 0.05$).

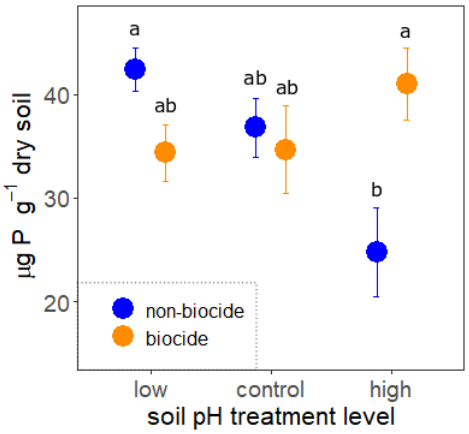


Figure 5.15 Effect of soil pH manipulation and biocide application on plant available P in grassland at the peak of the season (Day 70). Mean values of replicated measurements plotted. Error bar is ± standard error. Letters denote significant differences between means of soil treatment levels within each time point (pairwise comparisons $P < 0.05$).

Extracellular enzymes. Application of biocide did not affect potential activities of extracellular enzymes for C, N and P cycles in the soil (ANOVA, all $P > 0.1$) (Table 5.12).

Microbial biomass nutrients. Microbial biomass C:N showed an interactive effect of soil pH treatment and biocide application (ANOVA, $F_{2,24} = 7.0$, $P < 0.01$) however adjusting P value to account for multiple comparisons reduced the significance of the test to $P < 0.1$. When test with unadjusted P value is considered, microbial biomass C:N did not differ on pH plots without biocide application while it was higher on high pH plots when compared to control pH and low pH plots after biocide application (pairwise comparisons $P < 0.05$) (Figure 5.16). Furthermore, biocide application reduced microbial biomass C:N ratio on low pH plots (pairwise comparisons $P < 0.05$). This suggest that biocide had an opposite effect on microbial biomass C:N for different soil pH treatments whereby it reduced the ratio on low and control plots but did not affect the ratio on high pH plots.

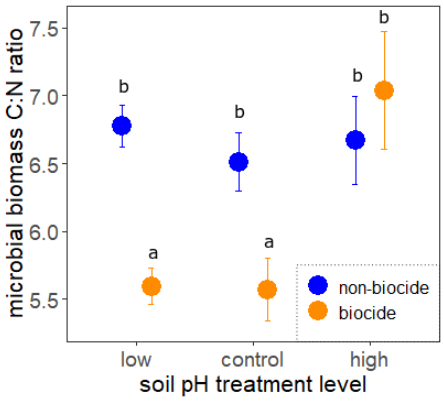


Figure 5.16 Effect of soil pH manipulation and biocide application on microbial biomass C:N ratio in grassland at the peak of the season (Day 70). Mean values of replicated measurements plotted. Error bar is ± standard error. Letters denote significant differences between means of soil treatment levels within each time point (pairwise comparisons $P < 0.05$).

Table 5.12 Soil pH manipulation, biocide treatment and their interaction on tested ecosystem characteristics in grassland.

Response variables	df			F			P [2]			Soil pH manipulation and biocide treatments [3]					
										Non-biocide			Biocide		
	pH	biocide	pH x biocide	pH	biocide	pH x biocide	pH	biocide	pH x biocide	low pH	control pH	high pH	low pH	control pH	high pH
Soil characteristics															
ammonium (ug NH_4^+-N g^{-1} dry soil) [1]	2	1	2	198.5	2.1	2.1	***	0.36	0.34	0 ± 0	0 ± 0	5.5 ± 1.1	0 ± 0	0 ± 0	3.5 ± 0.6
nitrate (ug NO_3^--N g^{-1} dry soil) [1]	2	1	2	2.3	0.8	0	0.30	0.56	0.99	0 ± 0	0 ± 0	0.76 ± 0.64	0.24 ± 0.17	0.12 ± 0.03	1.47 ± 1.3
Olsen P (ug PO_4^{3-}-P.g^{-1} dry soil)	2	1	2	1.3	0.6	7	0.52	0.64	*	42.4 ± 2.1	36.8 ± 2.8	24.7 ± 4.3	34.4 ± 2.7	34.7 ± 4.3	41 ± 3.5
DON (ug ON g^{-1} dry soil) [1]	2	1	2	10.9	0	0.7	***	0.95	0.67	5.5 ± 2.7	4.4 ± 0.3	8.5 ± 0.9	3.4 ± 0.8	5.4 ± 0.5	9.6 ± 1.2
DOC (ug OC g^{-1} dry soil) [1]	2	1	2	25.8	1	0.8	***	0.52	0.65	49.4 ± 20.1	35.7 ± 2.1	93 ± 12.2	37.6 ± 6.2	44.1 ± 4.4	121.6 ± 11.3
DOC:DON ratio	2	1	2	4.2	1.1	0.2	+	0.52	0.89	10.9 ± 2.5	8.1 ± 0.3	11.3 ± 1.7	12.4 ± 2	8.3 ± 0.4	13.3 ± 1.9
Microbial characteristics															
biomass C (µg biomass C g^{-1} dry soil)	2	1	2	0.1	1	0.2	0.97	0.52	0.87	210 ± 21	197 ± 11	219 ± 20	233 ± 22	230 ± 37	220 ± 21
biomass N (µg biomass N g^{-1} dry soil)	2	1	2	0.5	3.8	1.4	0.78	0.18	0.51	31.2 ± 3.6	30.5 ± 2.3	33.4 ± 4	41.5 ± 3.6	40.9 ± 5.9	32 ± 3.9
biomass C:N ratio	2	1	2	5.2	7.1	4.8	+	+	+	6.8 ± 0.2	6.5 ± 0.2	6.7 ± 0.3	5.6 ± 0.1	5.6 ± 0.2	7 ± 0.4
Plant biomass															
aboveground (g m^{-2})	2	1	2	2.3	0	1.3	0.31	0.95	0.52	386 ± 58	453 ± 27	545 ± 84	474 ± 29	399 ± 23	497 ± 52
Plant aboveground nutrients															
C (mg C g dry biomass^{-1})	2	1	2	8.9	37.5	0.7	**	***	0.67	451 ± 2	447 ± 1	445 ± 1	442 ± 1	442 ± 1	437 ± 2
N (mg N g dry biomass-^{1}) [1]	2	1	2	6.5	3.8	0.4	*	0.18	0.80	11.1 ± 0.5	11.1 ± 0.5	12.9 ± 1.1	9.6 ± 0.2	10.5 ± 0.5	12 ± 0.6
P (mg P g dry biomass^{-1})	2	1	2	5.3	27.7	0.7	*	***	0.67	3.1 ± 0.1	2.7 ± 0.1	2.6 ± 0.1	2.5 ± 0.1	2.2 ± 0.1	2.3 ± 0.1
C/N ratio	2	1	2	7.2	2.5	0.5	*	0.31	0.76	41.1 ± 2.1	40.9 ± 2.3	35.4 ± 2.7	46.1 ± 0.9	42.4 ± 2	36.8 ± 1.9
C/P ratio	2	1	2	3.5	19.3	0.4	0.15	***	0.81	147 ± 5	165 ± 9	171 ± 7	180 ± 8	200 ± 13	193 ± 5
N/P ratio	2	1	2	22	7.4	0.4	***	*	0.81	3.6 ± 0.1	4.1 ± 0.3	4.9 ± 0.2	3.9 ± 0.2	4.7 ± 0.1	5.3 ± 0.2
C (g C m^{-2})	2	1	2	2	0.1	1.2	0.36	0.86	0.52	174 ± 27	203 ± 12	242 ± 38	210 ± 12	176 ± 10	217 ± 23
N (g N m^{-2})	2	1	2	7.8	0.6	1.1	*	0.61	0.56	4.2 ± 0.6	5 ± 0.1	6.7 ± 0.7	4.6 ± 0.3	4.2 ± 0.3	6 ± 0.8
P (g P m^{-2})	2	1	2	1.3	4.6	1.2	0.52	0.13	0.52	1.2 ± 0.1	1.2 ± 0.1	1.4 ± 0.2	1.2 ± 0.1	0.9 ± 0.1	1.1 ± 0.1
Soil basal respiration															
(µg CO_2-C g^{-1} dry soil h^{-1}) [1]	2	1	2	29.2	0	0.1	***	0.94	0.95	1.5 ± 0.4	1.8 ± 0.4	6.9 ± 1.3	1.2 ± 0.1	2 ± 0.3	6.9 ± 1.4
Ecosystem respiration															
(mg CO_2-C m^{-2} h^{-1}) [1]	2	1	2	5.7	0.9	1.8	*	0.56	0.39	467 ± 45	672 ± 30	854 ± 136	543 ± 46	577 ± 79	665 ± 93
Net N mineralization															
(µg NH_4^+-N g^{-1} dry soil) [1]	2	1	2	61	7.5	1.6	***	*	0.46	0.8 ± 0.1	0.6 ± 0.1	8.8 ± 3.5	5.3 ± 3.3	1 ± 0.4	17.6 ± 2.1
(µg NO_3^--N g^{-1} dry soil) [1]	2	1	2	10.9	2	1.4	***	0.38	0.51	0.04 ± 0.01	0.02 ± 0.01	18.4 ± 7	0.68 ± 0.83	-0.06 ± 0.04	4.9 ± 3.4
(ng NH_4^+-N µg^{-1} microbial-C) [1]	2	1	2	17.9	5	1.4	***	0.12	0.51	3.7 ± 0.5	3.2 ± 0.3	45.2 ± 19.7	23.8 ± 13.6	4.4 ± 1.2	82.1 ± 10.7
(ng NO_3^--N µg^{-1} microbial-C) [1]	2	1	2	9.3	3.3	4.1	**	0.22	+	0.2 ± 0.1	0.1 ± 0	83.6 ± 30.3	4.5 ± 4.9	-0.3 ± 0.2	18.6 ± 12.4
Soil enzyme kinetics															
AG (nmol g^{-1} dry soil min^{-1}) [1]	2	1	2	20.5	2.7	0.4	***	0.30	0.80	0.2 ± 0.01	0.22 ± 0.02	0.35 ± 0.04	0.24 ± 0.03	0.23 ± 0.04	0.41 ± 0.02
BG (nmol g^{-1} dry soil min^{-1})	2	1	2	1.7	0.7	0.5	0.42	0.58	0.76	1.9 ± 0.2	2.8 ± 0.4	2.5 ± 0.5	2.3 ± 0.4	2.7 ± 0.4	3.3 ± 0.7
CHIN (nmol g^{-1} dry soil min^{-1})	2	1	2	0.4	0.2	1.2	0.80	0.79	0.52	0.75 ± 0.04	0.88 ± 0.17	0.76 ± 0.12	1 ± 0.13	0.76 ± 0.13	0.77 ± 0.10
ACE (nmol g^{-1} dry soil min^{-1})	2	1	2	1.2	1.5	1.6	0.52	0.46	0.46	37.4 ± 2.7	44.4 ± 2.7	36.7 ± 2.3	47.8 ± 2.5	42.1 ± 4.8	39.6 ± 5.3
LEU (nmol g^{-1} dry soil min^{-1}) [1]	2	1	2	57.5	0.8	1	***	0.56	0.58	2.3 ± 0.1	3.1 ± 0.3	6.3 ± 0.8	2.5 ± 0.4	2.9 ± 0.3	7.6 ± 0.7
LEU (pmol g^{-1} microbial-N min^{-1}) [1]	2	1	2	44.4	0.9	2.8	***	0.56	0.22	76 ± 9	103 ± 4	204 ± 37	61 ± 7	75 ± 8	252 ± 34
PHO (nmol g^{-1} dry soil min^{-1})	2	1	2	2.9	2.5	0.8	0.21	0.31	0.63	8.5 ± 0.5	11.9 ± 1.2	11.5 ± 1.1	11.1 ± 1.2	11.7 ± 1.2	13.9 ± 1.7
C:N enzyme kinetics ratio	2	1	2	28.3	0.3	0	***	0.75	0.99	0.86 ± 0.1	0.89 ± 0.07	0.39 ± 0.05	0.91 ± 0.06	0.91 ± 0.11	0.42 ± 0.05
C:P enzyme kinetics ratio	2	1	2	0.3	0.1	0.7	0.85	0.87	0.68	0.23 ± 0.03	0.24 ± 0.02	0.21 ± 0.03	0.20 ± 0.02	0.23 ± 0.01	0.23 ± 0.03
N:P enzyme kinetics ratio [1]	2	1	2	79.2	0.7	0.8	***	0.61	0.65	0.27 ± 0.02	0.27 ± 0.02	0.55 ± 0.05	0.22 ± 0.02	0.26 ± 0.02	0.56 ± 0.03

+ P<0.1, * P<0.05, ** P<0.01, *** P<0.001;

[1] response variable ln(y + 1) transformed [2] P values corrected by Benjamini-Hochberg adjustment procedure. [3] mean ± standard error. Tests were performed using two-way ANOVA.

5.3 Plant biomass

Plant aboveground biomass was not affected by biocide or soil pH application (ANOVA, $F_{2,24} = 0$, $P = 0.95$, $F_{2,24} = 2.3$, $P = 0.31$ respectively). Biocide application reduced plant aboveground biomass C and P concentrations (ANOVA, $F_{1,24} = 37.5$, $P < 0.001$, $F_{1,24} = 27.7$ respectively, pairwise comparisons $P < 0.001$, Table 5.12). Plant biomass P concentration decreased faster than biomass C concentration resulting in higher biomass C:P concentration ratio on biocide treated plots when compared to non-biocide plots (ANOVA, $F_{1,24} = 19.3$, $P < 0.001$, pairwise comparisons $P < 0.05$, Figure 5.17). Plant biomass N:P concentration ratio was also enhanced after biocide application (ANOVA, $F_{1,24} = 7.2$, $P < 0.05$, pairwise comparisons $P < 0.05$, Figure 5.17).

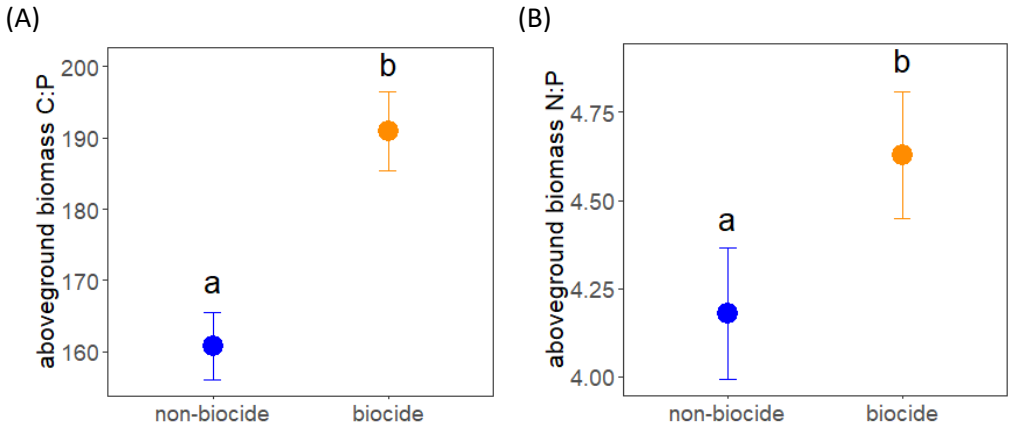


Figure 5.17 Effect of biocide application on plant aboveground biomass A) C:P concentration ratio and B) N:P concentration ratio in grassland. Mean values of replicate measurements plotted. Error bar is ± standard error. Letters denote significant differences between means of treatment levels (pairwise comparisons $P < 0.05$).

5.4 Discussion

Fungal community diversity responded to the biocide application with interactive effects (soil pH treatment x biocide application) on diversity indices (unique sequences, Simpson's and Shannon diversity) when only the effect on Shannon diversity was significant. Pairwise comparisons of Shannon diversity indices suggested that low pH and high pH treatments responded in the opposite direction to low pH treatment whereby low pH and high pH treatment diversity increased/tended to increase (respectively). Fungal community structure showed an interactive effect of soil pH treatment and biocide application including a tendency for differentiation between non-biocide low pH treatment and biocide high pH treatment (non-significant). Fungal phylotypes marked as indicator species responding to the biocide application showed complex and often opposite trends when they were expressed as relative abundances of respective fungal orders: *Chaetothyriales*, *Trichosporonales* and *Glomerellales* included relatively high proportion of indicator species in high pH treatment soil, while orders *Spiculogloeales* and *Geoglossales*, and orders *Chaetosphaeriales*, *Geoglossales* and *Glomerales* included a relatively high proportion of indicator species in control pH and low pH treatment soils respectively. Biocide application increased soil ammonium concentration at all pH levels as determined within 28 days after the application but not at day 70 while nitrate concentration was unaffected, and these ammonium increases were not mirrored by net N mineralization rates. Ecosystem respiration showed a trend for decrease on control and high pH plots 7 and 28 days after biocide application but was restored 70 days after the application. Plant aboveground biomass lowered its N concentration on all biocide plots.

Soil bacterial and fungal community diversity

It was found that fungal community in control pH soils tended not to respond to the biocide application in the same direction as low pH treatment fungal community contrary to hypothesis 1. Both, low pH and control pH treatments showed comparable biotic and abiotic ecosystem characteristics (including acidic soil pH) (Chapter 4) and thus their similar response to the biocide application was expected. However, comparison of fungal diversity indices responses to the biocide application demonstrated that fungal diversity in control pH treatment soils tended to response in the same direction as fungal diversity in high pH treatment soil. This trend was significant only for Shannon diversity index after removal of an outlier while full dataset showed nonsignificant pairwise differences.

Data further suggested that Shannon diversity tended to increase after biocide application on high pH treatment plots. Applied biocide was expected to cause cell death, releasing nutrients and making niches available for colonization. Increase of fungal diversity on high pH treatment plots was expected in the present research due to lower activity determined for fungi in non-acidic soils (Barcenas-Moreno et al., 2016) and such anticipated slower fungal growth on high pH treatment plots would reduce fungal competition for available resources leading to a greater number of fungal taxa coexisting in the soil. These taxa can originate from the soil community or can immigrate as fungi can be relatively easily transferred as spores on large distances (Shade et al., 2012). On the other hand, fungal diversity was expected to decrease on acidic pH plots due to relatively higher fungal activity found in acidic soils (Barcenas-Moreno et al., 2016) potentially resulting in competitive exclusion of species. Nevertheless, tendency for similar trends of fungal diversity in acidic control pH and high pH treatment soils did not confirm the above expectations.

Soil bacterial and fungal community composition

Biocide application including two fungicides affected fungal community with a relatively greater intensity than bacterial community, especially at higher taxonomic resolutions. Structure of fungal communities at class and order taxonomic resolutions showed an overall response to the biocide at all tested soil pH treatment levels while for bacterial community, these responses were in interaction (or tended to be in interaction) with soil pH treatment. At the lowest resolution (i.e. individual ASVs), both fungi and bacteria showed an interactive response to biocide and pH treatments. The biocide includes two fungicides, thus an effect predominantly on fungi was expected. On the other hand, effects of fungicides on bacteria were observed elsewhere (e.g. Feld et al., 2015; Fernández-Calviño et al., 2017). Bacteria was showed to increase respiration, which have been attributed to stress (Hassain et al., 2009). The stress response may be for instance due to disruption of fungal role of primary decomposers of plant derived compounds through extracellular enzyme synthesis and resulting lack of available substrates for bacterial uptake. Furthermore, fungi in association with plant roots are important for distribution of plant photosynthates through the soil structure as these compounds were shown to appear predominantly in fungal biomass in an undisturbed grassland (Hannula et al., 2017). Disruption of fungal network might affect distribution of these substances to bacteria. Bacterial activity can be also temporarily enhanced by released nutrients from fungi killed by applied fungicides (Hassain et al., 2009) potentially promoting fast growing (copiotrophic) taxa quickly capitalizing on available nutrients.

Fungal composition at the lowest taxonomic resolution showed an interactive effect of both treatments whereby biocide application resulted in a differentiation of low pH treatment community without biocide application from high pH treatment community after biocide application. Indicator

species analysis output encompassing relative contribution of fungal ASVs marked as indicator species to the overall relative abundance of the particular order showed following trends. Firstly, some orders included indicator species for biocide treatment only, and indicator species for more than one pH level within the order were observed such as *Geoglossales* (low pH and control pH), *Trichosporonales*, *Glomerellales* (both high pH) or *Glomerales* (low pH). Order *Archaeorhizomycetales* includes indicator species for plots without biocide (low pH).

Relative abundance of order *Trichosporonales* increased in high pH treatment soils (GLM, $dev_{2,19}$ = 32.7, $P < 0.05$, pairwise comparisons $P < 0.05$) and this order also contained a relatively large portion of indicator species for biocide treatment at high pH treatment level (Table 5.10). *Trichosporonales* (overall average relative abundance of 1.82 % ± 0.53 %) is a representative of basisiomycetous soil yeasts. Grasslands were described to harbour predominantly acomycetous yeasts (Yurkov et al., 2012; Yurkov, 2018), however, it was not the case in the present study which contained relatively large fraction of basisiomycetous yeasts (overall average relative abundance of 15 %) as opposed to very low ascomycetous yeast presence (overall average relative abundance of 0.02 %; not including black yeast like fungi). Basidiomycetous yeast species can utilize a wide spectrum of C substrates including complex compounds such as hemicellulose and lignin, while ascomycetous yeasts are characterized by copiotrophic lifestyle (Yurkov, 2017). Interestingly, the high pH soil treatment resulting in higher availability of soil substrates (mineral N, DOC) did not promote yeast with copiotrophic lifestyle from order Saccharomycetales but promoted basidiomycetous yeast. On the other hand, basidiomycetous yeast within plant rhizosphere was found to receive recent plant photosynthate (Hannula et al., 2012) which would suggest copiotrophic lifestyle. Promotion of basidiomycetous yeasts with ability to degrade complex plant substrates on high pH treatment soils might be related to a decrease of macroaggregation after lime application as reported by Egan et al. (2018) which would potentially increase availability of physically protected POM. Increased plant structural components decomposition (i.e. cellulose) would also result in a greater activity of β-glucosidase extracellular enzyme, however this was not observed. Relative abundance of *Trichosporonales* showed a tendency for increase after biocide application on all soil pH treatment plots (GLM, $dev_{1,19}$ = 32.7, $P_{unadjusted} < 0.001$), while other orders belonging to the same class did not show such trend. Slavikova and Vadkertiova (2003) reported differences in responses of different yeast species to two fungicides. *Trichosporonales* thus appears to benefit from application of the biocide as oppose to representatives of other orders, however these other orders did not show a negative response. The ability of yeasts to withstand attack of fungicide might be related to their ability to produce extracellular polysaccharides, which might function as a protective layer reducing penetration of fungicide towards cells.

Archaeorhizomycetales were found to be higher on low pH treatment plots when compared to high pH treatment plots soils (GLM, $dev_{2,19}$ = 26.4, $P < 0.05$, pairwise comparison $P < 0.05$) in contrary to Lanzen et al. (2015) who found positive correlation with soil pH in mountain grasslands. It is newly characterized group of fungi, which is assumed to prefer to live in a close vicinity of plant roots and was shown to be strongly associated with taxa from phylum *Glomeromycota* (Rosling et al., 2011; Chroma et al., 2016) however the nature of these interactions is unknown (Rosling et al., 2013). Its average relative abundance of 1.52 % ± 0.28 % in the present study of grasslands is similar to its abundance on selected sites across Europe (2 %) (while abundance on grasslands was up to 1 %) and co-occurrence analysis suggested that it is playing a non-negligible role in soil fungal communities as one of the keystone species (Chroma et al., 2016). It can grow on glucose and cellulose as sole substrates (Rosling et al., 2013) and was negatively correlated with soil basal respiration and soil total N content in a grassland survey in Spain (Epelde et al., 2017). It was suggested to play an important role in cycling of C derived from living or dead roots in soil (Rosling et al., 2013). Thus, potential negative effects of biocide (as suggested by interactive effect of soil pH manipulation and biocide application treatments, GLM, $dev_{2,19}$ = 8.3, $P_{unadjusted} < 0.05$; only non-significant tendency for

decrease of relative abundance on low pH treatment plots after biocide application) might result in an effect on soil C cycling as well as fungal community composition.

Secondly, other orders such as *Sordariales*, *Pleosporales* included indicator species for biocide treated as well as non-treated soils at the same pH level which may suggest a successional development within the order after biocide application. Third example included orders which encompassed indicator species for biocide treatment on one pH level while indicator species for biocide non-treated soils were found on another pH level, such as order *Chaetothyriales*. Taxa belonging to these three orders were found to be associated with plant organic matter degradation such as utilization of cellulose and cellobiose (Ma et al., 2013; Voriskova and Baldrian, 2013; Fan et al., 2019). Relative abundance of *Chaetothyriales* showed a tendency for higher relative abundance in low pH treatment soils when compared to high pH treatment soils (GLM, $dev_{2,19} = 10.4$, $P_{unadjusted} < 0.05$, posthoc comparison $P < 0.1$) which is in accordance with observed proliferation of members of this order in acidic soils and extremely oligotrophic environments by others (Männistö et al., 2018). Due to its melanized cell wall which can help the cell to withstand various environmental stresses (Cordero and Casadevall, 2017), *Chaetothyriales* can be expected to increase proliferation in biocide treated soils under low pH treatment, however it was not the case. Instead, *Chaetothyriales* showed a relatively high proportion of taxa marked as indicator species on high pH treatment plots. Overall, other orders also include indicator species but relative abundance of these orders or occurrence of indicator species within these orders are low.

It is worth mentioning, that mycorrhizal fungi from class *Glomerales* showed a tendency for positive response to biocide application (GLM, $dev_{1,19} = 7.5$, $P_{unadjusted} < 0.05$) which is on the opposite direction than expected (e.g. Bennett and Cahill 2016; Dostálek et al. 2013). Biocide was applied as one single application as opposed to small doses applied over the growing season as in the mentioned studies. Facilitation by plants might be behind a greater regrowth of their mycorrhizal symbionts after the initial decline by biocide action. Mycorrhiza is an important mechanism for increasing plant uptake of soil nutrients (Hodge and Storer, 2014), and thus, following its decline after biocide action plants might increase allocation of C substrates to promote their regrowth resulting in a larger growth than on control plots.

The fungal community analysis was performed 70 days after the biocide application. While immediate impact on fungal community was expected shortly after the application and also partially confirmed by a tendency for lower ecosystem respiration determined for control pH and high pH treatment plots, such a relatively long time since the application might result in regrowth of the community. Fungal communities in control and high pH treatment soils were suggested to respond similarly (as above) and thus both communities may have been affected similarly by biocide. However, the anticipated regrowth of the community might be more profound in high pH soils as demonstrated by similarity of biocide treated fungal community to non-biocide community at this pH level. If a disturbance is not strong enough to tip a system to a new stable state, it would return to its original position with time (Shade et al., 2012) showing resilience. However, tendency for differentiation between biocide and non-biocide fungal communities in control pH treatment soils might suggest slower or no resilience of control pH fungal community to a disturbance.

Moreover, the present experiment was in natural settings and early immediate changes of biocide applied in the begging of growing season may have indirectly affected plant growth through changes in soil nutrients and plant-microbe associations (Van Der Heijden et al., 2008) with possible consequences for feedback to microbial community (Haichar et al., 2014) later in the season. The changes observed at the peak season (70 days after biocide application) as reported above might be reflective of these feedbacks.

Soil N

Application of the biocide (active ingredients were tebuconazole and trifloxystrobin) increased availability of mineral N in the soil, and this increase was at time points of day 7 and day 28 after the application and was not determined on day 70 after the application. The increase was in the form of ammonium while nitrate stayed unchanged compared to non-biocide plots. Muñoz-Leoz et al. (2011) reported over 10-fold increase of ammonium concentration measured on day 7 after application of various doses of tebuconazole in a laboratory experiment. The ammonium concentration remained high until day 28 followed by a decrease towards the end of their experiment on day 90. Similarly, Cycoń et al. (2006) also reported positive effect of tebuconazole on ammonium concentration in the soil, however the change only lasted few days.

Ammonium increase after tebuconazole application was attributed to potential release of N from dead fungi or potential stimulation of ammonifying bacteria (Cycon et al., 2006). In the present experiment, microbial biomass N was more than ten times higher than the difference between soil ammonium on non-biocide and biocide plots. Thus, mineralization of dead microbial cells resulting in soil ammonium increase cannot be ruled out.

Feld et al. (2015) associated tebuconazole with a negative effect on nitrifying bacteria and archaea. However, ammonium increase was not accompanied by nitrate decline in the present experiment. Additionally, net N mineralization rate measured at time point of day 28 did not show an increase of ammonification or reduction of nitrification. Net N mineralization assays have to be interpreted with caution; N mineralized during the assay might be utilized by soil microorganisms which are released from competition with plants during the assay (Schimel and Bennett, 2004). Also, relative abundance of phylum *Nitrospirae*, which harbours nitrite oxidising bacteria, did not respond to fungicide application 70 days after the biocide application further suggesting that nitrification may not have been affected.

Interestingly, net ammonium mineralization increased on day 70 on biocide plots compared to non-biocide plots while soil ammonium did not change. However, the increase of ammonium determined by the assay may had been used by plants thus not increasing soil ammonium level in the field. Nevertheless, potential rate of extracellular enzyme for N cycle and soil organic N did not change in relation to biocide application. The unresponsiveness of β-1,4-N-acetylhexosaminidase enzyme process rate on biocide plots, which can degrade chitin from fungal cells, might suggest that the net ammonium increase on biocide plots on day 70 may not be related to decomposition of dead fungi as a result of biocide effect.

Although both fungicides applied contained N in their chemical composition, it was not expected that observed N increase in the soil originates solely from their degradation. Tebuconazole degradation in the soil was found to be of on average from 22.4 % (Bending et al. 2007) to 75 % (Papadopoulou et al., 2016) within 90 and 70 days respectively. Nevertheless less than 8 % of the fungicide's C was respired within 70 days in a lab study and a range of degradation products were found (El Azhari et al., 2018). Nearly all these degradation products contained triazole ring from the tebuconazole and this ring contains all the N present in the tebuconazole, thus, it can be assumed that very limited N was released from tebuconazole to the soil in their study. On the other hand, the commercial fungicides applied contained also an additive containing N. It can be assumed that some of this N would be released to the soil, however the additive has previously been found to bind to soil minerals (Čadková et al., 2012). The amount of N in the additive was 1.76 µg N g^{-1} dry soil. Trifloxytrobin also contained N at a rate of 1.85 µg N g^{-1} dry soil. Banerjee et al. (2006) reported 80 % degradation of trifloxystrobin in 7 days. Its degradation products, containing N from the original compound, were shown to have similarly fast degradation kinetics (Banerjee et al. 2006). It can be

thus expected that up to 1.5 µg N g^{-1} dry soil can be released from the tryfloxistrobin within the 7 days after application and further 2 µg N g^{-1} dry soil can be released from the remaining tryfloxistrobin and additives in the tebuconazole within 4 weeks after application. The potential N amount equals to 2.6 kg N ha^{-1} which is much lower as an average N deposition from agriculture activity expected on the site. Murphy et al. (2015) reported that addition of 10 and 30 ug NH_4NO_3 g^{-1} dry soil did not affect ammonium or nitrate concentration in the soil within a week lab incubation study. Hence, it was expected in the present experiment that such a small amount of N potentially released from the applied biocide will be quickly utilized by plants and would not affect soil N processes.

Ecosystem respiration

Results demonstrated a pH induced variability in the response of ecosystem respiration to the biocide application. Ecosystem respiration decreased on control and high pH treatment plots after biocide application for all three time points when the data were analysed together. When the data were analysed separately for each time point, the decrease of ecosystem respiration was determined on high pH treatment plots on day 7 and control pH treatment plots on day 28. Ecosystem respiration on low pH treatment plots was not affected by biocide application at all. This is surprising because low pH and control pH treatments showed similar biological, physicochemical and plant characteristics (Chapter 4) and thus their similar response was expected.

Others observed variable results of fungicide-induced respiration response. Increased soil field respiration, mainly in the peak season, was observed after multiple fungicide additions during three subsequent seasons targeting primarily AMF fungi in a semiarid grassland ecosystem (Zhang et al., 2016), however not in the first year of the experiment. Soil basal respiration was not negatively affected by application of a fungicide similar to the presented research in a wide range of doses tested in a lab study (Fernández-Calviño et al., 2017), while it decreased in another lab study using a similar tebuconazole dose to the present research (Wang et al., 2016).

Ecosystem respiration is a broad process. It can be expected that it is less affected by changes in microbial community composition due to potential functional redundancy in C mineralization (Rousk et al., 2009). Nevertheless, the observed decrease of ecosystem respiration may be related to fungicide induced microbial die off (Keiblinger et al., 2018) especially if the fungicide would nonspecifically target large portion of the fungal community. Potential increase of respiration due to microbial capitalization on released resources due to fungal die-off may not have been high enough in relation to respiration decrease in order to play a role in ecosystem respiration.

Moreover, fungal contribution to soil respiration was found to be higher in acidic soils than alkaline soils (Barcenas-Moreno et al. 2006), thus the applied biocide reducing fungal community would be expected to decrease respiration predominantly on acidic experimental plots in the present experiment. Respiration was lower on control pH treatment plots, which were acidic, however it did not change on low pH treatment plots. High stability of the respiration on the low pH treatment plots might be related to resistance of this microbial community to a disturbance. Acidic soils are usually characterized by high fungal:bacterial ratio. Higher fungal:bacterial ratio may be associated with higher resistance to a disturbance (De Vries & Shade, 2013).

Interestingly, the biocide effect on soil respiration was not determined on day 70 when the data was analysed separately at each time point. And similarly, soil basal respiration, which was measured only on day 70, was also not affected by biocide application. This may suggest a recovery of the ecosystem respiration towards day 70. However, indicator species analysis demonstrated positive response of relative abundance of individual phylotypes grouped at order taxonomic level to biocide

application. Such displacement of some phylotypes by others would mean that the recovered respiration was maintained due to functional redundancy of the taxa changed on biocide plots compared to non-biocide plots (Allison and Martiny, 2008). Alternatively, a portfolio effect, averaging positive responses of some taxa by negative responses of other taxa (Allison and Martiny 2008), may play a role in the recovery of ecosystem respiration.

Fungal phylum *Glomeromycota* and its orders tended to increase relative abundance on biocide plots. AMF, as represented by *Glomeromycota*, can account for up to 25 % of root respiration (Moyano et al. 2007; Nottingham et al. 2010). Relative abundance of *Glomeromycota* was below 0.25 % and thus such relatively low abundance may not have represented a strong contribution to ecosystem respiration.

Plant nutrients

Despite the increase of soil ammonium at day 7 and day 28, plant aboveground biomass yield and biomass N did not respond to the increase in the same direction. Plant aboveground yield did not change. This lack of response is similar to the response of the aboveground biomass on non-biocide high pH plots compared to non-biocide control pH and low pH plots whereby high N availability did not increase aboveground biomass yield (Chapter 4).

Overall, it was found that acidic control pH treatment and non-acidic high pH treatment grassland plots differing in key ecosystem abiotic and biotic characteristics (microbial community structure, N and C cycle rate, vegetation composition) expressed similar responses of some of these ecosystem components to biocide application (fungicide mixture) regardless of these differences. Fungal diversity showed a trend for an increase in control and high pH treatment soils after the biocide application while bacterial diversity was not affected. Fungal community structure has changed, showing an interactive effect of the biocide and soil pH treatments but pairwise comparisons only suggested a differentiation between fungal community on high pH treatment soils after biocide application from low pH treatment community without biocide application. Order *Capnodiales* tended to decease and orders *Trichosporonales* and *Glomerales* tended to increase after the biocide application on all soil pH treatment levels, and *Trichosporonales* and *Glomerales* included relatively high proportion of indicator species (determined as phylotypes) for biocide application on high pH and low pH treatment plots respectively. The tendency for similar responses of control and high pH treatments to the biocide application as found for fungal community diversity was further demonstrated for ecosystem respiration whereby it was decreased for control and high pH treatments after the biocide application. Stability of ecosystem respiration 70 days after the biocide application for all pH treatment levels, and for the whole period since the biocide application for low pH treatment only, together with changes in relative abundances of fungal taxa might suggest functional redundancy for OC transformations within the community. For instance, relative abundance of fungal orders important for C transformations in the soil, such as *Archaeorhizomycetales* and *Capnodiales*, tended to decrease in low pH treatment soils after biocide application while ecosystem respiration was unchanged. Biocide application increased soil ammonium immediately after the application for all pH treatments (as determined 7 and 28 days after the application) potentially due to release of nutrients from fungal biomass or disruption of nitrification. This increase of soil N availability early in the growing season did not result in enhancement of plant aboveground biomass yield or N concentration as determined 70 days after the application. Such differential responses in ecosystem and net ammonification between high pH and control pH treatment soils after the biocide application might suggest a differential stability of ecosystem function to perturbation in dependence of soil pH, however similarity of response between acidic control pH and non-acidic high pH treatment plots suggest more complex

interactions within the system are behind the stability of ecosystem function in the low pH treatment soils.

It has to be pointed out that the soil sampling design was not equal on biocide and control treatment plots as biocide treatment plots were samples from half the area as control plots. This may have introduced bias related to data which were derived from the soil analysis such as microbial community composition, soil nutrients and processes. Jangid et al. (2010) found lower bacterial diversity with smaller sample area possibly related to greater patchiness found in the larger area. In the present research, bacterial and fungal richness and diversity indices did not show reduction related smaller sample area (biocide treatment). This together with the relatively small area of the both treatment plots (4.5 m^2 vs. 2.25 m^2 for control and biocide application plots respectively) would suggest that the effect of sampling area differences will be likely minimal, however caution must be used comparing soil data with other studies.

Chapter 6: **Effect of plant species interactions differing in life strategy on soil N cycle, bacterial and fungal communities and ecosystem C flux.**

6.1 Introduction

Responses of plant communities to changes in environmental factors (such as climate change) most likely result in changes to community structure (Bardgett et al., 2008). Plant community structure change has been already documented in the case of increased N loading of ecosystem due to N deposition (Stevens et al., 2004). Changes in community structure can result in altered ecosystem processes when plant response traits are linked to effect traits by trade-offs or trait correlations (Lavorel and Garnier, 2002). Plants can affect ecosystem processes through different direct and indirect interactions with the soil microbial community, as microbes are key players in soil organic matter transformations (Bardgett and Wardle, 2012). Therefore, it is important to understand the mechanistic basis of these interactions and resulting effects on soil functioning, in order to be able to predict consequences of plant community change for multiple ecosystem provision (Grigulis et al., 2013) and feedback to the Earth-system (Orwin et al., 2010a).

Plant traits have been widely studied as they are a useful tool in explaining the consequences of plant community change on characteristics of ecosystems such as ecosystem processes (e.g. Violle et al., 2007; De Deyn et al., 2008; Orwin et al., 2010; Lavorel and Garnier, 2012). Orwin *et al.* (2010) showed that different plant species coexisting in grassland imposed changes on soil N and P cycles and microbial community and these changes were predictable from growth strategies of the plants as determined by growth rate and leaf and litter C:N ratio. Moreover, plant life strategies determined by collection of specific traits and their values were shown to express predictable responses in soil nutrient cycles, mainly N cycle (Grigulis et al. 2013; Legay et al., 2014). Fast growing plant species (exhibiting resource acquisitive growth strategies) are characterized by high leaf N and low leaf C:N ratio and typically associated with high soil mineral N and N cycling rates; whilst the opposite is true for slow growing species (resource conservating strategists). It has also been shown that plant functional traits associated with fast growing plant species are associated with bacterial dominated soil communities in grass species monocultures (Orwin *et al.*, 2010) and in grasslands on a reginal scale (De Vries *et al.*, 2012; Grigulis *et al.*, 2013). The differential plant effect on microbial communities of these life strategies can be expressed through differences in rhizodeposition between fast and slow growing plants (Kaštovská et al., 2014) or quality of plant litter inputs (De Deyn *et al.*, 2008).

Community weighed means of plant trait attributes have been shown to predict ecosystem characteristics (de Vries et al., 2012; Lavorel et al., 2011). However, mixed plant communities exhibit increased performance when compared to monocultures, pointing to complementary mechanisms (Spehn et al., 2000). Other mechanisms for the positive diversity effect include facilitation or dilution of pathogens (Van der Putten et al., 2013).

In order to increase understanding of relationship of plant interactions and resulting differences in plant composition and community traits with soil C and N processes, a mesocosm experiment was established utilising five locally abundant grass species from family *Poaceae*. Members of this plant family are cultivated as crops of high economic significance but also includes species which are present in natural grasslands, thus this family represents an important study area. In addition, earlier

studies of plant-soil interactions were often performed combining different plant functional groups, however, knowledge of variability of interactions within single functional group can be beneficial for further understanding of interaction in mixed communities.

6.2 Hypothesis

It was hypothesised that (1) Plant species with different growth rates and trait characteristics will be functionally differentiated along the resource acquisition-conservation spectrum, allowing prediction of their effect on soil processes in the monocultures. Specifically, resource acquisitive plant species characterized by higher biomass N content and quality as well as higher growth rate will be associated with faster C and N cycling in the ecosystem and higher proportion of copiotrophic members of soil microbial community than resource conservative plant species.

(2) Plant species interaction in species mixed communities will affect growth of individual plant species and will affect rate of soil processes at the ecosystem level.

6.3 Methodology

6.3.1 Experimental design

The species used were divided into two groups based on their growth rate characteristics: fast growing species (*Holculs lanatus*, *Lolium perenne*, *Poa trivialis*) and slow growing species (*Anthoxanthum odoratum*, *Festuca rubra*). Treatments consisted of full factorial combinations of mixtures of five, four, three and two species, and each species in monoculture. Bare soil mesocosms were also included in the treatments.

The mesocosms were set up in a fenced compartment at the Lancaster University field station at Hazlerigg, UK (54°1'N, 2°46' W, 94 m a.s.l) and were arranged in block design with 32 treatments replicated four times giving total of 128 mesocosms (Figure 6.1). Each mesocosm consisted of pots (38 cm × 38 cm area with 30 cm depth) filled with 10 cm of granite chippings followed by 20 cm of low-nutrient top-soil (pH of 5.6, 3.06 % C, 0.19 % N). The soil was from sandy-loam extensively managed pasture collected at the site and was sieved through 8 mm sieve to remove large stones and roots. Each mesocosm (excl. bare soil treatment) was planted with 36 3-month old plants during the last week of June 2016. Seedlings were planted in an evenly spaced grid in all mesocosms (each seedling 5 cm apart from its neighbours or sites of the pot). For the mixtures, seedlings were planted along the edges of the pot first following the same pattern towards the centre of the pot and they were planted in an order randomly selected for each block.

The plants were grown for two consecutive seasons before the final harvest. Mesocosms were watered using tap water when it was needed (May and June of the second seasons had large number of days without a significant rainfall and demanded watering more times a day).

6.3.2 Measurements

Sampling

The mesocosms were harvested in the week of 10 – 15 July 2017. Five soil cores of 3.5 cm diameter were taken from each mesocosms from an area of in-between plants distributed across whole mesocosm excluding places between outer plant row and pot site. The top 2 cm of each soil core was removed before taking 5 cm long soil core. The five cores from each mesocosm were homogenized by sieving through a 4-mm sieve and stored at 4 ˚C until analysed. A subsample for microbial analysis was taken and stored at – 20 ˚C within 72 h after sampling. Plant biomass was harvested by cutting plants 2.5 cm above the soil and sorted according to species for each species mixture mesocosm. Plant biomass in paper bags were then pre-dried at temperature below 65 ˚C and stored until analysed.

CO_2 fluxes

Fluxes of CO_2 were measured on three time points in the final season including 8 – 11 May, 13 – 15 June and 5 – 7 July, 2017, using portable gas analyser (EGM-4; PP Systems, Hertfordshire, UK), attached to a custom made chamber (Orwin et al., 2014). Net ecosystem exchange (NEE) was measured as the net CO_2 flux obtained using transparent chamber (92 % light transparency) and ecosystem respiration was measured as flux in darkened chamber. Rates of photosynthesis were calculated as the difference between NEE and ecosystem respiration (Orwin *et al.*, 2014). Soil temperature and photosynthetically active radiation PAR were measured at the same time. Gas flux calculations were adjusted to account for changes in air temperature and atmospheric pressure.

Soil abiotic characteristics

Measurements included concentrations of soil ammonium and nitrate, processes of net ammonification and net nitrification and gravimetric soil moisture content. Details of the measurements are listed in Methods chapter (Chapter 2).

Soil biotic characteristics

Bacterial and fungal community composition was determined employing 16S and ITS amplicon sequencing respectively. For bacteria, the V4 hypervariable region of 16S rRNA gene was amplified using 515f-806r primers (Walters et al., 2015) and for fungi, the ITS2 region was amplified using fITS7-ITS4r primer sequences (Ihrmark et al., 2012). Extraction of DNA, amplification of target gene sequences, sequencing and sequences processing and taxonomy assignment was performed as specified in Methods chapter (Chapter 2).

Plant aboveground biomass

Plant biomass was dried at 65 ˚C for three days before weight measurement of each species from each mesocosm was taken. The biomass of species belonging to same species mixture was combined for each unique mesocosm. The biomass was cut into smaller pieces to homogenize before two subsamples were taken, ball milled and homogenized to represent one aboveground biomass sample. The samples were analysed for C and N content. Details of analytical method described in Methods chapter (Chapter 2).

6.3.3 Data analysis

One-way ANOVA was used to analyse effect of plant species identity and plant life strategy on multiple ecosystem characteristics in monocultures and effect of plant species richness and interactive effect of plant species richness and plant occurrence on multiple ecosystem characteristics in mixtures. The ecosystem characteristics tested included soil N cycle variables (ammonium concentration, nitrate concentration, net ammonification and net nitrification), C ecosystem effluxes (ecosystem respiration, photosynthesis and net ecosystem exchange (NEE)) and aboveground biomass yield. Ecosystem characteristics for monocultures also included plant aboveground biomass nutrient concentration (C, N and C:N ratio) and plant aboveground nutrient pools (C and N). For the mixtures, the analysis was performed on three datasets. The first dataset contained all data including monocultures and mixtures. The second dataset included only mixtures to determine effect of species interactions in the mixtures. The third dataset included only two-, three- and four species mixtures to determine interactive effect of species mixture richness and species occurrence in the mixture.

To gain a further understanding of how species interactions affect ecosystem characteristics in the mixtures, logarithmic response ratio (LRR) for each ecosystem characteristic was calculated as ln(O/E), where O is observed value and E is expected value. The expected value was calculated based on monoculture response and the relative number of individuals of each species within the mixture (Orwin *et al.*, 2014). The LRR indicates whether functioning of particular ecosystem variable differed from the expected functioning calculated using value from monoculture (average value across the monocultures was used). LRR > 0 means positive diversity effect and LRR < 0 means negative diversity effect.

For those species with a negative effect of their occurrence on overall aboveground biomass of the mixtures, the effect of the species occurrence on aboveground biomass of individual species present in the mixture was tested using one-way ANOVA. The relationship between aboveground biomass yield and biomass of each species included in the mixture was tested using spearman's correlation test.

Models were examined for constancy of variance and normality of errors and ln(y) transformed was used to improve model fit. Kruskal-Wallis test was used in cases when ANOVA model fit was not improved by the transformation. All significant effects were tested for differences between the means using post-hoc Tukey test or Dunn's test if Kruskal-Wallis test was used. All models included block factor. Models for ecosystem C effluxes also included measurements of soil temperature (for ecosystem respiration and NEE) and photosynthetic assimilative radiation (PAR) (for NEE) to take into account variation of site characteristics during the measurements. For the model, testing interactive effect of species richness and species occurrence, improvement of the model fit was also performed by removing outliers determined by visual examination of model residuals, however if model could not be improved by removing of maximum of four data-points, Kruskall-Wallis test was used instead (this model included only species occurrence effect variable). The removal of data-points is marked in the result section.

For microbial data analysis, one-way ANOVA was used to examine effect of plant species identity and plant life strategy on microbial community diversity (richness, Shannon's and Simson's indexes), and relative abundance of microbial taxa.

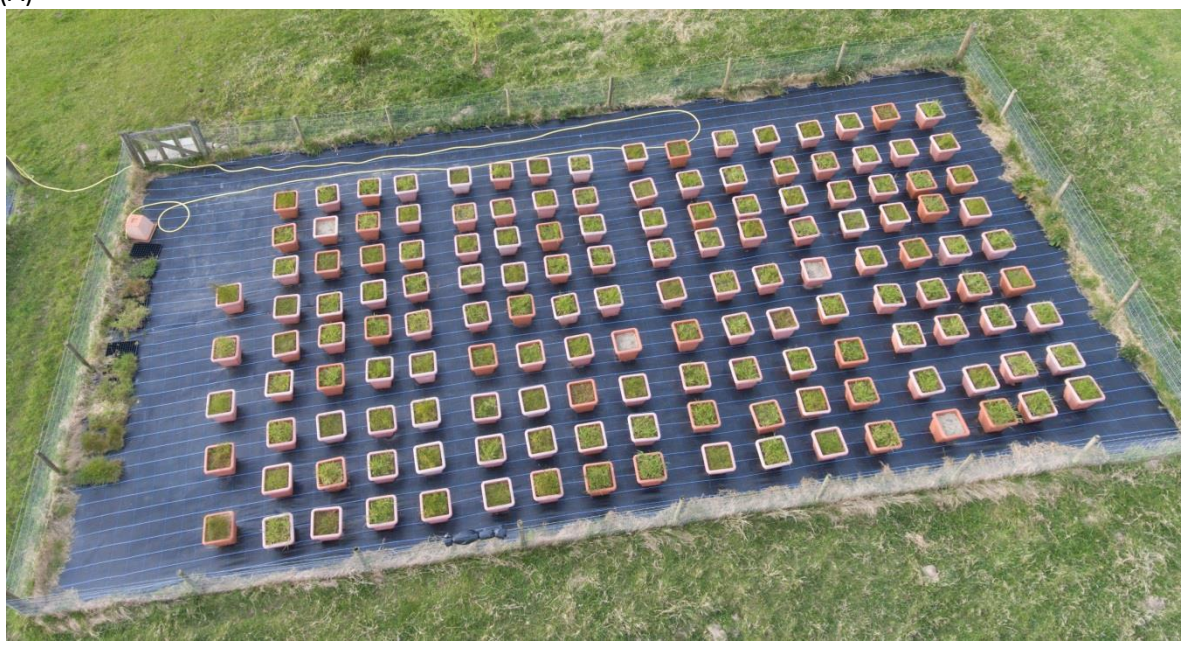
Response of microbial community composition at species taxonomic resolution to plant species in monocultures was assessed by multivariate generalised linear models (M-GLMs) using GLM framework from MVABUND 3.10.4 package in R (Wang et al., 2012). Details of the analysis are listed in method chapter (Chapter 2). Taxa with less than 5 reads were removed prior the analysis.

Microbial taxa were associated with plant species in monocultures using indicator species analysis (Dufrêne & Legendre, 1997) and grouped into cosmopolitan taxa (associated with all plant species), intermediate (associated with 2 – 4 plant species) and specialized (associated with only a single plant species). Details of the analysis are listed in method chapter (Chapter 2).

Fungal species were further functionally characterized using FUNGUILD database (Nguyen et al., 2016).

All analyses were conducted in R of version 3.5.0 (R Core Team, 2018).

(A)



(B)

(C)

Figure 6.1 Pictures of experimental site showing (A) aerial view (in May of the second season), (B) mesocosms in the end of the first growing season and (C) mesocosms in the second growing season (middle June).

6.4 Results

6.4.1 Monocultures

Soil N cycle

For soil N cycle measurements, only soil nitrate and net nitrification were affected by species identity in monocultures (ANOVA, $F_{5,90} = 34.8$, $P < 0.001$ and $F_{5,90} = 12.0$, $P < 0.05$ respectively) (Table 4.1, Figure 6.2(A-B)). The results also showed an effect of plant life strategy whereby fast growing plant species had higher soil nitrate and net nitrification in comparison to slow growing species (ANOVA, $F_{1,79} = 21.9$, $P < 0.05$ and $F_{1,79} = 5.4$, $P < 0.05$ respectively) (Table 4.1). Differences in ecosystem respiration between species in monocultures were not observed and only bare soil mesocosms showed lower ecosystem respiration than some or all species mesocosms in dependence of time point of the measurement (ANOVA, $F_{5,90} = 9.3$, $P < 0.001$; Kruskal Wallis, $chi^2 = 15.2$, d.f. = 5, $P < 0.05$ and $chi^2 = 13.5$, d.f. = 5, $P < 0.05$ in May, June and July respectively, pairwise comparisons $P < 0.05$; Table 4.1, Figure 6.3(A-C)).

Ecosystem C flux

Photosynthesis was affected by species identity at both time points whereby in May, photosynthesis was lower for *P. trivialis* in comparison to *A. odoratum* (Kruskal Wallis, $chi^2 = 11.5$, d.f. = 5, $P < 0.05$, pairwise comparisons $P < 0.05$) (Figure 6.3(D)) while in June, photosynthesis of *F. rubra* was higher in comparison to *P. trivialis* and *A. odoratum* (Kruskal Wallis, $chi^2 = 12.0$, d.f. = 5, $P < 0.05$) (Figure 6.3(E)). Photosynthesis was also affected by plant life strategy in May when it was higher for slow growing species in comparison to fast growing species (ANOVA, $F_{1,81} = 6.1$, $P < 0.5$) Table 4.1). In May, NEE (representing difference between ecosystem respiration and photosynthesis) was affected by both plant species (Kruskal Wallis, $chi^2 = 11.5$, d.f. = 5, $P < 0.05$, pairwise comparisons $P < 0.05$) (Figure 6.3(E)) and life strategy (Kruskal Wallis, $chi^2 = 6.1$, d.f. = 1, $P < 0.05$) (Table 4.1) whereby slow growing species showed lower NEE than fast growing species. NEE was affected by plant species also in June whereby *F. rubra* showed lower NEE in comparison to *P. trivialis* (Kruskal Wallis, $chi^2 = 10.6$, d.f. = 5, $P < 0.05$, pairwise comparisons $P < 0.05$) (Figure 6.3(G)).

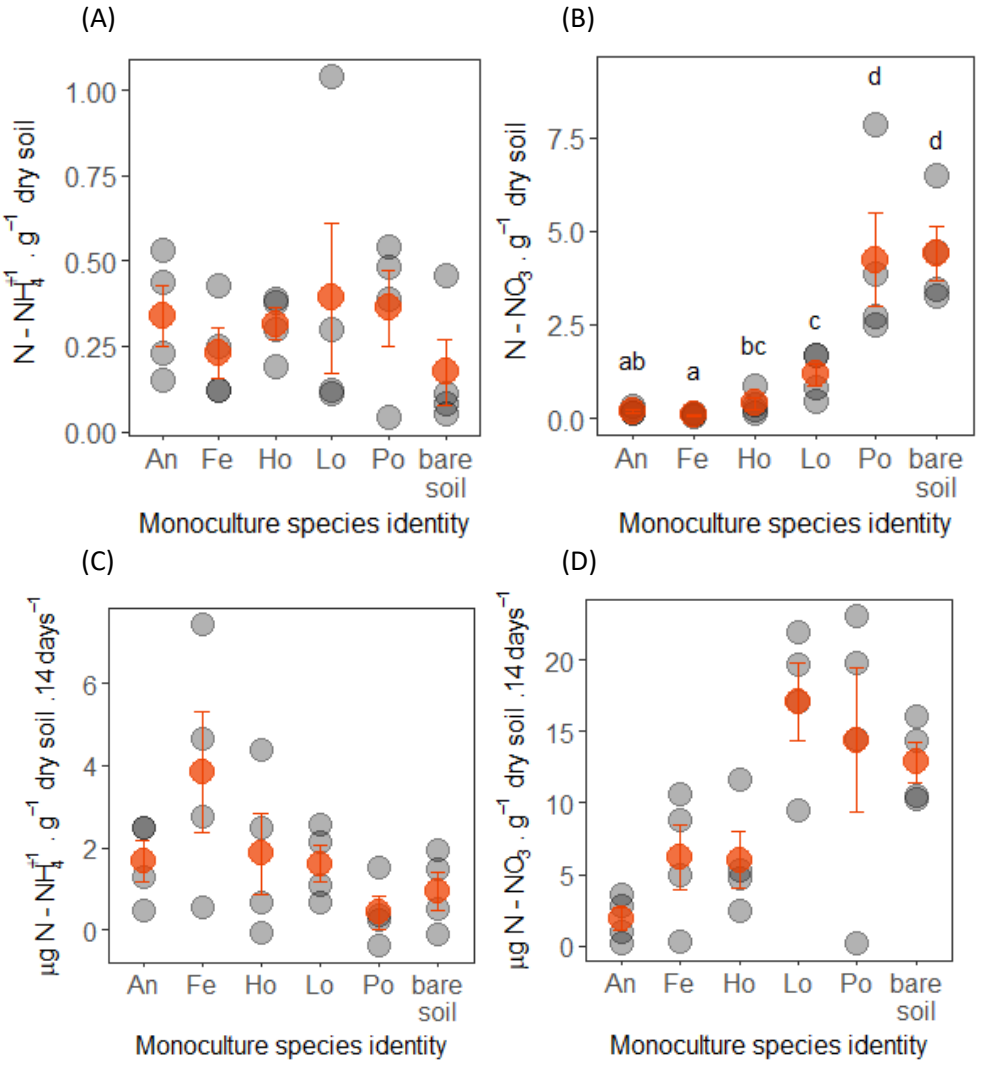


Figure 6.2 Effect of plant species identity on soil N cycle: concentration of soil (A) ammonium and (B) nitrogen, (C) net ammonification and (D) net nitrification in single species mesocosms. Orange points are means values and whiskers are s.e. Letters are significant differences between means ($P < 0.05$). An = *A. odoratum*, Fe = *F. rubra*, Ho = *H. lanatus*, Lo = *L. perenne* and Po = *P. trivialis*. Slow growing species are An and Fe, fast growing species are Ho, Lo and Po.

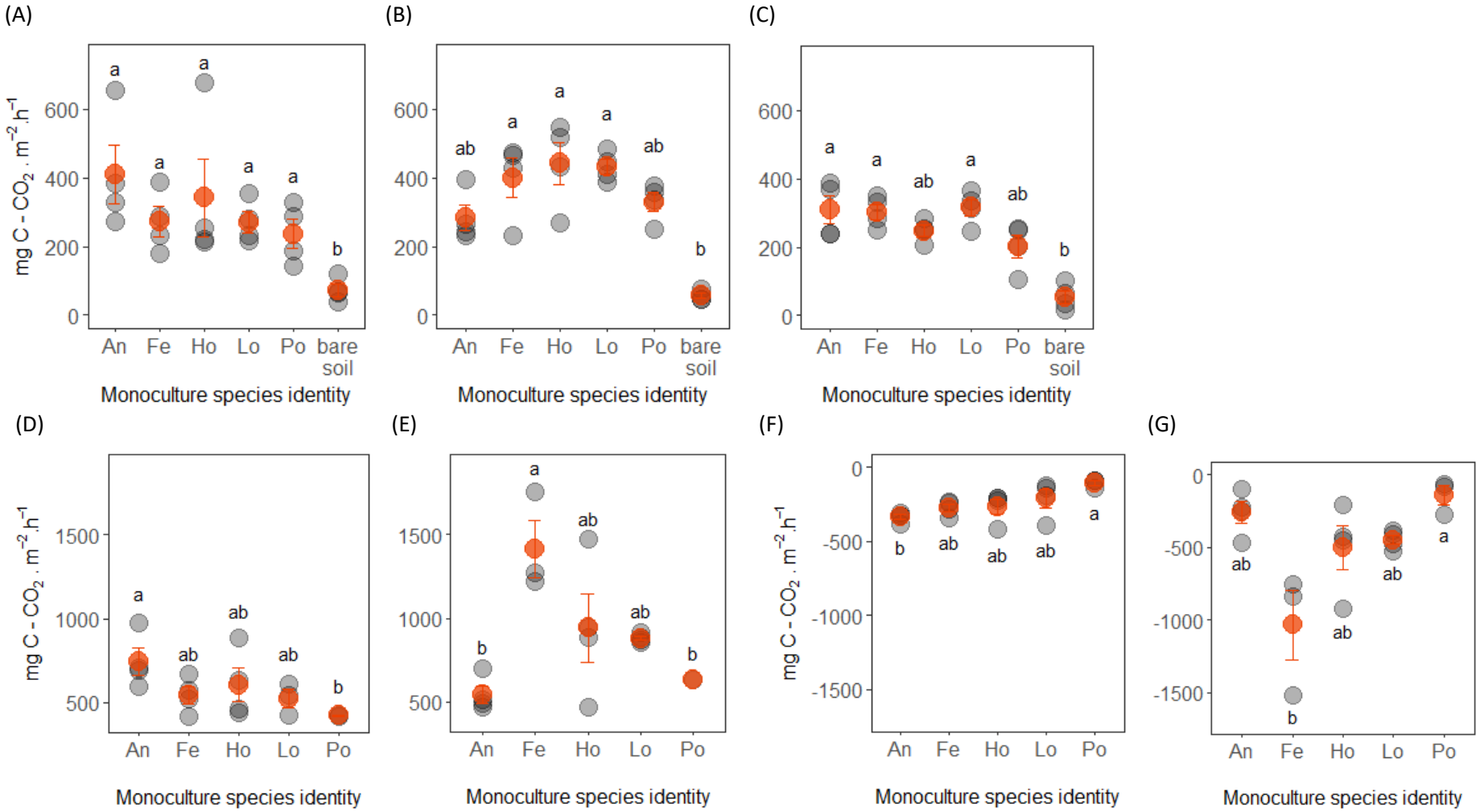


Figure 6.3 Effect of plant species identity on ecosystem C effluxes: ecosystem respiration in (A) May, (B) June and (C) July, photosynthesis in (D) May and (E) June and NEE in (F) May and (G) June in single species mesocosms. Orange points are means values and whiskers are s.e. Letters are significant differences between means ($P < 0.05$). An = *A. odoratum*, Fe = *F. rubra*, Ho = *H. lanatus*, Lo = *L. perenne* and Po = *P. trivialis.* Slow growing species are An and Fe, fast growing species are Ho, Lo and Po.

Plant characteristics

Plant aboveground biomass yield, C and N concentration and C:N ratio responded to species identity in the monocultures (Kruskal Wallis, $chi^2 = 12.2$, d.f. = 4, $P < 0.05$, $chi^2 = 11.3$, d.f. = 4, $P < 0.05$ and $chi^2 = 4.5$, d.f. = 4, $P < 0.05$, pairwise comparisons $P < 0.05$) (Table 6.1). For the biomass yield, *F. rubra* had higher biomass than *P. trivialis* (Figure 6.4). For the aboveground biomass C and N concentrations, the differences were only between *F. rubra* and *L. pratense* (Figure 6.5(A)) and *A. odoratum* and *P. trivialis* ($P < 0.05$) (Figure 6.5(B)) respectively. For the aboveground biomass C:N ratio, *A. odoratum* differed from *P. trivialis* (Figure 6.5(C)).

Plant life strategy affected plant aboveground biomass C and N (Kruskal Wallis, $chi^2 = 5.8$, d.f. = 1, $P < 0.05$; ANOVA, $F_{1,69} = 11.2$, $P < 0.01$) (Table 6.1). Fast growing plants showed lower aboveground biomass C and higher aboveground biomass N which resulted in lower aboveground biomass C:N ratio in comparison to slow growing plants (ANOVA, $F_{1,69} = 13.5$, $P < 0.01$).

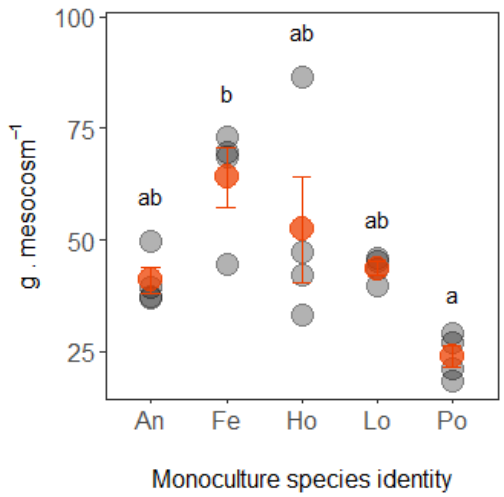


Figure 6.4 Effect of plant species identity on plant biomass yield in single species mesocosms. Orange points are means values and whiskers are s.e. Letters are significant differences between means ($P < 0.05$). An = *A. odoratum*, Fe = *F. rubra*, Ho = *H. lanatus*, Lo = *L. perenne* and Po = *P. trivialis.* Slow growing species are An and Fe, fast growing species are Ho, Lo and Po.

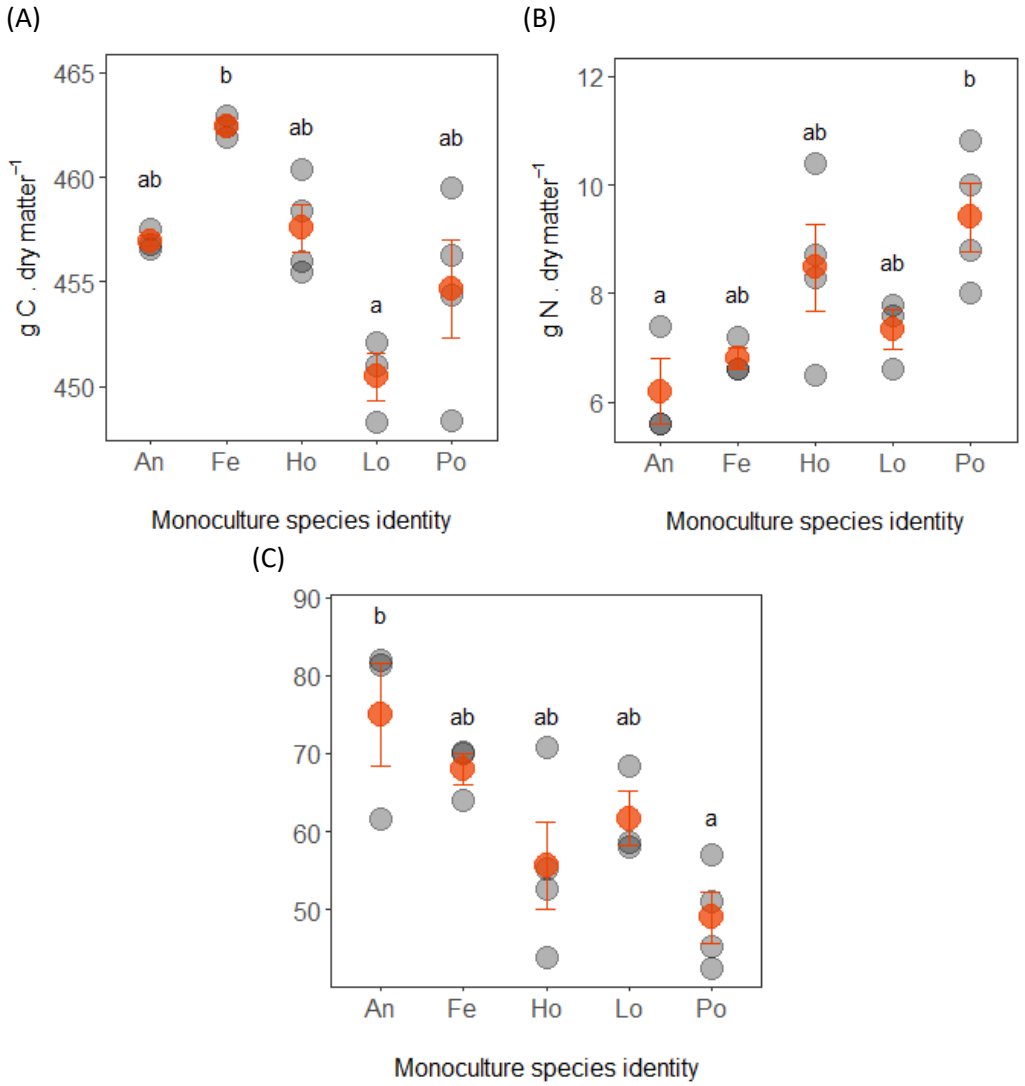


Figure 6.5 Effect of plant species identity on plant aboveground biomass nutrients: concentration of biomass (A) C and (B) nitrogen, and (C) biomass C:N concentration ratio in single species mesocosms. Orange points are means values and whiskers are s.e. Letters are significant differences between means ($P < 0.05$). An = *A. odoratum*, Fe = *F. rubra*, Ho = *H. lanatus*, Lo = *L. perenne* and Po = *P. trivialis.* Slow growing species are An and Fe, fast growing species are Ho, Lo and Po.

Both C and N aboveground biomass pools were affected only by plant species identity (Kruskal Wallis, $chi^2 = 11.4$, d.f. = 4, $P < 0.05$ and $chi^2 = 13.5$, d.f. = 4, $P < 0.01$ respectively, pairwise comparisons $P < 0.05$) (Table 6.1) whereby the difference in both pools was expressed only between *F. rubra* and *P. trivialis* (Figure 6.6).

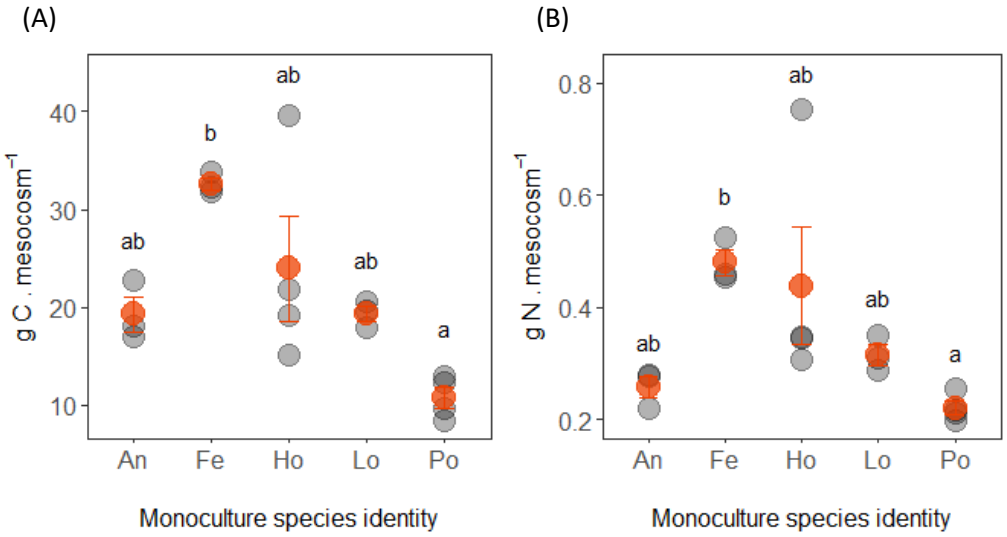


Figure 6.6 Effect of plant species identity on plant aboveground biomass nutrient pools of (A) C and (B) N in single species mesocosms. Orange points are means values and whiskers are s.e. Letters are significant differences between means (P < 0.05). An = *A. odoratum*, Fe = *F. rubra*, Ho = *H. lanatus*, Lo = *L. perenne* and Po = *P. trivialis.* Slow growing species are An and Fe, fast growing species are Ho, Lo and Po.

Table 6.1 Effect of Species identity on ecosystem characteristics determined in monocultures.

		Species effect [3]		Plant life strategy effect [4]
N cycle				
Ammonium (mg NH_4^+ - N g^{-1} dry soil)	[1]	0.7	[2]	0.2
Nitrate (mg NO_3^- - N g^{-1} dry soil)	[1]	**34.8** ***	[2]	**21.9** ***
net Ammonification (mg NH_4^+ - N g^{-1} dry soil .14 $days^{-1}$)		1.8	[2]	2.8 +
net Nitrification (mg NO_3^- - N g^{-1} dry soil .14 $days^{-1}$)	[1]	**12.0** *	[2]	**5.4** *
C cycle				
Ecosystem respiration in May (mg CO_2 - C $.m^{-2}.h^{-1}$)	[1]	**9.3** ***	[1]	2.9
Ecosystem respiration in June (mg CO_2 - C $.m^{-2}.h^{-1}$)	[2]	**15.2** *	[2]	1.5
Ecosystem respiration in July (mg CO_2 - C $.m^{-2}.h^{-1}$)	[2]	**13.5** *	[2]	1.5
Photosynthesis in May (mg CO_2 - C $.m^{-2}.h^{-1}$)	[2]	**11.5** *	[1]	**6.1** *
Photosynthesis in June (mg CO_2 - C $.m^{-2}.h^{-1}$)	[2]	**12.0** *	[1]	0.3
NEE in May (mg CO_2 - C $.m^{-2}.h^{-1}$)	[2]	**11.3** *	[2]	**6.1** *
NEE in June (mg CO_2 - C $.m^{-2}.h^{-1}$)	[2]	**10.6** *	[2]	0.6
Plant characteristics				
Plant aboveground biomass (g.$mesocosm^{-1}$)	[2]	**12.2** *	[2]	2.1
Aboveground biomass C (mg C $.g^{-1}$ dry biomass)	[2]	**11.3** *	[2]	**5.8** *
Aboveground biomass N (mg N $.g^{-1}$ dry biomass)		**4.5** *	[1]	**11.2** **
Aboveground biomass CN		**5.0** *	[1]	**13.5** **
Nutrient pools				
Aboveground nutrient C pool (g C .$mesocosm^{-1}$)	[2]	**11.4** *	[2]	3.3 +
Aboveground nutrient N pool (g N .$mesocosm^{-1}$)	[2]	**13.5** **	[2]	0.6

Values are test statistics from ANOVA (F values) or Kruskal-Wallis (ch^2 values) tests. Separate models were run for each effect variable. [1] data ln(y) transformed; [2] Kruskal-Wallis test was used; [3] data includes bare soil mesocosms; [4] data excludes bare soil mesocosms. *** P < 0.001, ** P < 0.01, * P < 0.05, + P < 0.1

Bacterial and fungal community

Sequencing of samples of monocultures yielded 1,075,176 and 973,646 reads for bacteria and fungi respectively (ASVs of < 5 reads removed, removed samples due to low read counts not included). Phylogenetic identification of the DNA amplicons was on average higher than 90 % at phylum, class and order taxonomic resolution for bacteria and it was higher than 90 % at phylum and class and it was 83 % at order taxonomic resolution for fungi (Table S6.1). Proportion of sequences with individual proportion greater than 0.01 % accounted for on average 95 % and 97 % of bacterial and fungal total reads respectively (Table S6.2). Distribution of individual phyla and classes within bacterial and fungal community in the soil of each mesocosm is shown in Figures 6.7 – 6.10.

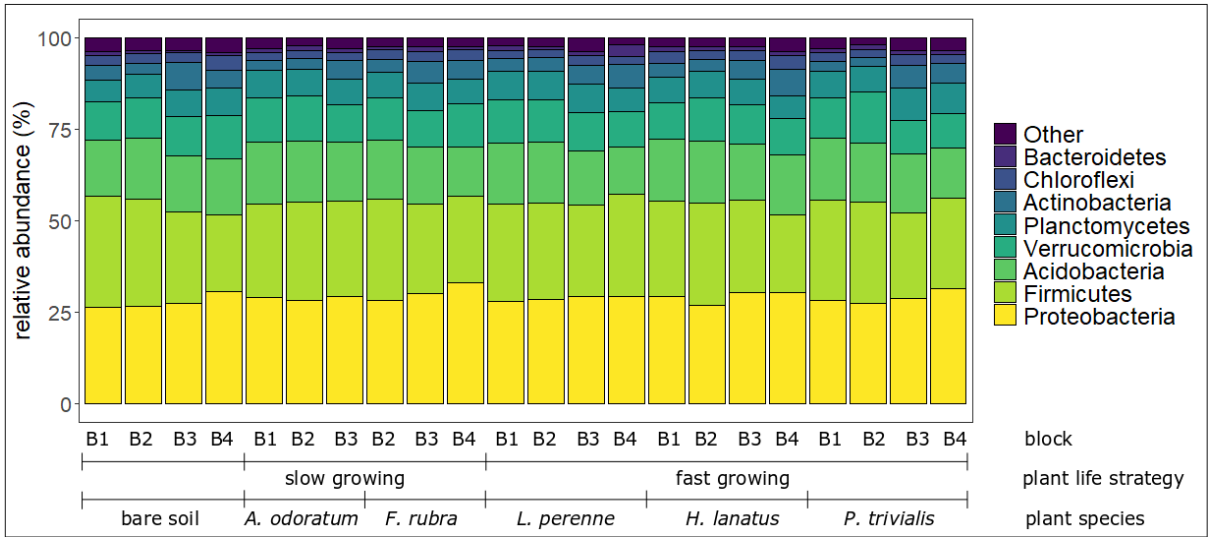


Figure 6.7 Bacterial phyla distribution in single species and bare soil mesocosms.

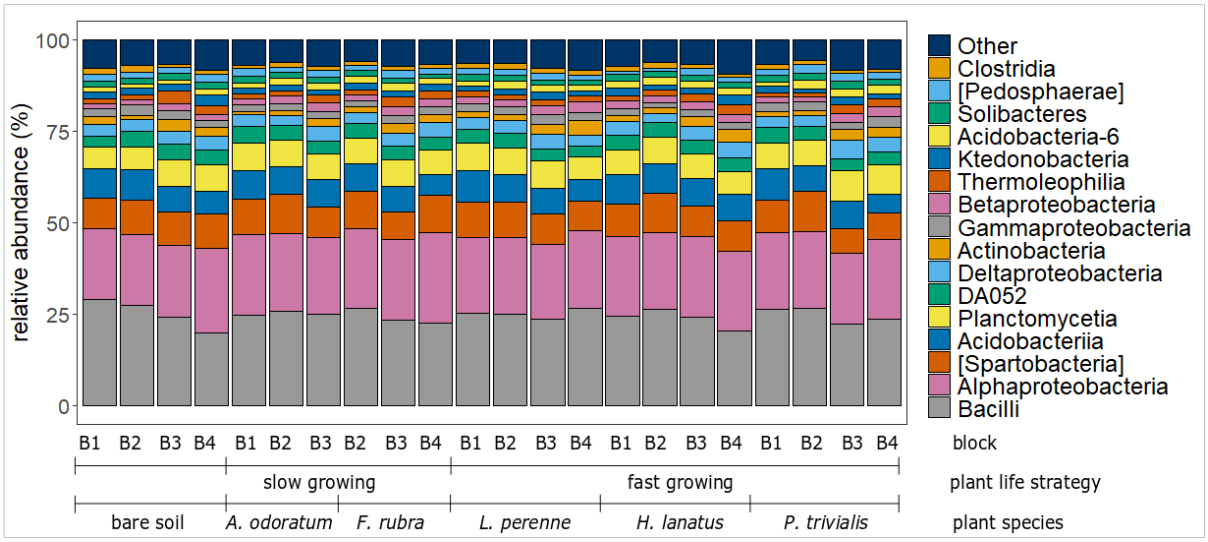


Figure 6.8 Bacterial classes distribution in single species and bare soil mesocosms.

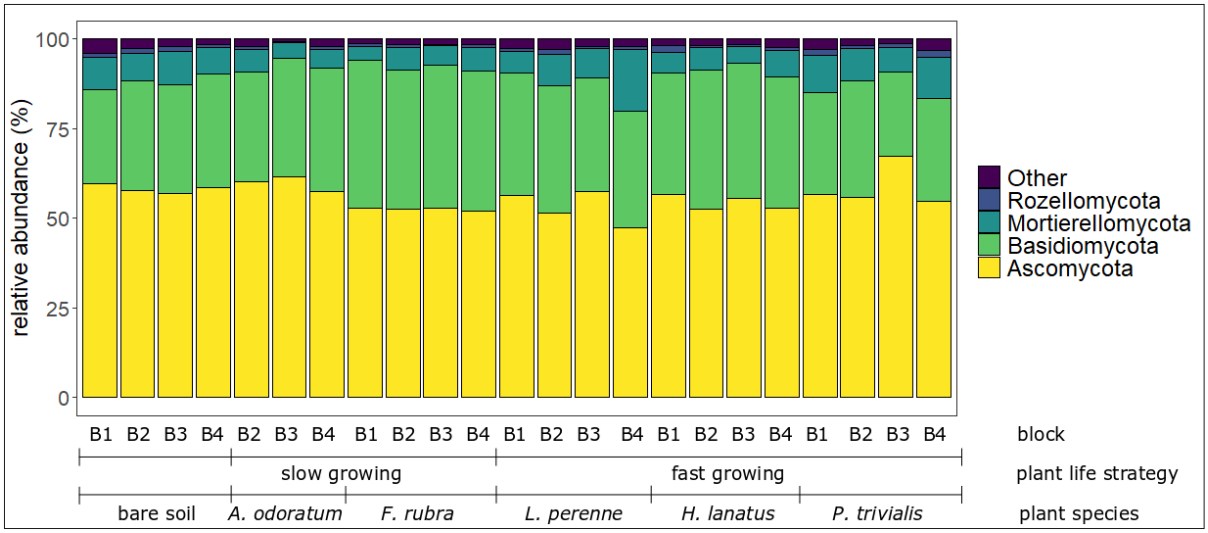


Figure 6.9 Fungal phyla distribution in single species and bare soil mesocosms.

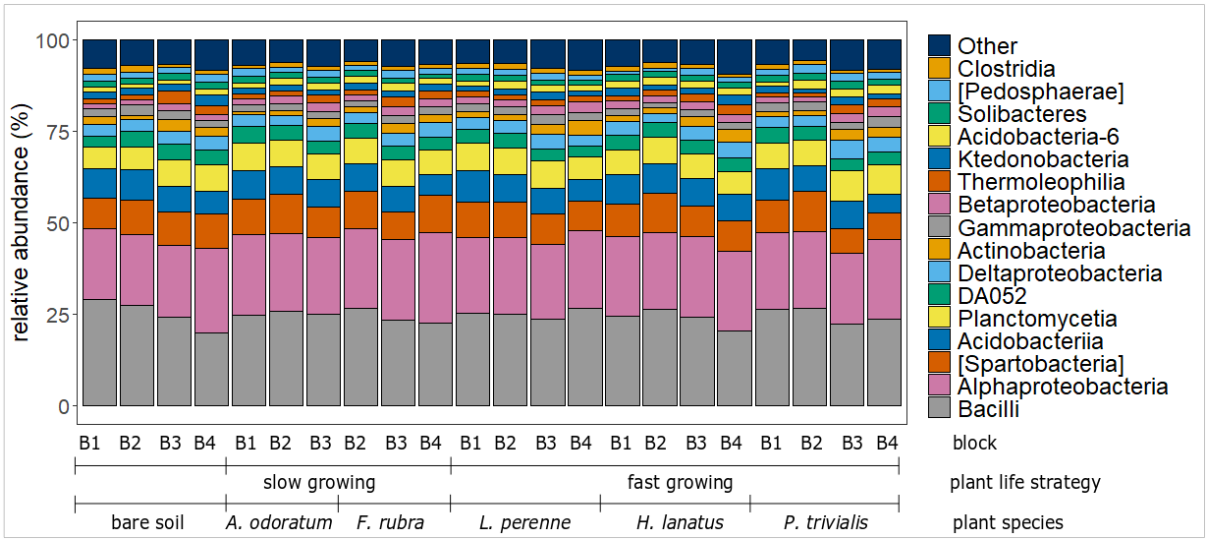


Figure 6.10 Fungal classes distribution in single species and bare soil mesocosms.

Microbial diversity indices including richness (number of individual ASVs), Simpson's and Shannon indices were not affected by plant species identity (Table 6.2) and plant life strategy for both bacteria and fungi (ANOVA, F test, all $P > 0.1$).

Table 6.2 Microbial diversity in the soil of plant species monocultures.

Microbial diversity	*A. odoratum*	*F. rubra*	*H. lanatus*	*L. perenne*	*P. trivialis*	bare soil
Bacteria						
Richness	610 ± 58	526 ± 24	584 ± 13	538 ± 19	566 ± 11	503 ± 22
Simpson's diversity	0.987 ± 0	0.986 ± 0	0.988 ± 0	0.987 ± 0	0.987 ± 0	0.987 ± 0
Shannon diversity	5.41 ± 0.04	5.30 ± 0.06	5.41 ± 0.06	5.36 ± 0.06	5.39 ± 0.03	5.33 ± 0.09
Fungi						
Richness	364 ± 39	340 ± 15	310 ± 35	387 ± 21.183	398 ± 41.574	344 ± 18
Simpson's diversity	0.968 ± 0.002	0.965 ± 0.001	0.966 ± 0.004	0.965 ± 0.003	0.972 ± 0.004	0.968 ± 0.005
Shannon diversity	4.43 ± 0.08	4.37 ± 0.05	4.30 ± 0.09	4.40 ± 0.05	4.52 ± 0.11	4.33 ± 0.1

Mean values ± s.e.

Microbial community structure

Both bacterial and fungal communities were affected by plant species identity (M-GLM, $Scores_{,21}$ = 12552, P < 0.05 and M-GLM, $Scores_{2,22}$ = 10710, P < 0.01 respectively) and plant life strategy (M-GLM, $Scores_{2,21}$ = 5675, P < 0.05 and M-GLM, $Scores_{2,22}$ = 4766, P < 0.01 respectively) (Figure 6.11). For the bacterial community at individual ASVs resolution, pairwise comparisons did not show any significant differences between different community associated with both plant species and plant life history strategies. Species pairs with four highest test statistics (M-GLM Wald test statistics) included pairs of bare soil mesocosms with *L. perenne*, *A. odoratum*, *F. rubra* and *P. trivialis* mesocosms. Similarly, the pair with highest test statistics for life history strategies included slow growing plants × bare soil mesocosm pair. For fungal pairwise comparisons, no significant differences between microbial communities associated with plant species and bare soil mesocosms were observed and contrary to bacteria, pairs with the highest three test statistics included combinations of *P. trivialis* with *F. rubra*, *A. odoratum* or *H. lanatus*. Pairwise comparison between fungal communities of plant life history strategies (incl. bare soil mesocosms) suggested difference between bare soil and slow growing plants (P < 0.05) and tendency for a difference between fast life strategy and slow life strategy plants (P = 0.052) and bare soil and fast life strategy plants (P = 0.071). When analysed without bare soil treatment, the model showed a difference between fungal communities of slow and fast growing plants (M-GLM, $Scores_{4,17}$ = 2393, P < 0.05).

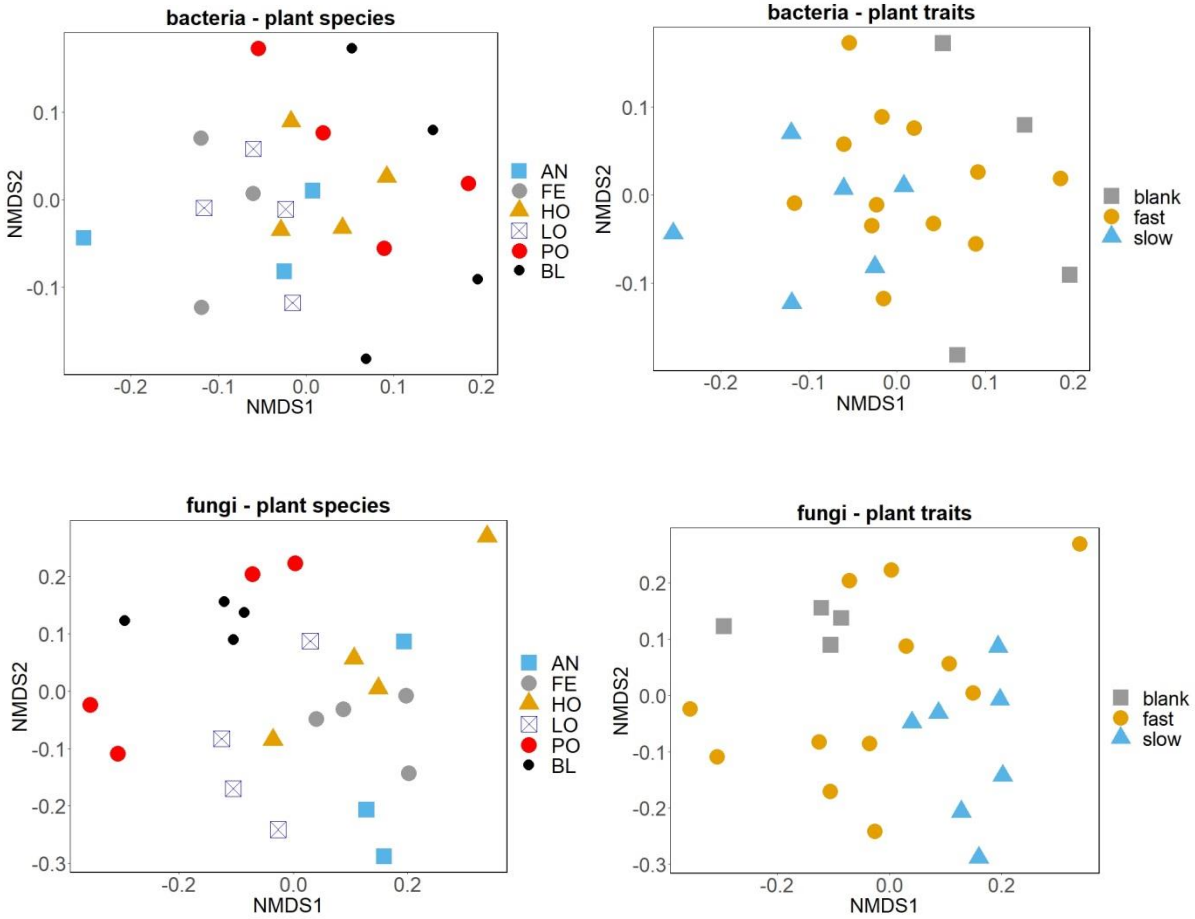


Figure 6.11 NMDS of bacterial and fungal community composition. AN = *A. odoratum*, FE = *F. rubra*, HO = *H. lanatus*, LO = *L. perenne* and PO = *P. trivialis.* Slow growing species are AN and FE, fast growing species are HO, LO and PO.

Bacterial community composition

Plant species and plant life strategy categories did not affect relative abundance of bacterial phyla of total relative abundance of each phylum greater than 1 % (ANOVA, F statistics, all P > 0.1) (Table 6.3) as well as bacterial classes within the selected phyla (ANOVA, F statistics, all P > 0.1) (data not shown). From bacterial orders, only relative abundances of *Rickettsiales* (*Alphaproteobacteria,* phylum *Proteobacteria*) and *Opitutales* (*Opitutae*, phylum *Verrucomicrobia*) were affected by plant species monocultures (ANOVA, $F_{21,5}$ = 14.6, P < 0.05 and $F_{21,5}$ = 9.3, P < 0.05 respectively, pairwise comparisons P <0.05) (Figure 6.12). Only order *Rickettsiales* responded to plant life strategy (ANOVA, $F_{21,2}$ = 14.7, P < 0.05, pairwise comparisons P <0.05) (Figure 6.13).

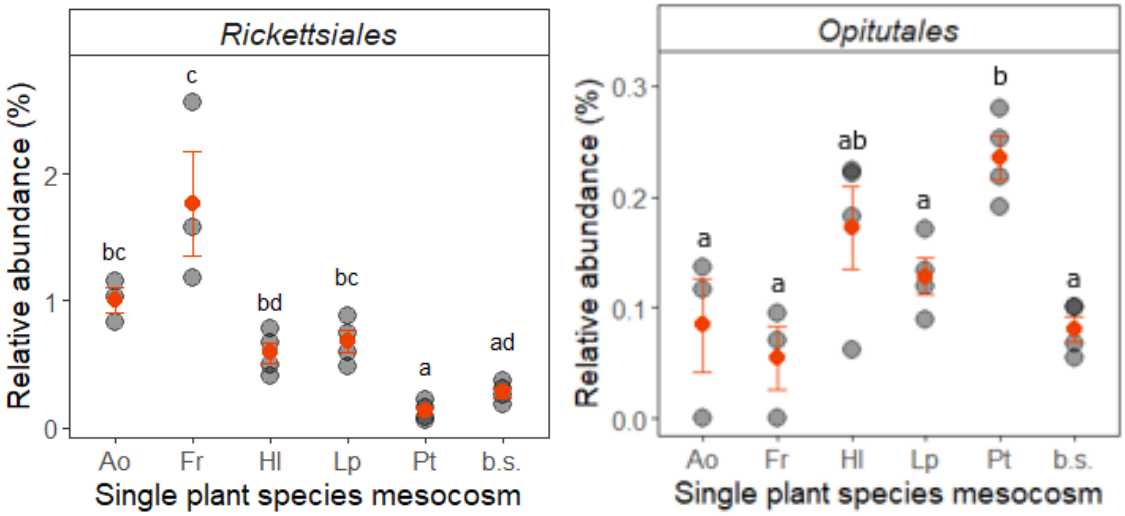


Figure 6.12 Relative abundance of bacterial orders in soil of single plant species mesocosms. Orange points are means values and whiskers are s.e. Letters are significant differences between means (P < 0.05). An = *A. odoratum*, Fe = *F. rubra*, Ho = *H. lanatus*, Lo = *L. perenne* and Po = *P. trivialis.* Slow growing species are An and Fe, fast growing species are Ho, Lo and Po.

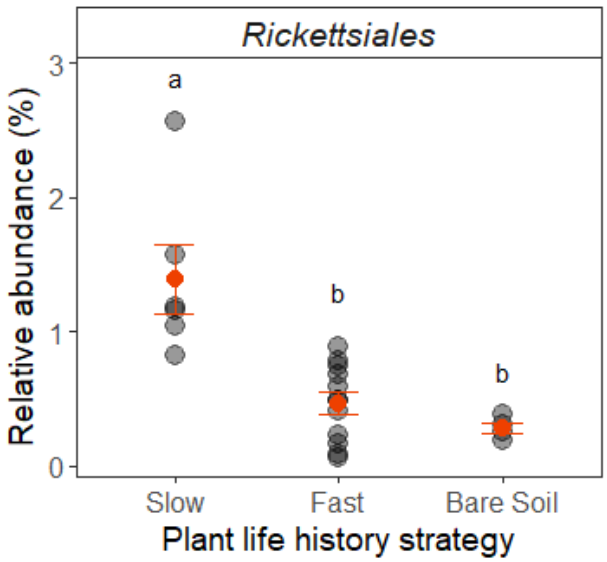


Figure 6.13 Relative abundance of soil bacterial orders across life history strategies of plants grown in monocultures in mesocosms. Orange points are means values and whiskers are s.e. Letters are significant differences between means (P < 0.05).

Contrary to bacteria, all fungal phyla of high relative abundance (> 1 %) were affected by plant species (ANOVA, F statistics, all $P < 0.05$) (Table 6.3). Plant species also affected relative abundance of all of the most abundant fungal classes (> 1 %; ANOVA, F statistics, all $P < 0.05$) except for *Leotiomycetes*, *Eurotiomycetes* and *Ascomycota_cls_Incertae_sedis* (all *Ascomycota*) (Table 6.4). Furthermore, plant species grouped according to their life strategy showed an effect on fungal phyla of *Basidiomycota* and *Morterellomycota* (ANOVA, $F_{22,2} = 3.6$, $P < 0.05$ and $F_{22,2} = 3.8$, $P < 0.05$ respectively, pairwise comparisons $P < 0.05$) but not *Ascomycota* (ANOVA, $F_{22,2} = 0.74$, $P = 0.48$) (Figure 6.14).

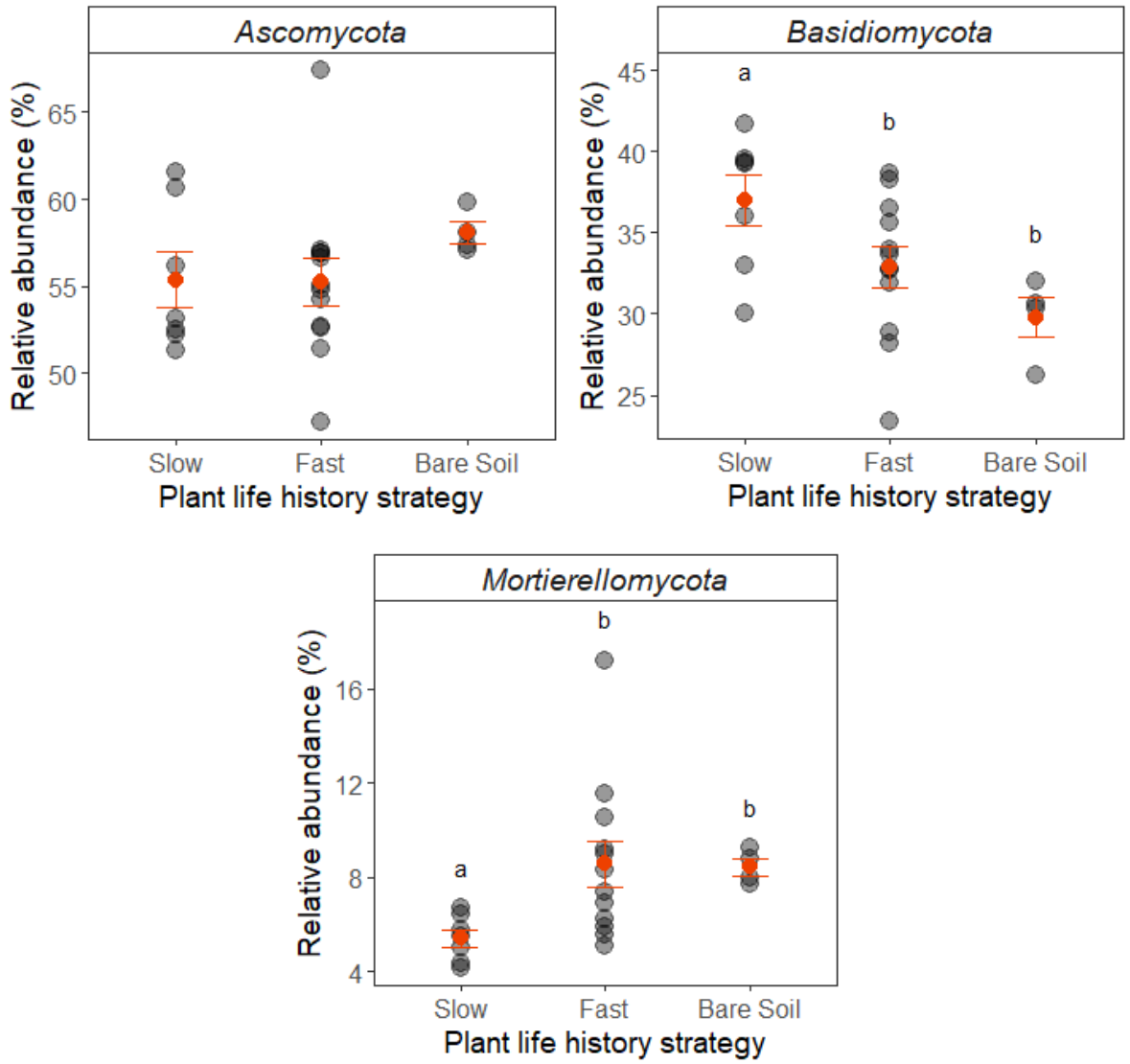


Figure 6.14 Relative abundance of soil fungal phyla across life history strategies of plants grown in monocultures in mesocosms. Orange points are means values of realtive abundance and whiskers are s.e. Letters are significant differences between means ($P < 0.05$).

Table 6.3 Relative abundance of bacterial and fungal phyla in single species mesocosms.

Microbial community	Relative abundance of bacterial phyla in single species mesocosms (mean ± s.e.)							one-way ANOVA	
Phylum	Mean [4]	*A. odoratum*	*F. rubra*	*H. lanatus*	*L. perenne*	*P. trivialis*	bare soil	F [3]	P
Bacteria									
Proteobacteria [1]	29.0	28.86 ± 0.34	30.57 ± 1.59	29.26 ± 0.8	28.75 ± 0.37	28.86 ± 0.83	27.8 ± 1.01	3.9	0.91
Firmicutes	25.8	26.17 ± 0.4	25.11 ± 1.36	24.95 ± 1.46	26.4 ± 0.69	25.75 ± 1.12	26.36 ± 2.16	0.3	0.93
Acidobacteria [1]	15.8	16.53 ± 0.21	15 ± 0.85	16.45 ± 0.39	15.22 ± 0.91	15.78 ± 0.68	15.63 ± 0.32	5.5	0.73
Verrucomicrobia [1]	11.0	11.53 ± 0.65	11 ± 0.52	10.58 ± 0.48	10.82 ± 0.48	10.91 ± 1.05	11.04 ± 0.32	2.2	0.91
Planctomycetes [1]	7.3	7.33 ± 0.21	6.75 ± 0.32	7.34 ± 0.2	6.87 ± 0.23	7.62 ± 0.38	7.83 ± 0.4	7.2	0.71
Actinobacteria [2]	4.5	1.2 ± 0.2	1.52 ± 0.2	1.52 ± 0.17	1.53 ± 0.18	1.5 ± 0.14	1.35 ± 0.23	1.3	0.50
Chloroflexi	2.7	2.24 ± 0.08	2.76 ± 0.07	3.01 ± 0.35	2.34 ± 0.17	2.48 ± 0.19	2.99 ± 0.32	1.9	0.49
Bacteroidetes [1]	1.2	1.2 ± 0.1	0.98 ± 0.12	1.17 ± 0.04	1.64 ± 0.53	1.13 ± 0.07	0.86 ± 0.1	6.4	0.71
Fungi									
Ascomycota [1]	55.8	59.47 ± 1.67	52.29 ± 0.36	54.27 ± 1.06	53.02 ± 2.32	58.41 ± 3.05	58.09 ± 0.6	13.4	*
Basidiomycota	33.6	33 ± 1.72 [bc]	39.95 ± 0.58 [a]	36.8 ± 1.14 [ab]	33.55 ± 0.82 [bc]	28.31 ± 1.93 [c]	29.8 ± 1.24 [c]	11.4	***
Mortierellomycota [2]	7.6	1.65 ± 0.11 [a]	1.7 ± 0.1 [a]	1.8 ± 0.08 [ab]	2.24 ± 0.22 [b]	2.24 ± 0.11 [b]	2.13 ± 0.04 [ab]	6.2	**

[1] Kruskal-Wallis test was used, [2] ln(y) transformed, [3] chi^2 if Kruskal-Wallis test used, [4] Mean relative abundance calculated using non-transformed. Relative abundance is percent abundance of overall sequence reads of rarefied sequence data. Letters are significant differences between means ($P < 0.05$).

Table 6.4 Relative abundance of fungal classes in single species mesocosms.

Phylum	Relative abundance of bacterial phyla in single species mesocosms (mean ± s.e.)							one-way ANOVA	
Class	Mean [4]	*A. odoratum*	*F. rubra*	*H. lanatus*	*L. perenne*	*P. trivialis*	bare soil	F [3]	P
Ascomycota									
Sordariomycetes	18.0	14.53 ± 0.12 [a]	13.63 ± 1.23 [a]	14.83 ± 0.7 [a]	18.23 ± 1.27 [ab]	23.02 ± 1.77 [b]	22.95 ± 0.87 [b]	13.2	***
Leotiomycetes [1]	12.0	10.88 ± 0.95	9.03 ± 0.57	10.92 ± 0.89	12.39 ± 1.2	15.42 ± 1.77	13.2 ± 0.81	12.1	+
Geoglossomycetes [2]	6.0	2.38 ± 0.21 [a]	2.04 ± 0.11 [ab]	2.03 ± 0.15 [ab]	1.77 ± 0.14 [ab]	1.3 ± 0.18 [b]	1.71 ± 0.23 [ab]	4.1	*
Eurotiomycetes [2]	5.5	1.95 ± 0.07	1.98 ± 0.06	2.06 ± 0.15	1.64 ± 0.07	1.69 ± 0.12	1.75 ± 0.08	3.2	+
Dothideomycetes [1]	3.5	5.23 ± 1.37 [bc]	2.02 ± 0.47 [ab]	1.57 ± 0.2 [a]	3.62 ± 0.63 [abc]	5.89 ± 0.9 [c]	3.24 ± 0.37 [abc]	15.6	*
Ascomycota_cls_Incertae_sedis [2]	2.4	0.96 ± 0.09	1.13 ± 0.17	1.37 ± 0.18	1.25 ± 0.11	1.32 ± 0.1	1.06 ± 0.09	1.1	0.47
Archaeorhizomycetes [2]	1.7	1.09 ± 0.32 [ab]	1.49 ± 0.11 [a]	1.01 ± 0.12 [ab]	0.8 ± 0.08 [bc]	0.44 ± 0.06 [c]	0.63 ± 0.11 bc	9.8	**
Lecanoromycetes [2]	0.6	0.14 ± 0.01 [ab]	0.13 ± 0.06 [a]	0.13 ± 0.02 [a]	0.1 ± 0.01 [a]	0.29 ± 0.09 [ab]	0.85 ± 0.44 [b]	2.5	0.11
Pezizomycetes [2]	0.5	0.13 ± 0.02	0.23 ± 0.1	0.42 ± 0.27	0.48 ± 0.19	0.53 ± 0.09	0.39 ± 0.09	0.7	0.64
Xylonomycetes [1]	0.2	0.18 ± 0.06 [b]	0.6 ± 0.3 [b]	0.1 ± 0.05 [ab]	0.04 ± 0.01 [ab]	0 ± 0 [a]	0 ± 0 [a]	19.3	*
Orbiliomycetes [1]	0.1	0.21 ± 0.06	0.11 ± 0.06	0.16 ± 0.07	0.07 ± 0.03	0.14 ± 0.03	0.06 ± 0.04	4.4	0.52
Basidiomycota									
Agaricomycetes	20.8	24.91 ± 1.97 [b]	29.22 ± 0.82 [b]	26.62 ± 1.35 [b]	17.88 ± 1.68 [a]	13.97 ± 2.04 [a]	13.3 ± 1.74 [a]	23.4	***
Tremellomycetes [1]	11.7	7.5 ± 0.46 [a]	9.95 ± 1.28 [abc]	9.36 ± 0.6 [ab]	14.47 ± 1.5 [bc]	12.51 ± 0.81 [ac]	15.09 ± 0.64 [c]	16.1	*
Microbotryomycetes [1]	0.6	0.32 ± 0.07 [a]	0.42 ± 0.06 [a]	0.45 ± 0.04 [a]	0.67 ± 0.18 [a]	0.85 ± 0.05 [a]	0.8 ± 0.13 a	14.0	*
Geminibasidiomycetes [1]	0.2	0.05 ± 0.02	0.16 ± 0.04	0.2 ± 0.15	0.16 ± 0.05	0.19 ± 0.07	0.23 ± 0.05	8.3	0.19
Spiculogloeomycetes [1]	0.1	0.11 ± 0.06	0.09 ± 0.05	0.03 ± 0.02	0.16 ± 0.03	0.14 ± 0.02	0.09 ± 0.05	6.6	0.30
Mortierellomycota									
Mortierellomycetes [2]	7.6	1.83 ± 0.1 [a]	1.87 ± 0.08 [a]	1.95 ± 0.07 [ab]	2.34 ± 0.2 [b]	2.34 ± 0.1 [b]	2.24 ± 0.04 [ab]	5.9	**
Rozellomycota									
Rozellomycota_cls_Incertae_sedis [1]	0.4	0.32 ± 0.05	0.3 ± 0.07	0.35 ± 0.03	0.37 ± 0.06	0.49 ± 0.13	0.54 ± 0.1	3.8	0.58
Chytridiomycota									
Rhizophydiomycetes [2]	0.2	0.12 ± 0.04 [a]	0.08 ± 0.02 [a]	0.1 ± 0.03 [a]	0.14 ± 0.02 [a]	0.14 ± 0.03 [a]	0.37 ± 0.05 [b]	10.8	**
Glomeromycota									
Archaeosporomycetes [2]	0.2	0.16 ± 0.08 [ab]	0.2 ± 0.04 [a]	0.17 ± 0.02 [ab]	0.29 ± 0.04 [a]	0.08 ± 0.01 [b]	0.06 ± 0.01 [b]	4.6	*

[1] Kruskal-Wallis test was used, [2] ln(y) transformed, [3] chi^2 if Kruskal-Wallis test used, [4] Mean relative abundance calculated using non-transformed data. Relative abundance is percent abundance of overall sequence reads of rarefied sequence data. Letters are significant differences between means ($P < 0.05$).

Table 6.5 Relative abundance of fungal orders in single species mesocosms.

Phylum		Relative abundance of fungalx phyla in single species mesocosms (mean ± s.e.)							one-way ANOVA	
Class	Order	Mean [4]	*A. odoratum*	*F. rubra*	*H. lanatus*	*L. perenne*	*P. trivialis*	bare soil	F [3]	P
Ascomycota										
	Hypocreales	8.45	7.26 ± 0.61	6.94 ± 0.74	6.65 ± 0.38	9.18 ± 1.31	10.38 ± 0.59	10.02 ± 0.61	4.4	+
	Sordariales	1.56	1.07 ± 0.07 ^{a}	1.09 ± 0.1 ^{a}	1.07 ± 0.06 ^{a}	1.33 ± 0.09 ^{a}	2.39 ± 0.25 ^{b}	2.26 ± 0.13 ^{b}	20.1	***
Sordariomycetes	*Coniochaetales*	1.02	0.61 ± 0.05 ^{a}	0.82 ± 0.1 ^{a}	0.71 ± 0.08 ^{a}	1.12 ± 0.08 ^{bc}	1.44 ± 0.14 ^{c}	1.33 ± 0.08 ^{c}	12.3	**
	Chaetosphaeriales [1]	0.41	0.41 ± 0.14	0.27 ± 0.06	0.28 ± 0.07	0.58 ± 0.13	0.47 ± 0.09	0.47 ± 0.07	8.4	0.30
	Microascales [2]	0.31	0.12 ± 0.04 ^{a}	0.29 ± 0.05 ^{bc}	0.2 ± 0.01 ^{ab}	0.21 ± 0.03 ^{ab}	0.32 ± 0.04 ^{bc}	0.39 ± 0.01 ^{c}	11.7	**
	Glomerellales	0.22	0.06 ± 0.04	0.06 ± 0.02	0.93 ± 0.76	0.06 ± 0.03	0.1 ± 0.03	0.07 ± 0.0	1.1	0.62
Leotiomycetes	*Leotiomycetes_ord_Incertae_sedis* [1]	7.3	7.52 ± 1.17	6.33 ± 0.31	6.88 ± 0.64	7.22 ± 0.43	7.65 ± 0.8	8.23 ± 0.41	5.6	0.53
	Helotiales [1]	4.65	3.32 ± 0.35	2.67 ± 0.32	4.02 ± 0.55	5.12 ± 1.07	7.67 ± 2.11	4.77 ± 0.49	12.5	0.10
Geoglossomycetes	*Geoglossales* [2]	5.95	2.38 ± 0.21	2.04 ± 0.11	2.03 ± 0.15	1.77 ± 0.14	1.3 ± 0.18	1.71 ± 0.23	4.1	+
	Chaetothyriales [2]	1.66	1.04 ± 0.04	0.89 ± 0.06	1.22 ± 0.09	0.85 ± 0.03	0.82 ± 0.11	0.93 ± 0.1	3.5	+
Eurotiomycetes	*Eurotiales* [1]	1.61	1 ± 0.38	1.17 ± 0.23	2.06 ± 1.05	1.09 ± 0.26	2.25 ± 0.36	1.93 ± 0.31	8.2	0.30
	Onygenales [1]	0.45	0.49 ± 0.08	0.38 ± 0.06	0.35 ± 0.05	0.39 ± 0.09	0.59 ± 0.1	0.54 ± 0.06	6.0	0.51
Dothideomycetes	*Pleosporales*	2.78	4.54 ± 1.3 ^{ac}	1.72 ± 0.4 ^{a}	1.14 ± 0.1 ^{b}	2.66 ± 0.61 ^{abc}	4.91 ± 0.53 ^{c}	2.17 ± 0.39 ^{ab}	6.4	*
	Capnodiales [1]	0.36	0.23 ± 0.01	0.06 ± 0.02	0.26 ± 0.11	0.3 ± 0.1	0.56 ± 0.14	0.71 ± 0.63	9.7	0.22
Archaeorhizomycetes	*Archaeorhizomycetales* [1]	1.67	2.22 ± 0.85	3.49 ± 0.54	1.78 ± 0.33	1.2 ± 0.16	0.55 ± 0.09	0.89 ± 0.23	13.9	+
Ascomycota_cls_Incertae_sedis	*Ascomycota_ord_Incertae_sedis* [2]	2.41	0.96 ± 0.09	1.13 ± 0.17	1.37 ± 0.18	1.25 ± 0.11	1.32 ± 0.1	1.06 ± 0.09	1.1	0.62
Pezizomycetes	*Pezizales* [2]	0.53	0.13 ± 0.02	0.23 ± 0.1	0.42 ± 0.27	0.48 ± 0.19	0.53 ± 0.09	0.39 ± 0.09	0.7	0.68
Xylonomycetes	*GS34* [1]	0.15	0.18 ± 0.06 ^{ad}	0.6 ± 0.3 ^{ab}	0.1 ± 0.05 ^{abd}	0.04 ± 0.01 ^{abd}	0 ± 0 ^{d}	0 ± 0 ^{c}	19.3	*
Basidiomycota										
Agaricomycetes	*Agaricales* [1]	19.77	23.98 ± 1.99 ^{ab}	28.68 ± 0.74 ^{a}	25.8 ± 1.37 ^{ab}	16.87 ± 1.62 ^{ab}	12.61 ± 2.19 ^{b}	11.71 ± 1.78 ^{b}	18.4	*
	Sebacinales [2]	0.24	0.21 ± 0.08	0.1 ± 0.02	0.2 ± 0.05	0.34 ± 0.07	0.25 ± 0.11	0.11 ± 0.03	1.8	0.41
Tremellomycetes	*Tremellales* [2]	5.95	1.58 ± 0.16 ^{a}	1.88 ± 0.15 ^{abc}	1.71 ± 0.05 ^{ab}	2.15 ± 0.07 ^{c}	1.96 ± 0.06 ^{abc}	2.08 ± 0.06 ^{bc}	5.7	*
	Filobasidiales [1]	5.07	3.12 ± 0.35 ^{a}	3.87 ± 0.31 ^{a}	4.23 ± 0.46 ^{ab}	5.83 ± 0.41 ^{ab}	5.76 ± 0.57 ^{ab}	7.15 ± 0.18 ^{b}	17.0	*
	Trichosporonales [1]	0.63	0.39 ± 0.09	0.27 ± 0.1	0.58 ± 0.28	0.98 ± 0.43	0.6 ± 0.17	0.87 ± 0.35	3.5	0.67
Microbotryomycetes	*Microbotryomycetes_ord_Incertae_sedis* [2]	0.36	0.15 ± 0.02 ^{a}	0.24 ± 0.03 ^{ab}	0.22 ± 0.04 ^{ab}	0.3 ± 0.03 ^{bc}	0.45 ± 0.07 ^{c}	0.38 ± 0.03 ^{bc}	8.2	**
	Leucosporidiales [2]	0.22	0.11 ± 0.06	0.13 ± 0.03	0.16 ± 0.02	0.24 ± 0.13	0.21 ± 0.06	0.28 ± 0.07	0.8	0.65
Geminibasidiomycetes	*Geminibasidiales* [1]	0.17	0.05 ± 0.02	0.16 ± 0.04	0.2 ± 0.15	0.16 ± 0.05	0.19 ± 0.07	0.23 ± 0.05	8.3	0.30
Mortierellomycota										
Mortierellomycetes	*Mortierellales* [1]	7.6	5.29 ± 0.62	5.54 ± 0.53	6.08 ± 0.51	10.11 ± 2.44	9.55 ± 1.01	8.44 ± 0.35	14.7	+
Rozellomycota										
Rozellomycota_cls_Incertae_sedis	*GS11* [1]	0.39	0.32 ± 0.05	0.3 ± 0.07	0.35 ± 0.03	0.36 ± 0.07	0.48 ± 0.13	0.54 ± 0.1	3.7	0.65
Chytridiomycota										
Rhizophydiomycetes	*Rhizophydiales* [2]	0.18	0.12 ± 0.04 ^{a}	0.08 ± 0.02 ^{a}	0.1 ± 0.03 ^{a}	0.14 ± 0.02 ^{a}	0.14 ± 0.03 ^{a}	0.37 ± 0.05 ^{b}	10.8	**
Glomeromycota										
Archaeosporomycetes	*Archaeosporales* [2]	0.18	0.16 ± 0.08 ^{ab}	0.2 ± 0.04 ^{a}	0.17 ± 0.02 ^{ab}	0.29 ± 0.04 ^{a}	0.08 ± 0.01 ^{b}	0.06 ± 0.01 ^{b}	4.6	*

[1] Kruskal-Wallis test was used, [2] ln(y) transformed, [3] chi^2 if Kruskal-Wallis test used, [4] Mean relative abundance calculated using non-transformed data. Relative abundance is percent abundance of overall sequence reads of rarefied sequence data. Letters are significant differences between means ($P < 0.05$).

Fungal classes within some fungal phyla followed contrasting dynamics (Table 6.5). From phylum *Ascomycota*, classes *Geoglossomycetes* and *Archaeorhizomycetes* showed overall tendency for higher relative abundance while *Sordariomycetes* showed overall tendency for lower relative abundance for plant species of *A. odoratum*, *F. rubra* and partially also *H. lantus*. Similarly, for phylum *Basidiomycota*, class *Agaricomycetes* showed a tendency for higher relative abundance while *Tremellomycetes* showed a tendency for lower relative abundance in the soil of these plant species mesocosms. Despite *H. lanatus* (fast growing plant) tended to respond in the similar direction to *A. odoratum* and *F. rubra* (both slow growing plants), when plant species were grouped according to their life strategy, an effect of the life strategy on six of the most abundant (> 1 %) fungal classes was observed including classes *Sordariomycetes*, *Leotiomycetes*, *Archaeorhizomycetes*, *Agarocimycetes*, *Tremellomycetes* and *Mortierellomycetes* (ANOVA, $F_{22,2} = 15.9$, $P < 0.01$, $F_{22,2} = 12.5$, $P < 0.05$, $F_{22,2} = 15.3$, $P < 0.01$, $F_{22,2} = 15.9$, $P < 0.01$, $F_{22,2} = 17.6$, $P < 0.001$ and $F_{22,2} = 12.2$, $P < 0.05$ respectively, pairwise comparisons $P < 0.05$) (Figure 6.15). It is interesting to point out that bare soil mesocosms responded in the same direction to the fast growing plant species.

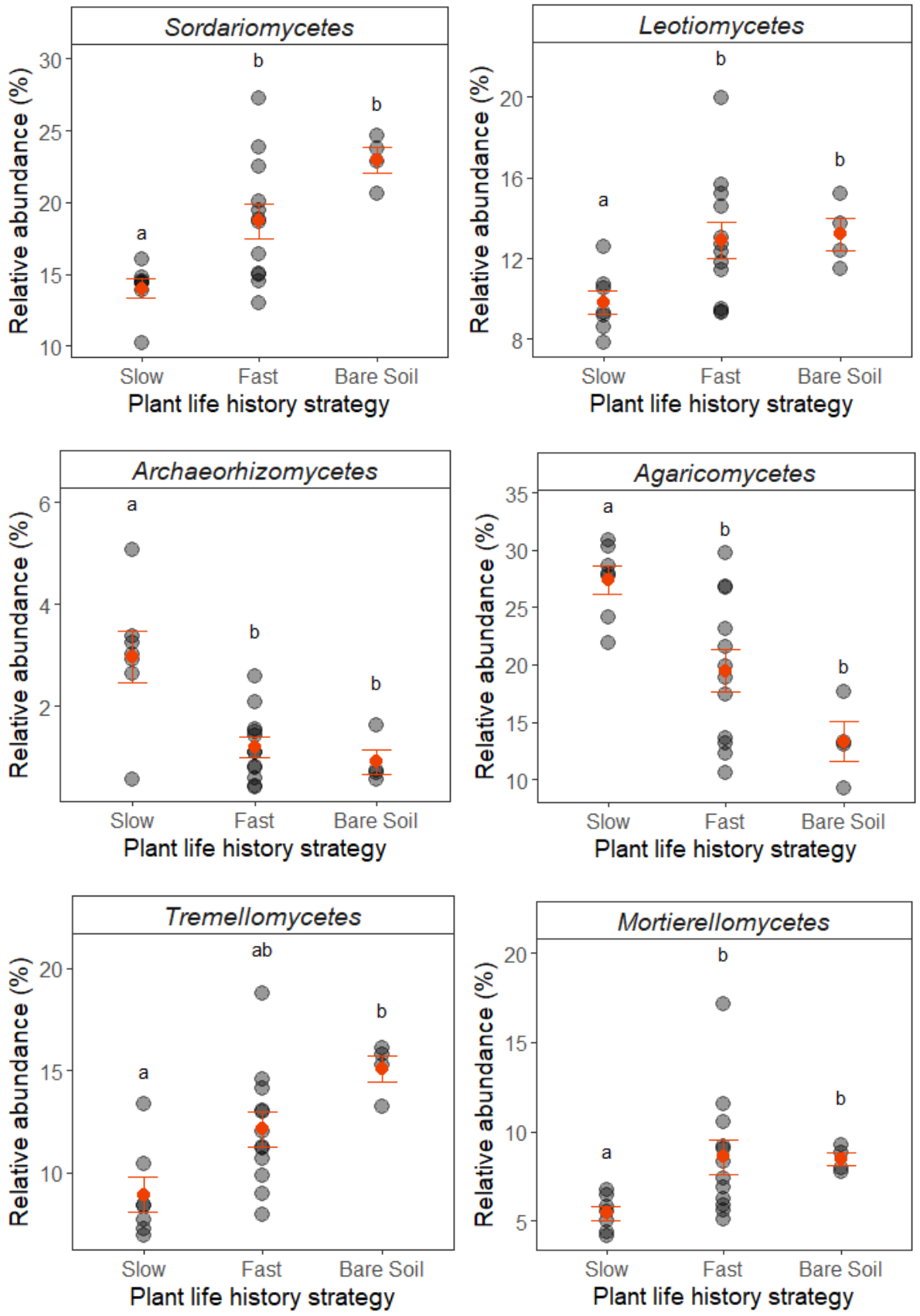


Figure 6.15 Relative abundance of soil fungal classes across life history strategies of plants grown in monocultures in mesocosms. Orange points are means values of realtive abundance and whiskers are s.e. Letters are significant differences between means ($P < 0.05$).

Plant species identity impact on relative abundance of fungal orders was stronger than plant life strategy as only order *Filobasidiales* responded to plant life strategy (ANOVA, $F_{22,2} = 24.5$, $P < 0.01$, pairwise comparisons $P < 0.05$) (Figure 6.16).

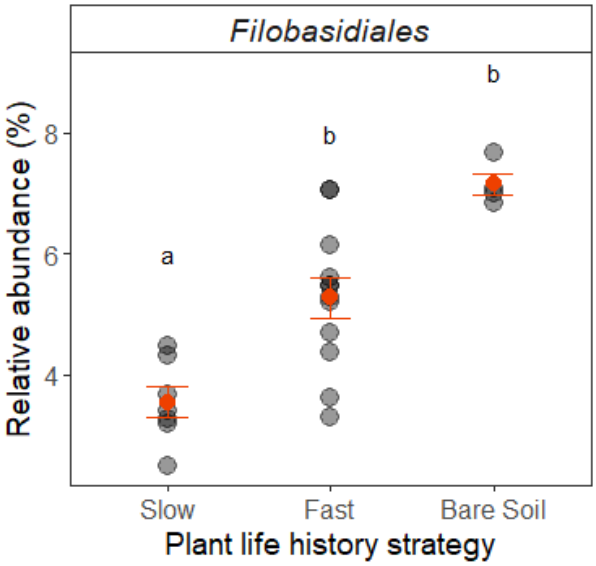


Figure 6.16 Relative abundance of soil fungal order *Filobasidiales* across life history strategies of plants grown in monocultures in mesocosms. Orange points are means values of realtive abundance and whiskers are s.e. Letters are significant differences between means ($P < 0.05$).

Cosmopolitan soil microbial taxa were represented by higher relative abundance of *Acidobacteria* and *Basidiomycota*, while more specialized taxa were represented by higher proportion of *Planctomycetes* and *Ascomycota* (Figure 6.17(A-B)). Additionally, cosmopolitan fungal taxa were represented by a higher proportion of saprotrophs while specialized taxa were represented by a greater proportion of pathotrophs and symbiotrophs (Figure 6.17(C)).

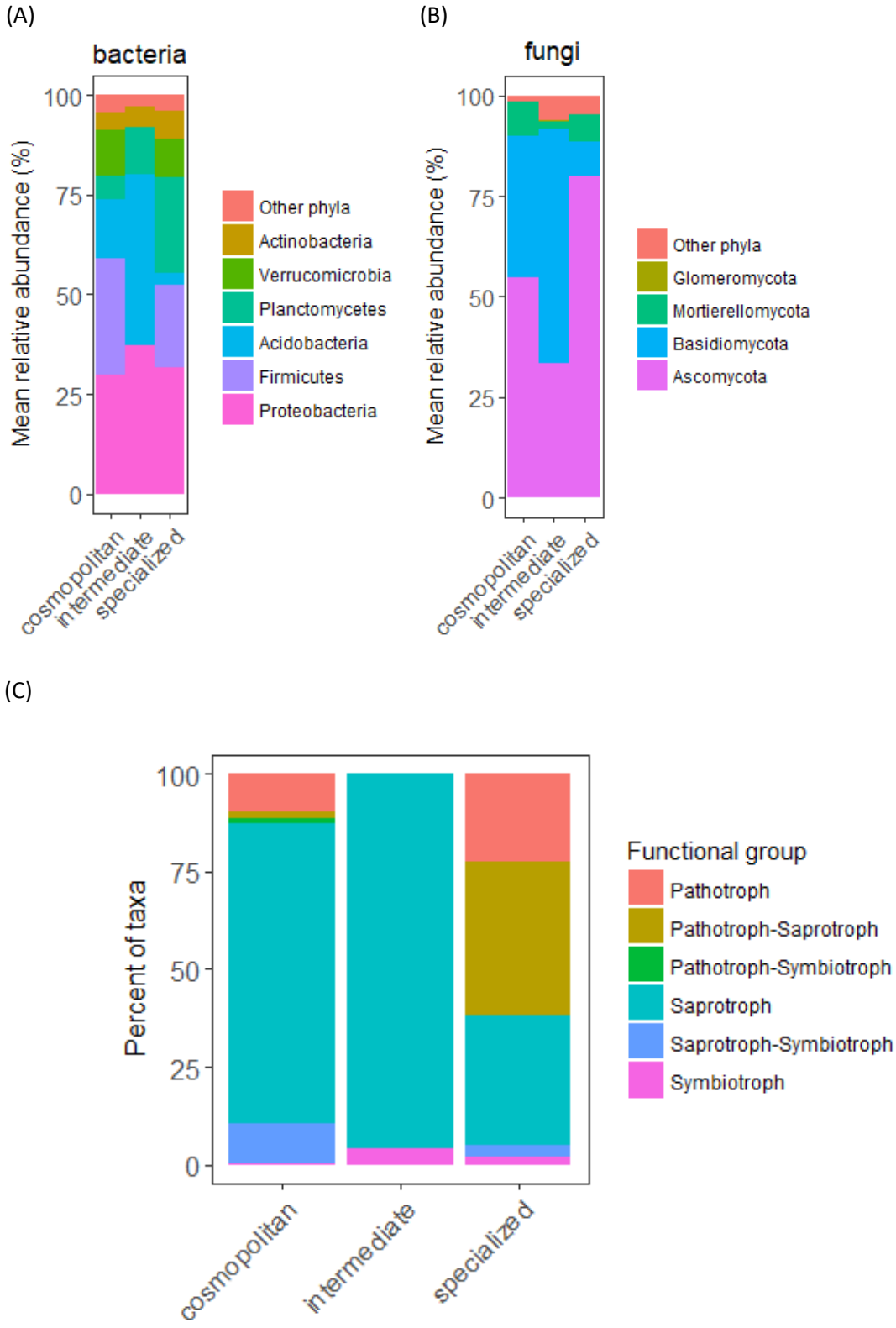


Figure 6.17 Effect of plant species identity on soil microbial communties in plant monocultures.
Composition of cosmopolitan soil taxa (those taxa associated with all plant species), intermediate (taxa associated with only 2 to 20 plant species), and specialized (taxa that associate with only a single plant species) for (A) bacteria and (B) fungi. (C) The composition of functional groups of fungal taxa identified as being cosmopolitan, intermediate, and specialized across plant species.

6.4. 2 Species richness and species occurrence in species mixtures

Soil N cycle

Species richness affected nitrate concentration and the effect was significant only when monocultures were included in the model (Kruskal-Wallis, $chi^2 = 17.5$, d.f. = 4, $P < 0.01$, posthoc comparisons $P < 0.05$) (Table 4.3) whereby nitrate was higher in monocultures compared to each of the mixtures (Figure 6.18(B)). Ammonium concentration and both net ammonification and net nitrification processes were not affected by species richness (ANOVA/Kruskal-Wallis, all $P > 0.05$, Table 4.3, Figure 6.18). When the effect of occurrence of individual plant species in the mixtures was studied (Table S4.7), presence of *A. odoratum* decreased both nitrate (Kruskal-Wallis, $chi^2 = 4.7$, d.f. = 1, $P < 0.05$) and net nitrification (ANOVA, $F_{1,84} = 62.3$, $P < 0.001$). Presence of *F. rubra* decreased nitrate concentration (Kruskal-Wallis, $chi^2 = 13.3$, d.f. = 1, $P < 0.001$). Presence of *L. perenne* increased net nitrification (ANOVA, $F_{1,81} = 21.6$, $P < 0.001$), while presence of *P. trivilalis* increased both nitrate (Kruskal-Wallis, $chi^2 = 4.9$, d.f. = 1, $P < 0.05$) and net nitrification (ANOVA, $F_{1,84} = 6.6$, $P < 0.05$).

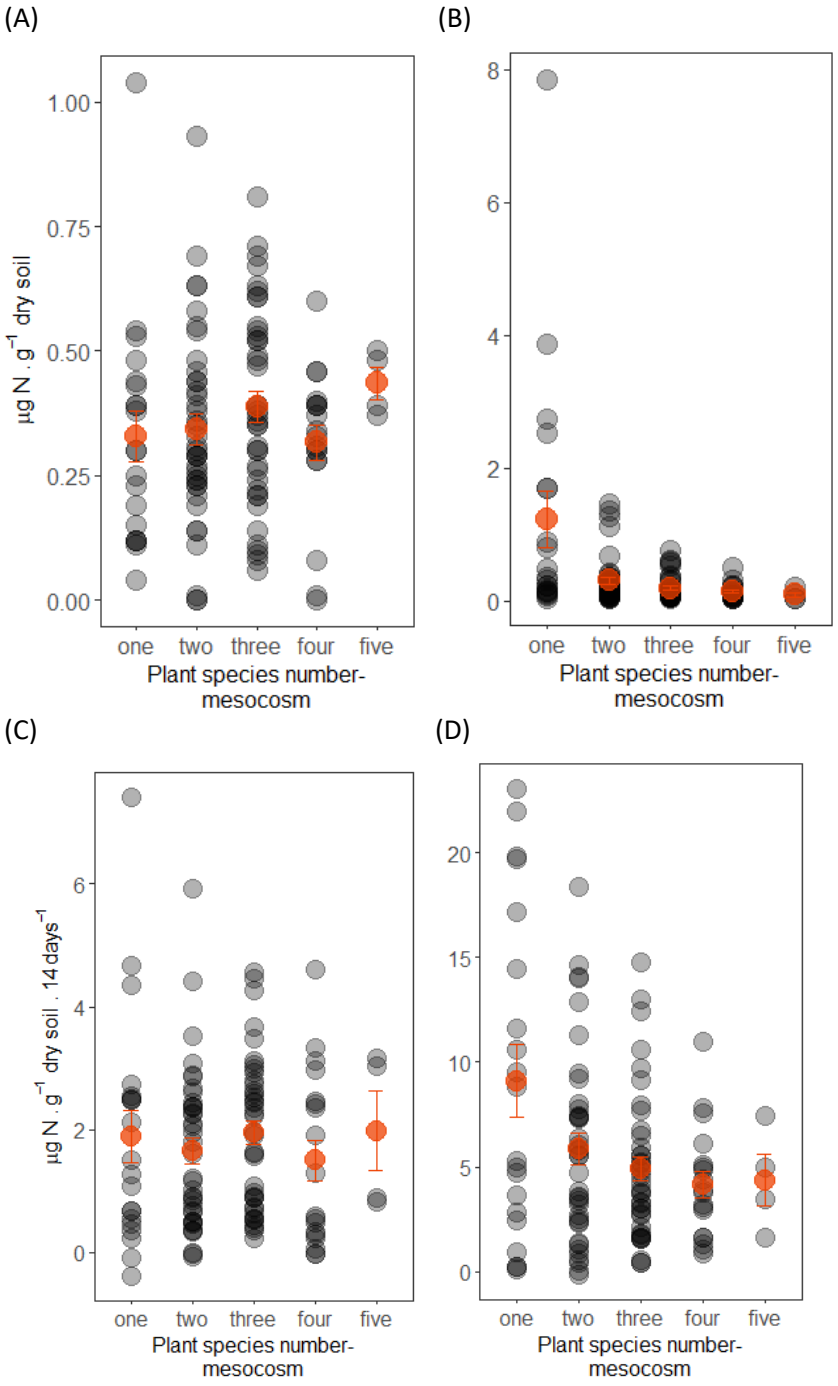


Figure 6.18 Effect of number of plant species in the mesocosms on soil N cycle: concentration of (A) ammonium and (B) nitrate in the soil, (C) net ammonification and (D) net nitrification. Red points represent mean and bars are ± s.e.

Ecosystem C efflux

Plant species richness did not affect ecosystem C effluxes in the mixtures (ANOVA, all $P > 0.05$, Table 6.6) (Figure 6.19). On the other hand, strong effects of particular species occurrence were observed on both measurements of C flux ecosystem respiration and photosynthesis (ANOVA, Table 6.7). Presence of *L. perenne* increased ecosystem respiration in June and July (ANOVA, $F_{1,81} = 6.0$, $P < 0.05$ and $F_{1,81} = 10.7$, $P < 0.01$ respectively), while in May, it increased respiration in three-species mixtures (ANOVA, $F_{1,81} = 6.0$, $P < 0.05$, posthoc comparisons $P < 0.05$). Presence of *H. lanatus*

decreased respiration in July (ANOVA, $F_{1,81} = 5.0$, $P < 0.05$). *P. trivialis* negatively affected ecosystem respiration in July only when present in four-species mixtures (ANOVA, $F_{1,81} = 8.1$, $P < 0.001$, posthoc comparisons $P < 0.05$).

Photosynthesis was affected only in June by species occurrence when it was negatively affected by presence of *A. odoratum* or *H. lanatus* (ANOVA, $F_{1,74} = 9.9$, $P < 0.01$ and $F_{1,74} = 7.0$, $P < 0.01$ respectively). It was enhanced by presence of *F. rubra* in two-species mixtures in June (ANOVA, $F_{2,72} = 4.5$, $P < 0.05$, posthoc comparisons $P < 0.05$).

Table 6.6 Effect of plant species combinations on ecosystem functions.

	Species richness (incl. monocultures)		Species richness (excl. monocultures)	
N cycle				
Ammonium (mg NH_4^+ - N g^{-1} dry $soil^{-1}$)	[1)]	3.8	[1)]	3.1
Nitrate (mg NO_3^- - N g^{-1} dry soil)	[1)]	**17.5** **	[1)]	6.5 $^{+}$
net Ammonification (mg NH_4^+ - N g^{-1} dry soil 14 $days^{-1}$)	[1)]	3.7	[1)]	3.6
net Nitrification (mg NO_3^- - N g^{-1} dry soil 14 $days^{-1}$)		3.9	[2)3)]	0.2
C cycle				
Ecosystem respiration in May (mg CO_2 - C m^{-2} h^{-1})		1.5	[1)]	1.2
Ecosystem respiration in June (mg CO_2 - C m^{-2} h^{-1})	[3)]	1.0	[2)3)]	0.1
Ecosystem respiration in July (mg CO_2 - C m^{-2} h^{-1})	[3)]	0.6	[1)3)]	0.3
Photosynthesis in May (mg CO_2 - C .m^{-2} h^{-1})	[3)]	7.6		0.1
Photosynthesis in June (mg CO_2 - C m^{-2} h^{-1})	[3)]	0.8	[1)]	3.1
Plant growth				
Total plant aboveground biomass (g)		8.8 $^{+}$	[1)]	3.9

Data analysed using ANOVA. Separate models were run for each effect variable. [1)] Kruskal-Wallis model used; [2)] data ln(y) transformed; [3)] outliers determined by checking model fit were removed from the model and the model was re-run. Data shows test statistic (F values) and P values. Data in bold are significant at $P < 0.05$. $^{+}$ $P < 0.1$, ** $P < 0.01$

Table 6.7 Effect of species richness and species presence-absence (occurrence) in the mesocosms on multiple ecosystem functions.

		Species richness x *A. odoratum*				Species richness x *F. rubra*				Species richness x *H. lanatus*				Species richness x *L. perenne*				Species richness x P. trivialis		
		Richness	Species presence	Richness x Species presence		Richness	Species presence	Richness x Species presence		Richness	Species presence	Richness x Species presence		Richness	Species presence	Richness x Species presence		Richness	Species presence	Richness x Species presence
N cycle		F	F	F		F	F	F		F	F	F		F	F	F		F	F	F
Ammonium (mg NH_4^+ - N g^{-1} dry soil)	1)	2.0	0.8	2.6 +	1)	1.9	0.0	0.8	1) 3)	1.9	2.5	0.0	1) 3)	1.8	0.3	0.4	1) 3)	1.9	0.1	1.3
Nitrate (mg NO_3^- - N g^{-1} dry soil)			**4.7 ***				**13.3 *****				1.9				3.8 +				**4.9 ***	
net Ammonification (mg NH_4^+ - N g^{-1} dry soil 14 $days^{-1}$)	1)	**5.8 ****	0.0	0.4	1)	**5.9 ****	0.8	1.2		**5.8 ****	0.1	0.8	1)	**5.1 ****	0.3	2.9 +		**4.8 ***	0.0	0.5
net Nitrification (mg NO_3^- - N g^{-1} dry soil 14 $days^{-1}$)		**3.5 ***	**62.3 *****	1.8		0.9	0.9	0.3	3)	0.9	1.3	0.2	1) 3)	**3.4 ***	**21.6 *****	1.0	1) 3)	1.4	**6.6 ***	0.9
C cycle																				
Ecosystem respiration in May (mg CO_2 - C m^{-2} h^{-1})	1)	0.1	0.0	2.6 +	1)	0.6	0.001	**3.2 ***	1) 4)	0.8	0.4	0.3	1)	0.3	**4.7 ***	**5.1 ****	1)	0.4	0.0	2.8 +
Ecosystem respiration in June (mg CO_2 - C m^{-2} h^{-1})	1)	0.3	3.0 +	1.4	1)	0.3	2.2	0.5	1)	0.3	1.0	0.9	1)	0.3	**6.0 ***	0.0	1)	0.3	0.0	1.3
Ecosystem respiration in July (mg CO_2 - C m^{-2} h^{-1})	1)	0.2	2.3	0.8	1)	0.2	1.8	0.0	1)	0.5	**5.0 ***	1.0		0.3	**10.7 ****	0.2	1) 4)	0.4	1.5	**8.1 *****
Photosynthesis in May (mg CO_2 - C m^{-2} h^{-1})	1)	0.3	0.5	1.0	1)	0.3	0.295	1.6	1)	0.3	0.3	0.8		0.3	2.0	0.7	1)	0.3	0.4	0.3
Photosynthesis in June (mg CO_2 - C m^{-2} h^{-1})	1)	3.0 +	**9.9 ****	2.1	1)	**3.4 ***	**15.0 *****	**4.5 ***		0.9	**7.0 ****	0.2		0.8	1.2	0.0		0.8	0.6	0.5
Plant characteristics																				
Plant aboveground biomass (g $mesocosm^{-1}$)		1.1	1.2	0.7	1)	1.1	**43.6 *****	2.4 +	1)	1.0	**4.6 ***	1.1		0.8	0.4	0.1		1.1	1.0	0.0

Species occurrence effect: positive effect; positive effect

Data analysed using ANOVA. Separate models were run for each combination of species richness and presence of particular plant species. Effects of species richness, plant species presence and their interaction were determined using data from two-, three- and four- species mixtures. All response data were log(y) transformed. [1)] points determined as outliers by model checking were removed from the model to improve model fit; [2)] Kruskal-Wallis model fit only to the effect of species occurrence because removing outliers from the model did not improve model fit; 3) small non-normality of errors remained; [4)] removal of outliers only slightly improved model fit. Data shows test statistic (F values or ch^2 if Kruskal-Wallis test was used) and P values. Data in bold are significant at $P < 0.05$. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, + $P < 0.1$

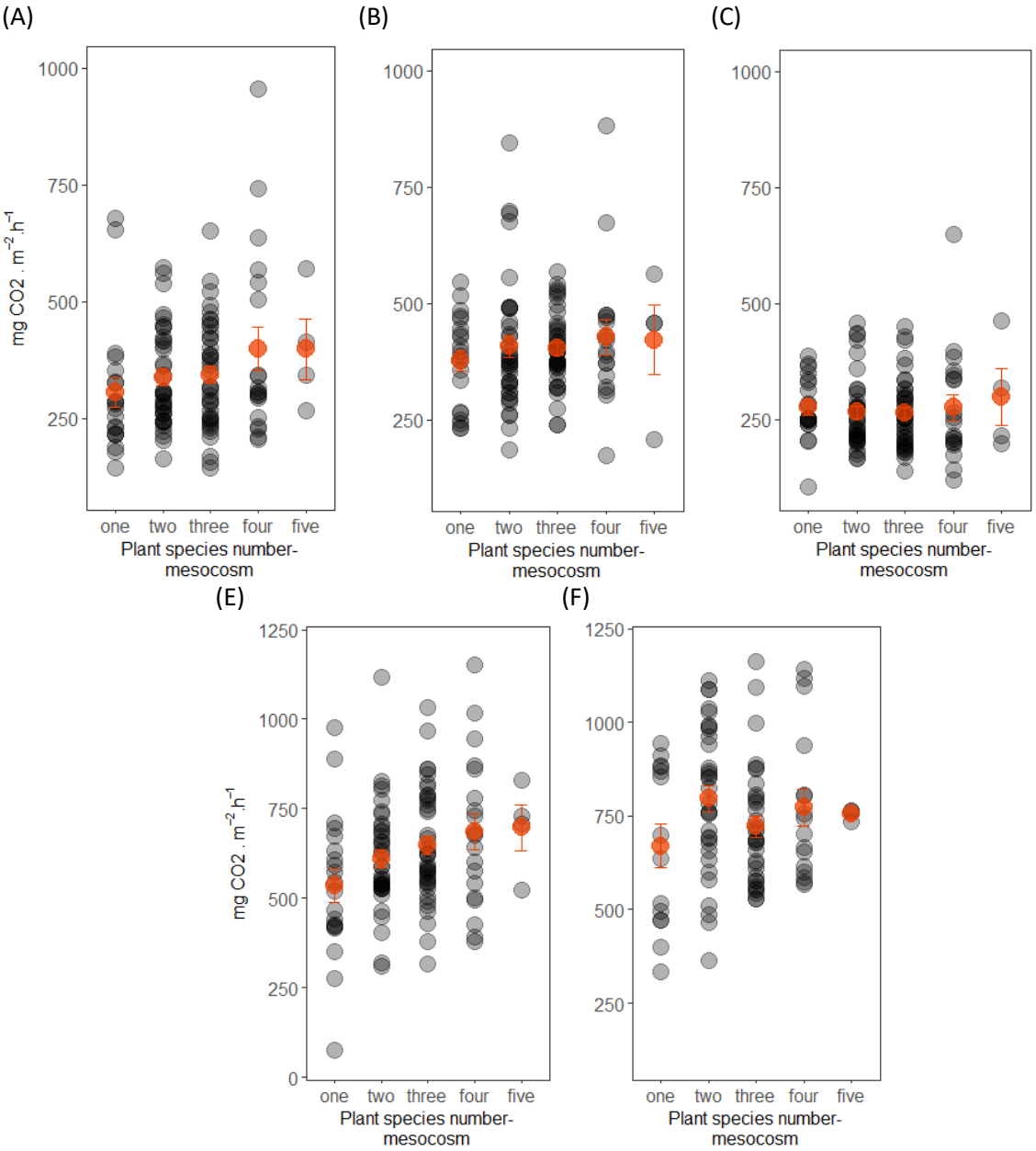


Figure 6.19 Effect of number of plant species in the mesocosms on ecosystem C cycle: ecosystem respiration in (A) May and (B) June and (C) July and photosynthesis in (D) May and (E) June. Red points represent mean and bars are ± s.e.

Plants

Plant species richness did not show an effect on aboveground biomass yield (Kruskal-Wallis, chi^2 = 8.8, d.f. = 4, P = 0.07; Table 6.9; Figure 6.20). Only aboveground biomass of *F. rubra* and *H. lanatus* in the mixtures positively correlated with overall aboveground biomass of the mixtures (Spearman's correlation, rho = 0.49, P < 0.001 and rho = 0.55, P < 0.001 respectively) (Table 6.8). For an effect of species occurrence in the mixtures on aboveground biomass yield, only presence of *F. rubra* increased the yield (ANOVA, $F_{1,89}$ = 20.5, P < 0.001). For the effect of occurrence of species on biomass of other species in the mixture, presence of *H. lanatus* decreased aboveground biomass of all other species which were also present in the mixture (ANOVA, all P < 0.05, Table 6.9), while presence of *F. rubra* did not affect aboveground biomass of other species also present in the mixture (ANOVA, all P > 0.05, data not shown).

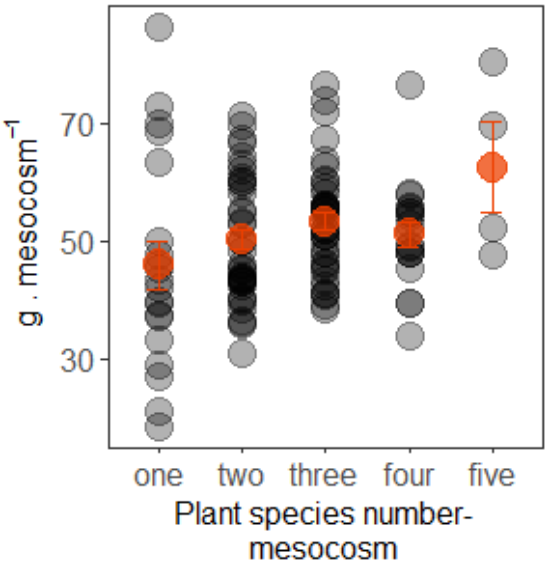


Figure 6.20 Effect of number of plant species in the mesocosms on plant aboveground biomass yield. Red points represent mean and bars are ± s.e.

Table 6.8 Relationship of aboveground biomass yield of species in the mixtures with the overall aboveground biomass yield of plant species mixture.

	rho
A. odoratum	-0.08
F. rubra	**0.49** ***
H. lanatus	**0.55** ***
L. perenne	0.16
P. trivialis	0.00

Spearman's correlation test used. Values are strength of correlation and values in bold is significant at P < 0.05.
*** P < 0.001

Table 6.9 Effect of presence of *Holcus lanatus* on aboveground biomass of other plant species in species mixtures.

		Species richness	*H. lanatus* presence	Species richness × *H. lanatus* presence
A. odoratum	1)	**18.1** ***	**27.0** ***	0.6
F. rubra	1)	**29.6** ***	**107.0** ***	1.0
L. perenne	2)	NA	**30.4** ***	NA
P. trivialis	2)	NA	**10.6** **	NA

Data are F test statistic or chi^2 (if Kruskal-Wallis test was used). Values in bold are significant at P < 0.05.
*** P < 0.001, ** P < 0.01 1) ln(y) transformed; 2) Kruskal-Wallis test was used

Species richness only affected LRR of soil nitrate (Kruskal-Wallis, $chi^2 = 14.7$, d.f. = 3, $P < 0.01$, Table 6.10) (Figure 6.21). Three-, four- and five- species mixtures showed lower values of the LLRs for nitrate than two-species mixture (posthoc comparisons $P < 0.05$) meaning that they showed lower nitrate in the soil than would be expected based on monocultures of these species. LRR for two-species mixture was lower than 0 (t-test, $t(37) = 9.3$, $P < 0.001$). For the effect on LRRs of individual species biomass in the mixtures, only LRRs for *H. lanatus* and *P. trivialis* were affected by species mixtures (Kruskal-Wallis, $chi^2 = 18.7$, d.f. = 3, $P < 0.001$ and $chi^2 = 10.8$, d.f. = 3, $P < 0.05$ respectively) (Figure 6.22). *H. lanatus* showed an overperformance in species mixtures when compared to mean of the monocultures regardless of species richness (posthoc comparisons $P < 0.05$). On the other hand, *P. trivialis* showed strong underperformance in the mixtures compared to the mean of monocultures, whereby four-species mixtures showed lower LRR than two-species mixtures (posthoc comparisons $P < 0.05$).

Table 6.10 Determination of strength and direction of plant species richness effect, as measured by log response ratio (LRR).

		Species richness
N cycle		
Ammonium (mg NH_4^+ - N g dry $soil^{-1}$)	1)	2.0
Nitrate (mg NO_3^- - N g dry $soil^{-1}$)	1)	**14.7** **
net Ammonification (mg NH_4^+ - N g dry $soil^{-1}$ 14 $days^{-1}$)	1)	1.2
net Nitrification (mg NO_3^- - N g dry $soil^{-1}$ 14 $days^{-1}$)	1)	2.8
C cycle		
Ecosystem respiration in May (mg CO_2 - C m^{-2} h^{-1})		0.6
Ecosystem respiration in June (mg CO_2 - C m^{-2} h^{-1})	1)	1.8
Ecosystem respiration in July (mg CO_2 - C m^{-2} h^{-1})		0.1
Photosynthesis in May (mg CO_2 - C m^{-2} h^{-1})	1)	2.2
Photosynthesis in June (mg CO_2 - C m^{-2} h^{-1})		7.5 $^{+}$
Plant growth		
Total plant aboveground biomass (g)	1)	4.0
A. odoratum aboveground biomass (g)	1)	1.1
F. rubra aboveground biomass (g)		0.5
H. lanatus aboveground biomass (g)	1)	**18.7** ***
L. perenne aboveground biomass (g)	1)	3.6
P. trivialis aboveground biomass (g)	1)	**10.8** *

LRR = ln (observed value in mixed communities/expected value in monoculture). 1) Kruskal-Wallis model used. Data from all mesocosms used. Values are F statistic from ANOVA or chi^2 from Kruskal-Wallis. Data in bold are significant at $P < 0.05$. $^{+}$ $P < 0.1$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

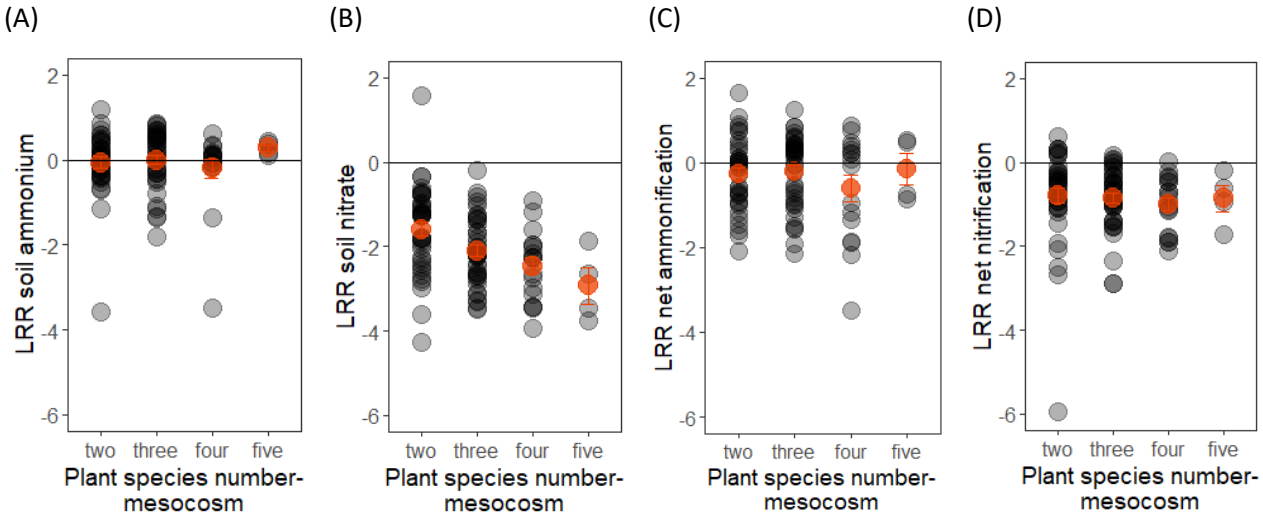


Figure 6.21 Effect of number of plant species in the mesocosms on LRR of soil N cycle: (A) ammonium and (B) nitrate in the soil and (C) net ammonification and (D) net nitrification. Red points represent mean and bars are ± s.e.

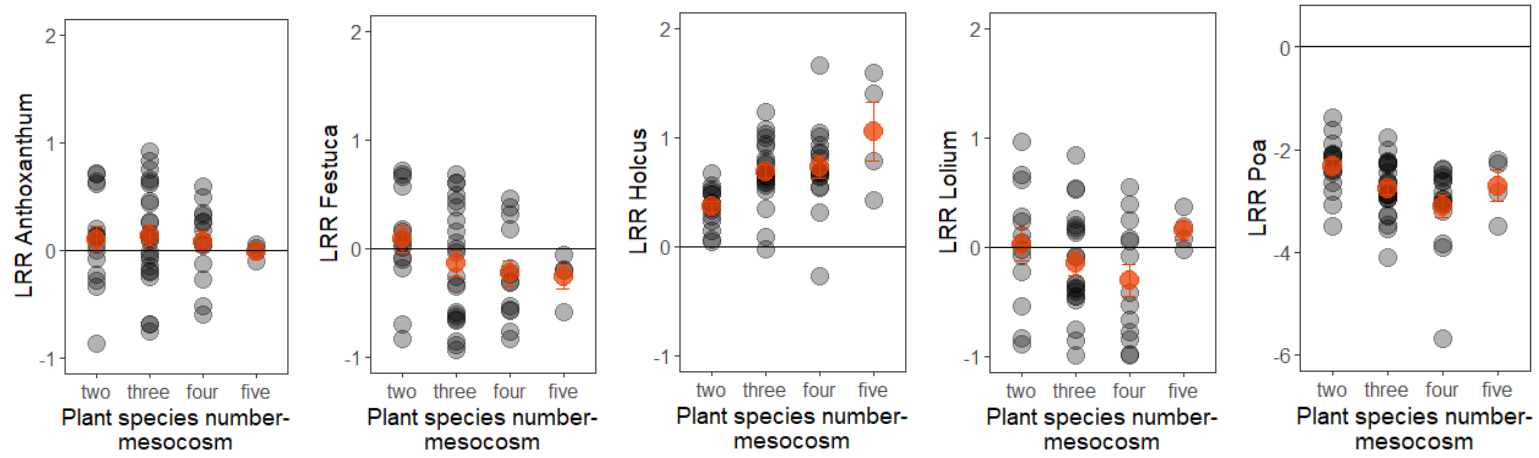


Figure 6.22 Effect of number of plant species in the mesocosms on LRR of aboveground biomass yield of single plant species. Red points represent mean and bars are ± s.e. Anthoxanthum = *A. odoratum*, Festuca = *F. rubra*, Holcus = *H. lanatus*, Lolium = *L. perenne* and Poa = *P. trivialis*

6.5 Discussion

Plant species expressed differences in their traits which characterize their life strategy for nutrient acquisition. These traits were aboveground biomass N concentration and C:N ratio. Plant species characterized as fast growing had higher biomass N and lower biomass C:N in comparison to species characterized as slow growing. Based on their strategy, plants also showed predictable impacts on soil mineral N availability whereby fast growing plants increased soil nitrate and these effects on N cycle were also shown when they were grown in plant mixtures. Plant life strategy was not associated with plant aboveground biomass in monocultures. In mixtures, fast growing *H. lanatus* was shown to be a strong competitor, decreasing biomass of other species in the mixture, while presence of slow growing *F. rubra* increased overall biomass of the mixture but did not affect biomass of other species. Measurements of C effluxes did not show a pattern distinguishing between plant life strategy effects in both monocultures and mixtures despite an increase of ecosystem respiration with presence of fast growing *L. perenne* in the mixtures. The bacterial community did not respond strongly to different plant species in monocultures, but the fungal community showed a response and data suggested differences between plant species. Class *Basidiomycota* increased its relative abundance in slow growing species soil and the most abundant classes of *Ascomycota* responded in the opposite direction.

6.5.1 Monocultures

C and N cycles

Plant species differed in their effects on attributes of N cycle when they showed an effect on nitrate and net nitrification while ammonium and net ammonification was not impacted. *L. perenne* and *P. trivilialis* showed higher soil nitrate than *A. odoratum*, *F. rubra* and *H. lanatus*, and also non-significantly higher values for net nitrification than the other plant species. These results would suggest an increase of rate of N cycling in the soil of these species. Higher level of potential N mineralization was found for *L. perenne* in comparison to *A. odoratum* in a mesocosm study by De Deyn *et al.* (2012). Harrison and Bardgett (2010) also found differences in effect of species on soil N attributes and the difference was between legumes and species from other functional groups tested. However, they did not find differences in nitrate among *F. rubra*, *A. odoratum* and *L. perenne* while difference between *L. perenne* and *F. rubra* was found in the present study. Their study was only a short term in contrary to the present study spanning two growing seasons. This may point towards a need for longer time plants to develop soil feedbacks resulting from inherent plant strategies (Orwin *et al.*, 2010).

The present results also suggested an effect of plant life strategy on soil N cycle which was shown by higher soil nitrate and net nitrification for fast growing plant species in comparison to slow growing species, in accordance with hypothesis 1. This agrees with theory and studies showing relevance of plant leaf economic spectrum (Wright et al., 2004) for dynamics of soil N cycle (Orwin *et al.*, 2010; Grigulis *et al.*, 2013; Kastovska *et al.*, 2014) whereby fast growing plant species (as determined by high leaf N and low leaf C:N) are associated with greater soil N availability. Orwin et al. (2010) showed that grass species with high leaf and litter N content grown in monoculture plots for 7 years were associated with higher soil inorganic N pool and to a lesser extent net N mineralization and nitrification. In grassland multisite comparison study, leaf N content showed a positive correlation with litter decomposition rate in two out of three sites (Grigulis et al., 2013) suggesting greater release of nutrients into the soil for more nutrient exploitative plants. They further argued that more exploitative plat strategies are expected to result in greater plant biomass but poor C and nutrient

retention as they are associated with microbial communities performing rapid rates of mineralization and nitrification. On the other hand, potential nitrification rate was not associated with any of plant traits measured (e.g. leaf N and C:N ratio) but solely with availability of soil ammonium (Legay et al., 2014) suggesting that plant effects on soil N cycling might be indirect. Similarly, Grigulis et al. (2013) found that soil processes related to N cycling (e.g. potential N mineralization) were largely explained by microbial parameters and not plant traits.

Greater soil N availability is generally considered to result from decomposition of high litter quality (low C:N ratio) however, aboveground litter was removed in the present study, and while root decomposition effect cannot be discounted, it can be anticipated that different plant life strategies will affect soil N cycle through their association with soil microbial community. For instance, Orwin et al. (2010) showed that fast growing plants were associated with higher bacterial:fungi ratio. Bacterial food web was found to be associated with greater soil N cycling in a large-scale survey (De Vries et al., 2013). Plants through rhizodeposition release a substantial portion of primary productivity (Pasch and Kuzyakov, 2017; Hirte et al., 2018) and its quantity as well as quantity would affect microbial community structure and activity (Bardgett and Wardle, 2010) and microbial community attributes were found strong drivers of soil N cycle (Grigulis et al., 2013). Legay et al. (2014) hypothesized that root C:N ratio might influence exudate C:N ratio and labile organic compounds C:N ratio which might affect soil denitrification. The mechanism for higher soil N availability for fast growing plant species may thus be related to generally expected higher rhizodeposition by fast growing species, which have been also shown by Kastovska *et al.* (2014), when the increased rhizodeposition may promote N mineralization in the soil (Dijkstra et al., 2013).

However, it was also shown, that the effect of plant life strategy was strongly supported by only two fast growing species but not by *H. lanatus* which did not differ from both slow growing species. This may suggest that life strategy concept is a continuum of plant species allocation in a two-dimensional space than a strict separate grouping into fast- and slow- growing plants. Alternatively, N cycle attributes related to soil mineral N and net mineralization rates in the peak season may not fully capture the dynamics of N transformations and availability (Schimel and Bennet, 2004). N demand of plants have been shown to differ temporarily during season with the highest plant demand in spring which then decreased towards autumn (Kastovska *et al.*, 2014) thus the higher levels of soil N in the present study may simply reflect seasonal changes due to plant phenology.

Interestingly, bare soil mesocosms had higher nitrate compared to most of species but *P. trivialis*. The high soil nitrate concentration might have reflected that the soil for the mesocosms was taken from intact grassland and N transformation processes may have continued without plant presence in until the following season and energy source for microbial community was provided from root decomposition. Disturbance related to soil processing during mesocosms set up may have made these roots available for decomposition. Lack of plant N demand would then result in accumulation of nitrate. On the other hand, highest soil nitrate concentration for bare soil than for most plant species and differences in soil nitrate among plant species might also suggest differential plant nitrate uptake between the species. This would also mean lower N pool in plant aboveground biomass for *P. trivialis* which showed similar nitrate as bare soil and higher than the other species. However, this was only true for *F. rubra* while the other species had similar N pool in aboveground biomass as *P. trivialis*. Nevertheless, the differences might be present for belowground biomass N pool which was not determined.

Aboveground biomass C:N ratio was affected by plant species and plant life strategy whereby it was higher for *A. odoratum* in comparison to *P. trivialis* and it was higher for slow growing plant species compared to fast growing species. It confirms that evolutionary trade-offs in plat growth strategies results in differences in biomass nutrients typically assessed by leaf characteristics (Wright et al.

2004) but assessed as whole aboveground plant biomass characteristics in the present study. Plant biomass C and N stoichiometry reflects relative strength of plant C and N metabolism (Luo et al., 2017). It can affect decomposer food web in the soil through differences in response of soil fungal and bacterial communities to plant litter of different C:N ratio as shown by (Rousk and Bååth, 2007) whereby fungal growth was promoted more by substrate with higher C:N than lower C:N and bacteria showed an opposite response. Furthermore, changes in soil microbial food web dominance can have consequences for ecosystem processes when fungal dominated food web has been linked to conservative nutrient cycling (De Vries et al., 2011; De Vries et al., 2013) and is expected to promote soil C storage (J. Six et al., 2006). On the other hand, C:N litter ratio as a measure of litter quality can affect fate of litter degradation in the soil and differentially contribute to SOC storage whereby higher quality litter might be more efficiently assimilated into microbial biomass resulting in a greater microbial biomass and microbial by-products available for stabilization (Cotrufo et al., 2013).

Species did not differ in ecosystem respiration at any time point tested and overall effects on the respiration measured in the three time points during plant growth towards peak of season were only due to higher ecosystem respiration for plant species mesocosms in comparison to bare soil mesocosms. Similarity in ecosystem respiration between grass species of *A. odoratum* and *L. perenne* grown in monocultures have been shown by others (Orwin *et al.*, 2010; De Deyn et al., 2012). On the other hand, differences in ecosystem respiration between grassland species were found when different functional groups were compared. Legumes showed a higher respiration in comparison to grasses and forbs (Orwin *et al.*, 2010; De Deyn *et al.*, 2012). But also differences within C3 grasses have been shown by (Orwin *et al.*, 2010) comparing *Festuca ovina* to *L. perenne*. They showed that F. ovina was weakly associated with higher root biomass and with high microbial biomass C and biomass which could have been behind the positive effect on ecosystem respiration rates. This suggests that rates of C cycling are unlikely to be predictable based solely on leaf and litter quality (Orwin et al., 2010).

Microbial community response in monocultures

Data suggested that response of bacterial community structure was primarily related to differences between bare soil and planted mesocosms. These differences were shown to be associated with changes of relative abundance of microbial taxa associated with plant roots such as *Alphaproteobacteria* and *Acidobacteria* by Thompson et al. (2013). However, relative abundances of *Alphaproteobacteria* (ANOVA, $F_{5,13} = 4.3$, $P_{adjusted} = 0.24$) and *Acidobacteria* were not affected in the present study. Similarly, plant life strategies would show different effect on soil bacterial communities as they shown an effect on soil N availability. They would also promote copiotrophic bacterial taxa due to anticipated differences in exudation pattern between fast and slow growing plant species (Kastovska *et al.*, 2014). Nevertheless, this was not suggested by the present data. Only orders *Opiputalles* (*Opitutae*) and *Rickettsiales* (*Alphaproteobacetria*) showed responses. *Opiputalles* responded to plant species. Hester et al. (2018) found this order to be positively affected by N addition treatment and it was also weakly correlated with N_2O emissions. Its members were described as polysaccharide-utilizing bacteria capable of nitrate reduction to nitrite (Chin et al., 2001). They have been found associated with rhizosphere of diverse plants such as sugarcane and wetland plants (Soil et al., 2018). Order *Rickettsiales* showed a response to both plant species and plant life strategy. Members of *Rickettsiales* are mainly associated with arthropods hosts but were also found as algae endosymbionts and cannot survive in the long term outside of their host (Kawafune et al., 2012), nevertheless, they are not typically abundant in the soil.

Lower overall response of bacterial community to plant species in monocultures can be due to relatively low impact of plant species on soil nutrient level in the mesocosms. Despite change of soil N cycle attributes between fast growing and slow growing plants were found, the overall quality and quantity of soil organic matter may have been unaffected during the relatively short duration of the experiment. As such, bacterial response to these relatively small changes may have been overruled by similarity of organic matter characteristics in the mesocosms of different plant species (Millard and Singh, 2010). Moreover, only transient changes in relative abundance may have been masked by presence of relic DNA (Carini et al., 2016).

The fungal community showed changes in both community structure and relative abundance of fungal taxa at order and individual ASVs resolution. Changes in fungal community with plant alteration can be expected as close association of the fungal community with the plant community have been shown before (e.g. Cassman *et al.*, 2016) and can be related to plant-fungal relationship in the form of mutualistic or pathogenic associations (Putten *et al.*, 2013) or to plant litter driven fungal saprotrophs (Millard and Singh, 2010). Indeed, indicator species analysis showed that true pathotrophs increased their relative abundance within taxa associated only with single plant species.

6.5.2 Plant species mixtures

Growing plant species in the mixtures reduced soil N availability. Soil nitrate concentration was lower in species mixtures mesocosms in comparison to monocultures. Furthermore, mixtures showed lower nitrate levels than would be expected based on monocultures determined using LRR analysis whereby two-species mixtures showed highest LRR values. Decrease of N availability in species mixtures may point towards enhanced nutrient use efficiency resulting from higher plant diversity (Hooper and Vitousek, 1998). Lower soil nitrate resulting from higher species diversity has been frequently observed in grassland biodiversity experiments (Tilman et al., 2014). For instance, higher diversity plant species plots showed lower soil nitrate in the 'small' Cedar Creek experiment and also lower nitrate was found to be associated with higher plant species number in nearby native grassland (Tilman et al., 2014). In the 'big' Cedar Creek experiment, Tilman et al. (1997) also observed lower nitrate with higher plant species diversity and suggested that the underlying reason was functional differences among plant species. Similarly, Hooper and Vitousek (1998) observed that plant functional composition was more important for soil nitrate than functional group richness. All these experiments and others (Hector et al., 1999) reported higher aboveground biomass yield with more diverse plant communities (both species and functional richness). Positive impact of higher diversity on the biomass yield can result from multiple causes including greater use of limiting resources, decreased herbivory and disease, and nutrient-cycling feedbacks that increase nutrient stores and supply rates over the long term (Tilman et al., 2014). However, no increase of the biomass yield was observed for higher species rich mixtures in the present experiment, despite a non-significant trend showing biomass increase with species mixture. The observed lower soil nitrate with species mixtures than in monocultures might suggest either greater plant nitrate uptake or higher investment of resources into roots during interspecific competition in mixtures (Baxendale et al., 2014). Alternatively, lower nitrate may result from lower N mineralization (e.g. nitrification). N in the form of nitrate is usually present in the soil under high N availability because nitrifiers are poor competitors for ammonium (Verhagen et al., 1995; Schimel and Bennet, 2004) and thus higher plant N uptake as a result of higher biomass with greater species diversity would increase plant-microbe competition for soil N. Nevertheless, the present data did not support this hypothesis despite only non-significant trends and suggesting higher treatment replication may be beneficial for significance of observed trends.

Contrasting effect of different plant life strategies on soil nitrate in the mixtures was observed. Presence of either *A. odoratum* or *F. rubra*, slow growing plant species, had a negative impact on soil

nitrate when the former species affected both nitrate and net nitrification and the latter species affected only nitrate. Opposite effect was seen for fast growing *L. perenne* and *P. trivialis*, whereby presence of the former species positively affected soil nitrate and the presence of the latter species positively affected both nitrate and nitrification. This shows that species characterized as slow growing expressed negative effect while species characterized as fast growing expressed positive effect on the components of N cycle. These species showed the same effect on soil nitrate in monocultures as well as in the mixtures. On the other hand, *H. lanatus*, characterized as fast growing species (Baxendale et al., 2014), did not show an effect on N cycle, which may be due to its fastest maturation. At the time point when the soil nitrate was measured, plants of *H. lanatus* may not have been sufficiently connected with the microbial community due to reduced rhizodeposition as a result of senescing of aboveground biomass. This can be further confirmed by negative effect of *H. lanatus* on photosynthesis observed in June.

Ecosystem respiration was enhanced by presence of *L. perenne* in the species mixtures when it positively affected the respiration in June and July for all mixtures and for three-species mixtures in May. It is reasonable that *L. perenne*, which is characterized as fast growing species, would increase ecosystem respiration due to positive effect of fast growing species on root exudation (Kastovska *et al.*, 2013) and resulting higher soil respiration due to priming effect (Kuzyakov *et al.*, 2000), however *L. perenne* did not show higher ecosystem respiration in monoculture. *L. perenne* promoted soil nitrate both in monocultures and mixtures, and such effect might result from increased rhizodeposition which would be also associated with higher respiration, although this was not the case in *L. perenne* monoculture. Differential response in monoculture and species mixtures might originate from plant-soil feedback differences between when it was grown in monoculture and species mixtures. Plant-soil feedbacks can be used to explain vegetation dynamics (Van der Putten et al., 2013). *L. perenne* may show a negative soil feedback in monoculture, for instance through root pathogens, reducing root growth and thus negatively affecting respiration in monocultures. While in mixtures, pathogens may be reduced due to dilution effect (Van der Putten et al., 2013) and thus *L. perenne* may express fully its effect on ecosystem functioning in mixtures. It may also be that *L. perenne* may show a different competitive strategy in species mixtures than in monocultures. Baxendale et al. (2014) showed that fast-growing species responded by greater root growth in mixtures while increased root growth was not observed in its monoculture. They argued that this differential response was not likely due to soil symbionts or pathogens. Similar for the present experiment, *L. perenne* might show an increased root growth in species mixtures than in the monoculture and greater root biomass and related microbial community might increase ecosystem respiration.

Aboveground biomass yield was not affected by plant community richness. This is in contrary to other studies showing positive effect of plant community richness on aboveground yield (e.g. Tilman et al., 2014 and references therein). On the other hand, species identity had an impact on aboveground biomass yield of total plan community and also of other species in the mixture. The total yield was higher when *F. rubra* was present in the mixture. Biomass of *F. rubra* correlated with overall aboveground biomass of the mixture but it did not change its biomass based on what would be expected from its biomass in monoculture. It also did not affect aboveground biomass of any other species in the mixtures which altogether suggests that the increase of the overall biomass was solely due to biomass of *F. rubra*, which also showed the highest mean biomass in the monocultures (however non-significant). The overall aboveground biomass yield in the mixture also correlated with the biomass of *H. lanatus* in the mixture. *H. lanatus* strongly over-performed in all mixtures in comparison to monocultures and it also decreased aboveground yield of all other species in the mixtures which resulted in no effect of *H. lanatus* on aboveground yield in the mixtures.

Overall, plant species expressed trends in their traits (aboveground biomass N concentration and C:N ratio) in accordance with their life strategy affiliation, however relatively large data variation of the biomass data did not result in significant differences between all species with opposite life strategies. The large variation may have been also due to the fact that traits determined for life history characterization are typically measured on leaves while whole overall biomass was used in the present research. It was also found that species with opposite life strategies resulted in an opposite effect on soil N cycle, higher N availability with fast growing plants, as predicted by the theory. Detailed analysis of soil N cycle rate and ecosystem N pools would inform about explicit mechanism behind observed differences and specifically, whether they are due to lower plant uptake or variation in N mineralization rates. Such analyses might include use of stable N isotopes and EEAs for determination of N transformation rates and partitioning of N uptake between plants and microbes.

Moreover, greater plant species diversity resulted in lower soil mineral N concentration and non-significant increase in aboveground biomass yield. This suggests greater nutrient use efficiency with species mixtures resulting in higher yield. Analysis of plant belowground biomass yield and nutrient concentration would provide additional support for hypothesized higher yield and N use efficiency with greater species diversity and would increase understanding of differences in plant-soil interactions between monocultures and mixed species communities. Such knowledge might be beneficial for increasing agricultural sustainability and reducing its environmental impact when for instance higher plant diversity of fodder crops would result in a decrease of available nitrate minimising risk of its loss through leaching. If the increased N use efficiency is due to a greater belowground plant biomass and/or greater flow and stabilization of organic compounds in the soil, then this can have a positive effect on soil C sequestration. It can be also that the increased N use efficiency is due to a presence of specific plant species as demonstrated here when presence of *A. odoratum* or *F. rubra* was associated with lower soil nitrate availability without negative effect on aboveground yield. These are slow growing species thus future research on plant-soil interactions should benefit from measurements of plant traits and characterization of plant life strategies. The research also demonstrated benefits of studying plant interactions through comparison of effects in monocultures vs. mixtures as it was demonstrated here when a difference between measured values in species mixtures and expected values recalculated from monocultures strengthened obtained results from species mixtures for lower soil nitrate concentration in species mixtures. Microbial analysis might include determination of frequencies of specific genes involved in N cycle and comparing them with N cycle rate for greater insight into N dynamics in the soil as well as analysis of active microbial community and identifying specific community members receiving plant derived photosynthates might further the knowledge of soil C cycle and its drivers.

Chapter 7: **General discussion**

Grasslands represent an important ecosystem globally and due to the size, large soil C pool, and the other services they provide. Improvement of their management can be used as a tool for climate change mitigation though soil C sequestration (Conant et al., 2017). Thus, this thesis aimed at increasing understanding of SOC storage in grasslands.

Many grasslands have lost C from the soil due to intensive land use (Lorenz and Lal, 2018). For instance, Bellamy et al. (2005) reported loss of soil C in grassland in England and Wales during past 20 years. Thus, due to past C loss, grasslands may have a high potential for storage of a substantial fraction of atmospheric CO_2 as protected or stable C in the soil (i.e. soil C sequestration) (Lorenz and Lal, 2018).

Agricultural soil management to increase SOC will have other benefits (Smith, 2012) such as restoration of degraded land contributing to higher biomass production and further increasing soil C sequestration within the ecosystem (Lal, 2004), promoting soil biodiversity, reducing erosion, runoff and water pollution, and helping to buffer agricultural systems against impact of climate change (Paustian et al., 2016). Increasing soil C stocks can thus reduce the vulnerability of managed soils to future global warming (Smith, 2012). Higher production from agroecosystems due to benefits from increased SOC may also result in lower need for new conversions of natural ecosystems into managed land. Agricultural improvement may in some instances (such as irrigation in arid regions or increasing soil nutrients in nutrient poor soils) lead to promotion of SOC, specifically when these measures lead to increase of biomass production (Lal, 2018). For instance, Ward et al. (2016) found higher soil C stock under grasslands of intermediate management intensity when compared to both, high intensive and extensive grasslands. However, full cycle analysis is needed in order to account for indirect C losses such as those from fertilizer manufacturing or whether organic matter added to the soil at one location does not reduce C at other location (Lal et al., 2004) in order to assess whether such management improvement result in reduction of atmospheric CO_2.

7.1 Measures for improved soil C sequestration in grasslands

Soil C stock increases in managed ecosystems can be achieved by optimising 'best management practices' (Smith, 2012). In general, measures increasing plant growth would result in higher potential for increases in soil C stocks (Conant et al., 2017) due to greater inputs from the primary productivity into the soil. Lal (2018) listed three basic strategies for enhancement of SOC sequestration in managed ecosystems including increasing input of biomass C, decreasing losses of SOC by erosion and decomposition, and increasing the mean residence time of C by its stabilization in the soil. However, the potential of the soil and other components of the ecosystem to act as a C sink will also depends on the historic and present land use, the magnitude of antecedent C depleted from soil, properties of the soil profile, climate, and management (Lal, 2018). Conversion of cropland to grasslands might be the easiest strategy as grasslands usually store more SOC than croplands (Guo and Gifford, 2002) but such land use change might not be the best option when the gained soil C increase is low as there is a growing need for food production. On the other hand, conversion of degraded marginal land into grasslands has been widely recommended (e.g. Smith, 2012).

Apart from changes in grazing management and introduction of irrigation, common grassland management practises affecting SOC include repeated additions of lime and fertilizers into the soil (Lorenz and Lal, 2018). Increasing nutrient availability can be regarded as a useful measure to increase SOC in the ecosystems where nutrient availability is limiting primary productivity. Natural ecosystems are generally N limited (Vitousek and Horvath, 1991) and N additions were found to

increase aboveground primary productivity of most of ecosystems (except deserts) by 29 % (53 % increase for temperate grasslands) (LeBauer and Treseder, 2008). Many other studies have demonstrated such increases (e.g. Xia and Wan, 2008). Fornara et al. (2013) further showed an increase of SOC after 19 years of N addition to mesotrophic grassland. Increases of primary productivity would then result in greater inputs of OC into the soil from rhizodeposition and aboveground and belowground litter which is a prerequisite for greater SOC storage. It should be noted that N addition may not always increase plant aboveground productivity where multiple nutrient co-limitations may be limiting the productivity (Fay et al., 2015).

Chapter 4 of this thesis studied effect of liming followed by increases of available N in the soil, and despite an increase of soil N availability, plant biomass yield (both aboveground and belowground) was not enhanced. Fay et al. (2015) showed combined N and P addition increasing aboveground biomass, however Chapter 4 did not suggest that plants on the limed plots were P limited. Chapter 4 also showed plant composition change after liming and a trend of decreasing plant C:N ratio. Changes in community plant traits, such as leaf C:N ratio, can be related to changes of life strategies of the plants within the community, whereby higher leaf C:N ratio determines nutrient acquisitive strategists associated with open soil N cycle (Grigulis et al., 2013) with potential for N losses from the ecosystem. Indeed, Chapter 6 found higher soil mineral N associated with fast growing plant species (nutrient acquisition strategists). Moreover, species identity affected aboveground biomass growth of the other species in species mixtures whereby *Holcus lanatus* decreased biomass of the other species in the mixture (Chapter 6). *Holcus lanatus* increased its plant cover after liming in Chapter 4. It is thus possible that its negative impact on growth of other species in the community was behind the lack of positive response of aboveground biomass to increase of soil mineral N in Chapter 4.

Moreover, relatively small increases of mineral N after two fungicides applied in the beginning of the growing season in the same grassland as in Chapter 4 also did not lead to increases of plant aboveground biomass at the peak of the season on any of the pH treatment plots (Chapter 5). At the same time, decrease of ecosystem respiration was observed on plots where fungicide was applied. Such a decrease may be related to positive impact of N on alleviation of microbial N limitation and reduction of microbial decomposition of SOC for obtaining limiting N. Thus management measure including low N applications may be beneficial for reduction of SOC stock decomposition. However, such low N doses may negatively impact plant species diversity (Stevens et al., 2004) with potential consequences for ecosystem services delivery.

Liming effect on SOC stock showed contradictory results. Long-term grassland liming of 129 years (Park Grass experiment, U.K.) increased SOC stock as determined in topsoil, while the cause of enhanced SOC stock was greater biological activity in the limed soils (Fornara et al., 2011). However, grassland liming over the period of around 20 years (Nash's field, U.K.) did not show an increase of SOC stock (Heyburn et al., 2017; Egan et al., 2018). Both of the studies suggested importance of organic C associations with soil mineral fraction after liming. Similarly, Chapter 3 showed an increase of soil OC concentration in a regenerating grassland chronosequence spanning over 150 years in a chalk soil while majority of SOC was related to OC associated to soil mineral matrix suggesting importance of OC associated with mineral soil particles and derived protection from decomposition (Schmidt et al., 2011). Some literature (e.g. O'Brien and Jatrow, 2013) emphasizes that overall increase of SOC is associated also with increase of POM within the soil as plant OC inputs in the form of litter are protected by soil aggregation and slowly decomposed into smaller particles as their physical protection mechanisms are destabilized (Lehman and Kleber, 2015). Chapter 3 supports this theory of slow decomposition of POM due to physical protection as coarse POM was higher in old regenerating grasslands than younger sites and litter was also higher in older grasslands. This is in contrast to what data from Fornara et al. (2011) and Egan et al. (2018) suggest. However whilst

Fornara et al. (2011) did not show differences in light organic C fractions within the soil, they mentioned that a thick vegetation mat present in the unlimed soils was not present in limed soils suggesting its decomposition. Higher OC within the limed soil may thus be associated with decomposition of aboveground litter. Data presented by Egan et al. (2018) showed greater SOC associated with lower soil aggregate fractions on limed plots suggesting lower protection of OC (especially POM) in larger macroaggregates. This may suggest that the SOC storage mechanism might be different in limed soils than in unlimed soils. In chapter 4, liming induced dissolution of SOC (Bailey et al., 2019) promoting microbial activity and despite greater ecosystem respiration observed, which might be also affected by a tendency for higher ecosystem photosynthesis on limed plots potentially increasing rhizodeposition, the SOC as determined by LOI method did not suggest an effect of liming. It would be interesting to further determine whether internal changes within the soil C pools were affected. Chapter 3 suggested that silt fraction separated into coarse and fine sub-fractions based on size differed in their responses to land management history. It would be interesting to further study whether liming which is known to increase dissolution of SOC and was shown to increase breakage of larger soil aggregates (Egan et al., 2018), will also affect POM and whether increased decomposition of aboveground plant litter after liming would be associated with an increase of OC in any of the mineral associated OC sub-pools (thus increasing its long term persistence potential).

7.2 Plant community relationship with soil C sequestration.

Plant traits are believed to be strongly involved in mechanism controlling soil C sequestration and plant life history strategies and have been recommended to play an important role in this process through control of inputs of soil organic matter as well as their fate in the soil (De Deyn et al., 2008). Fast growing plant species contribute larger amount of organic matter to the soil and this organic matter is typically of lower C:N ratio than slow growing species. Lower C:N ratio was suggested to be transformed into microbial biomass more efficiently as microbial biomass C:N ratio is lower than that of plants (Cotrufo et al., 2013). Also, complex plant compounds with higher C:N might require greater microbial investment into degradative enzymes thus lowering microbial C use efficiency. On the other hand, microbial communities promoted by fast growing plants with typically more bacteria-oriented food webs (Orwin et al., 2010; Grigulis et al., 2013) and also with fast growing bacterial taxa promoting more N open cycle (Fierer et al., 2007) may negatively impact SOC due to greater losses of soil N which may be originating from decomposition of SOC.

Different plant species belonging to *Poa* family were grown in monocultures (Chapter 6) and while their aboveground C:N ratio was differentiated as expected based on their life strategies, their aboveground biomass yield did not differ between their life strategies (analysis included plant species grouped according to their life strategies). When plant species were grown in species mixtures, no effect of the presence of fast growing plant species in the mixtures on aboveground plant biomass was observed. Instead, *Holcus lanatus* showed strong aboveground competitive strategy when its presence reduced biomass of other species in the mixtures. On the other hand, presence of slow growing *Festuca rubra* was beneficial for biomass yield. These results points towards importance of plant species interactions in species mixtures in grasslands. Grassland productivity in less intensive systems might be achieved through selected species mixtures which will assure biomass yield as well as will promote plant species biodiversity. Chapter 6 also showed that species mixtures had lower soil mineral N availability suggesting greater plant N use efficiency while presence of fast growing species positively impacted mineral N availability in mixtures. Thus future research should target which plant species due to their positive impacts on biodiversity and nutrient use efficiency are best suited for less intensive grassland management systems.

Sustained biomass yield without N addition might not be a reliable strategy for SOC sequestration. Biomass removal can reduce soil C sequestration (Skinner, 2008). Chapter 4 showed higher aboveground N pool after liming which would largely originated from decomposition of SOC suggesting that biomass harvest without replenishment of removed N will likely reduce C stock. Thus, grassland species mixtures for extensive management should include plant species promoting biological N fixation either through symbiosis (e.g. legumes) or as free living diazotrophs. Positive effects of including legumes in species mixtures have been frequently reported (e.g. De Deyn et al., 2011; Yang et al., 2019).

7.3 Ecosystem restoration and soil C stock recovery

Ecosystem recovery is typically a top down approach when plant community restoration is targeted and rest is assumed to follow (Strickland et al., 2017). Regeneration of cropland soils by sowing seeds to resemble native ecosystem showed relatively fast restoration of soil structure and increase of SOC stock (Matamala et al. 2008; O'Brien and Jastrow, 2013). Turnover of annual plant species was attributed to rapid increase of SOC after the conversion as observed by Hernandez et al. (2012). Greater increases of SOC after land conversion into restoration sites after SOC depletion from the previous intensive land use let O'Brien and Jastrow (2013) to argue that C sequestered early in the restoration have the greatest opportunity to be stabilized in the soil. C stabilization in the later stages of restoration is typically slow when for instance several hundreds of years were predicted for complete recovery of C stock in degraded soils by Matamala et al. (2008). Chapter 3 studied grassland regeneration chronosequence and results suggested that older grasslands have higher mass of coarse silt and reduced mass of fine silt fractions than younger grasslands and cropland soils. Fine silt fractions together with clay and POM are building blocks of coarse silt fraction (Virto et al., 2008, 2010; Totsche et al., 2018). It was thus further suggested in Chapter 3 that the lower relative abundance of coarse silt within microaggregates is related to both lower POM in this fraction and lower aggregation of fine silt and clay into coarse silt. O'Brien and Jastrow (2013) argued that lack of soil C stock increase in later stages of grassland restoration may be related to lower availability of OC of the right size or chemistry. Their grassland chronosequence was restored using native species seed mix while the chronsequence presented in Chapter 3 was left to natural succession with differences of plant species between the stages of restoration whereby younger grasslands included species typical for intensive agricultural sites and older grasslands included species typical of native chalk grasslands (Redhead et al., 2014). It can be thus speculated to what extent the species composition will affect the rate of soil C stock recovery and the role of plant traits in this process. Yang et al. (2019) showed that restoration of late-successional grassland plant diversity resulted in greater soil C sequestration due to high both aboveground and belowground plant biomass suggesting importance of diversity as well as community composition on soil C sequestration. Similarly, Chapter 6 of the present thesis suggested greater benefits of species mixtures for N use efficiency. Nevertheless, O'Brien and Jastrow (2013) also targeted restoration of native plant diversity while showing a plateauing of C accrual in different soil aggregate sub-fractions determined. Yang et al. (2019) further suggested that plant functional composition may be important for soil C sequestration such as presence of C4 grasses and legumes. It will be thus interesting to compare impacts of different approaches to grassland ecosystem restoration on soil aggregate sub-fractions (such as coarse and fine silt and clay) and POM and associated C pools in order to increase understanding of soil C sequestration and develop the best restoration management strategy for C sequestration apart from other benefits from restored biodiversity.

Soil microbial community is an important driver of soil processes affecting overall ecosystem (Van der Heijden et al., 2008). Thus, development of microbial community composition during ecosystem

restoration will be of importance for recovery of soil processes and especially those related to delivery of OC for stabilization in case of restoration targeting C sequestration. Microbial role in ecosystem recovery have been questioned as its community composition may simply follow plant community development (Harris, 2009). Soil abiotic factors are strong drivers of microbial composition (Fierer, 2017) and soil environmental conditions maybe also strongly related to plant impacts such as role of roots in soil moisture gradient. Moreover, plants have been shown to be strong drivers of soil microbial community composition through inputs of organic matter into the soil (e.g. Orwin et al., 2010). However, differences in relationship between plant and microbial community compositions of bacteria and fungi might exist. Cassman et al. (2016) showed that grassland composition was associated with fungal community structure while bacterial community structure was more related to soil abiotic conditions. This was partially showed in the present research whereby plant species in monocultures showed a tendency for differentiation of their fungal communities while bacterial community tended to show differentiation between planted and bare mesocosms (Chapter 6). On the other hand, microbial role in ecosystem restoration is realized through plant-soil feedback as microbial community can be a strong driver of plant community composition (Putten et al., 2013) when for instance mycorrhizal fungi community was shown to affect plant community structure through plant-plant interactions (Bennett and Cahill, 2016) and soil inoculation using different soil microbial communities can drive development of the restoring plant community (Wubs et al., 2016). These findings point toward an important role of microbial community in plant community composition.

Nevertheless, determination of a target soil microbiome of restored sites is difficult, especially due to large microbial diversity and variation of microbial composition among sites making site comparisons difficult to interpret. Furthermore, potential stochastic development of microbial communities (Hirsch et al., 2017) might further make these comparisons hard to interpret. The present research also did not observe trends predicted by literature when for instance sites with greater nutrient and substrate availability have been associated with greater abundance of *Alphaproteobacteria* (Leff et al., 2015) which was not shown for higher available N plots in the present research (Chapter 4). However, relative abundance of phylum *Verrucomicrobia* was suggested as grassland restoration indicator in the present study (Chapter 3). It will be important to further determine the role of members of this phylum in ecosystem development and potential implication of its increasing abundance on soil C sequestration. Greater understanding of changes in soil microbial community composition during ecosystem regeneration might be achieved through relating these changes to microbial community traits (Wood and Franks, 2018). To achieve this, it might be important to determine drivers of microbial diversity at the microscale including associated functional attributes as well as relationship of these microbial functional traits with soil organic C quality and quantity at the microscale in order to test whether greater C in certain pools is associated with specific microbial traits.

Further to increase understanding of soil C sequestration, higher level interactions might be studied. Visual assessment of the soil cores sampled in Chapter 4 suggested reduction of litter layer on the top of the soil for limed soils when compared to control and low pH treatments as well as a greater mole activity on the limed plots. It can be speculated that litter removal from the soil surface might be largely due to combined effect of litter burying by soil from mole hips and increased activity of earthworm in the limed soils, their higher presence may in fact cause increased mole activity in these plots (Edwards et al., 1999). This points towards inclusion of other members of the soil food web in order to understand drivers of soil C cycling. Although only bacterial and fungal communities were studied in the present thesis, archaeal populations are important members of soil microbial community (Bates et al., 2011) and drivers of soil N cycle (Song et al., 2016) which is strongly linked to C cycle. Archaeal determination might be also included in future research, especially if their composition can be assessed using the same 16S primers as bacteria.

7.4 Measurements of success of soil C sequestration

Monitoring of soil C stock is important for assessment of the success of applied improvements to the grassland management. It is also important to award obtained C sequestration if this is performed as a paid service (e.g. offsetting industrial CO_2 releases). Grasslands are supporting livelihood of a billion of people especially with lower incomes (Lorenz and Lal, 2018) and thus improvements in how they use their grasslands might be an opportunity for those communities. C sequestration schemes can increase their income as well as sustainability of the grasslands which will delivery required services for the future (incl. soil C sequestration). Thus, knowledge derived from better understanding of soil C storage and applied to development of monitoring of C stock is important for overall promotion of grassland C sequestration as a vital strategy for mitigating climate change as well as adapting to future climate change.

Apendix

Table S5.1 Identification of sequences at different taxonomic resolutions.

Taxonomic resolution	Proportion of identified sequences (%) [mean ± s.d.]	
	Bacteria	Fungi
Phylum	99.9 ± 0.04	97.0 ± 6.5
Class	99.4 ± 0.4	88.5 ± 8.3
Order	97.1 ± 0.7	82.4 ± 9.0
Family	76.4 ± 2.3	73.1 ± 10.5
Genus	44.9 ± 2.6	67.2 ± 10.6
Species	6.9 ± 1.9	51.2 ± 12.0

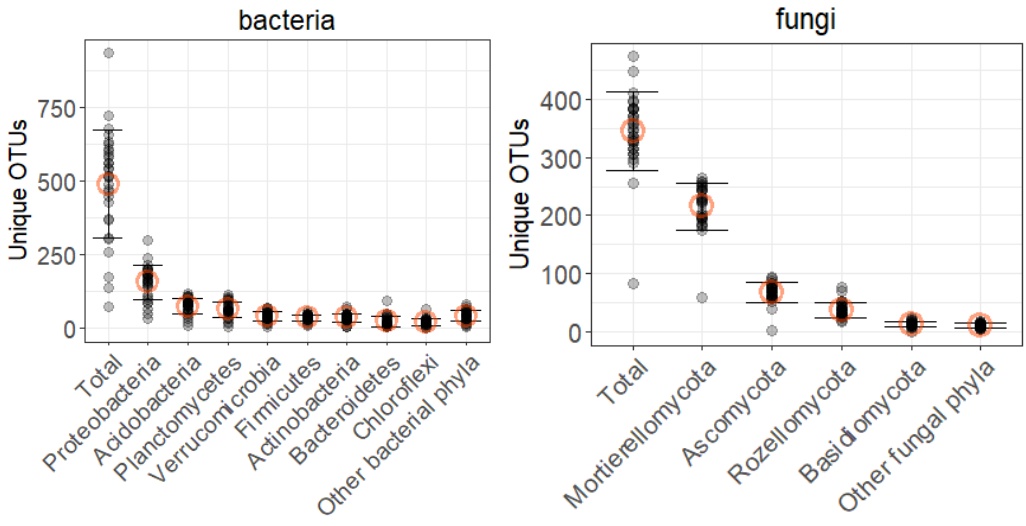


Figure S5.1 Overall community composition of soil bacterial and fungal communities in experimental grassland. Bacterial and fungal phyla of relative abundance higher than 1% displayed

Table S5.2 Mean relative abundance of bacterial phyla on experimental grassland plots.

Phylum	Non-biocide			Biocide		
	low pH treatment	control pH treatment	high pH treatment	low pH treatment	control pH treatment	high pH treatment
Proteobacteria	36.73 ± 1.29	34.08 ± 0.8	34.02 ± 0.46	36.81 ± 0.49	36.09 ± 0.75	34.73 ± 0.66
Firmicutes	17.04 ± 1.45	15.51 ± 1.66	23.43 ± 1.99	15.21 ± 1.39	15.43 ± 1.49	22.08 ± 1.86
Verrucomicrobia	14.56 ± 1.4	18.19 ± 1.31	11.19 ± 0.65	16.29 ± 0.86	17.89 ± 0.87	9.95 ± 0.88
Acidobacteria	14.03 ± 1.04	13.33 ± 0.54	12.36 ± 1.01	14.23 ± 0.46	12.74 ± 0.78	12.16 ± 0.87
Planctomycetes	6.14 ± 0.43	8.17 ± 0.31	5.66 ± 0.49	6.87 ± 0.33	8 ± 0.73	5.98 ± 0.42
Actinobacteria	6.41 ± 1.88	4.59 ± 0.42	5.97 ± 0.84	5.45 ± 0.37	4.69 ± 0.45	7.07 ± 0.45
Bacteroidetes	1.26 ± 0.17	2.1 ± 0.16	3.42 ± 0.6	1.04 ± 0.13	1.74 ± 0.24	3.48 ± 0.57
Chloroflexi	1.37 ± 0.32	1.48 ± 0.08	2.29 ± 0.2	1.26 ± 0.11	0.87 ± 0.11	2.91 ± 0.16
Gemmatimonadetes	0.57 ± 0.2	0.36 ± 0.08	0.39 ± 0.06	0.63 ± 0.09	0.48 ± 0.07	0.5 ± 0.07
WS3	0.26 ± 0.12	0.62 ± 0.04	0.4 ± 0.09	0.14 ± 0.04	0.43 ± 0.16	0.36 ± 0.1
Chlamydiae	0.39 ± 0.07	0.28 ± 0.05	0.27 ± 0.05	0.34 ± 0.05	0.3 ± 0.02	0.2 ± 0.05
WPS-2	0.28 ± 0.05	0.33 ± 0.05	0.17 ± 0.04	0.36 ± 0.06	0.28 ± 0.04	0.14 ± 0.05
TM6	0.16 ± 0.06	0.31 ± 0.05	0.06 ± 0.03	0.32 ± 0.03	0.37 ± 0.07	0.05 ± 0.01
Elusimicrobia	0.07 ± 0.02	0.2 ± 0.1	0.27 ± 0.09	0.1 ± 0.03	0.11 ± 0.04	0.23 ± 0.06
Nitrospirae	0.1 ± 0.04	0.15 ± 0.02	0.07 ± 0.02	0.12 ± 0.02	0.14 ± 0.02	0.13 ± 0.04
Cyanobacteria	0.21 ± 0.04	0.07 ± 0.05	<0.01	0.23 ± 0.03	0.12 ± 0.04	<0.01
FCPU426	0.43 ± 0.07	0.25 ± 0.02	0.03 ± 0.01	0.6 ± 0.16	0.33 ± 0.02	0.02 ± 0.01

Table S5.3 Mean relative abundance of bacterial classes on experimental grassland plots.

Phylum	Class	Non-biocide			Biocide		
		low pH treatment	control pH treatment	high pH treatment	low pH treatment	control pH treatment	high pH treatment
Proteobacteria	*Alphaproteobacteria*	29.1 ± 1.73	25.48 ± 0.66	22.85 ± 0.54	28.48 ± 0.6	27.57 ± 0.68	21.56 ± 0.39
	Deltaproteobacteria	3 ± 0.3	4.3 ± 0.31	5.55 ± 0.34	3.21 ± 0.26	4.15 ± 0.26	6.68 ± 0.57
	Gammaproteobacteria	3.47 ± 0.29	2.35 ± 0.15	2.73 ± 0.38	3.76 ± 0.25	2.4 ± 0.13	2.92 ± 0.31
	Betaproteobacteria	1.63 ± 0.27	2.35 ± 0.12	3.32 ± 0.35	1.93 ± 0.25	2.39 ± 0.11	4.01 ± 0.35
Firmicutes	*Bacilli*	16.33 ± 1.5	14.8 ± 1.63	22.6 ± 2.03	14.47 ± 1.35	14.73 ± 1.43	21.22 ± 1.89
	Clostridia	0.96 ± 0.19	0.92 ± 0.1	1.12 ± 0.14	0.98 ± 0.07	0.9 ± 0.08	1.14 ± 0.08
Verrucomicrobia	*[Spartobacteria]*	12.07 ± 1.61	14.84 ± 1.1	9.65 ± 0.83	13.27 ± 0.87	14.49 ± 0.86	7.88 ± 1.03
	[Pedosphaerae]	2.31 ± 0.47	3 ± 0.45	1.32 ± 0.28	2.74 ± 0.27	3.03 ± 0.22	1.52 ± 0.12
	Opitutae	0.26 ± 0.05	0.42 ± 0.1	0.06 ± 0.02	0.36 ± 0.08	0.47 ± 0.05	0.14 ± 0.03
	Verrucomicrobiae	0.01 ± 0.01	0.09 ± 0.01	0.22 ± 0.06	0.01 ± 0.01	0.03 ± 0.01	0.43 ± 0.11
Acidobacteria	*Acidobacteriia*	7.1 ± 0.64	4.99 ± 0.21	3.29 ± 0.44	6.73 ± 0.19	5.06 ± 0.4	2.58 ± 0.62
	Acidobacteria-6	1.37 ± 0.29	2.94 ± 0.37	4.87 ± 0.76	1.23 ± 0.24	2.3 ± 0.16	5.52 ± 0.41
	iii1-8	0.24 ± 0.09	0.32 ± 0.05	0.53 ± 0.09	0.28 ± 0.04	0.32 ± 0.02	0.55 ± 0.12
	Acidobacteria-5	0.15 ± 0.06	0.33 ± 0.06	0.36 ± 0.05	0.32 ± 0.03	0.24 ± 0.06	0.3 ± 0.06
	[Chloracidobacteria]	0.03 ± 0.02	0.17 ± 0.04	0.16 ± 0.04	0.05 ± 0.01	0.11 ± 0.03	0.33 ± 0.04
	DA052	3.96 ± 0.37	3.15 ± 0.22	2.02 ± 0.31	4.4 ± 0.41	3.12 ± 0.3	1.56 ± 0.24
	Solibacteres	1.3 ± 0.17	1.49 ± 0.05	0.9 ± 0.11	1.35 ± 0.17	1.7 ± 0.09	1.13 ± 0.13
Planctomycetes	*Planctomycetia*	6.03 ± 0.41	7.65 ± 0.24	5.54 ± 0.48	6.67 ± 0.35	7.43 ± 0.65	5.71 ± 0.41
	Phycisphaerae	0.12 ± 0.05	0.31 ± 0.06	0.04 ± 0.02	0.24 ± 0.05	0.29 ± 0.04	0.16 ± 0.01
Actinobacteria	*Actinobacteria*	2.66 ± 0.85	1.81 ± 0.17	3.21 ± 0.46	2.45 ± 0.16	1.77 ± 0.18	3.67 ± 0.21
	Thermoleophilia	3.4 ± 0.79	2.45 ± 0.24	2.02 ± 0.3	2.78 ± 0.29	2.63 ± 0.28	2.54 ± 0.3
	Acidimicrobiia	0.45 ± 0.28	0.38 ± 0.06	0.79 ± 0.17	0.31 ± 0.04	0.34 ± 0.03	0.92 ± 0.14
Bacteroidetes	*[Saprospirae]*	0.83 ± 0.07	1.02 ± 0.17	2.31 ± 0.4	0.62 ± 0.06	0.89 ± 0.04	2.18 ± 0.34
	Cytophagia	0.08 ± 0.04	0.49 ± 0.06	0.38 ± 0.08	0.09 ± 0.05	0.35 ± 0.1	0.59 ± 0.14
	Sphingobacteriia	0.32 ± 0.07	0.29 ± 0.08	0.32 ± 0.07	0.31 ± 0.07	0.22 ± 0.05	0.36 ± 0.13
	Flavobacteriia	0.01 ± 0.01	0.29 ± 0.04	0.37 ± 0.14	0.01 ± 0.01	0.28 ± 0.11	0.31 ± 0.06
Chloroflexi	*Ellin6529*	0.36 ± 0.16	0.45 ± 0.07	0.84 ± 0.26	0.13 ± 0.03	0.26 ± 0.08	1.21 ± 0.1
	Ktedonobacteria	0.6 ± 0.15	0.44 ± 0.11	0.7 ± 0.1	0.64 ± 0.04	0.36 ± 0.06	0.55 ± 0.08
	Anaerolineae	0.06 ± 0.03	0.16 ± 0.04	0.34 ± 0.1	0.14 ± 0.04	0.07 ± 0.01	0.73 ± 0.17
	TK10	0.16 ± 0.06	0.28 ± 0.06	0.14 ± 0.04	0.2 ± 0.04	0.14 ± 0.03	0.18 ± 0.04
Gemmatimonadetes	*Gemmatimonadetes*	0.5 ± 0.18	0.34 ± 0.06	0.27 ± 0.06	0.6 ± 0.09	0.48 ± 0.08	0.4 ± 0.07
WS3	*PRR-12*	0.27 ± 0.12	0.63 ± 0.03	0.4 ± 0.09	0.14 ± 0.05	0.44 ± 0.16	0.37 ± 0.1
Chlamydiae	*Chlamydiia*	0.4 ± 0.07	0.28 ± 0.05	0.27 ± 0.05	0.34 ± 0.05	0.31 ± 0.02	0.2 ± 0.05
TM6	*SJA-4*	0.26 ± 0.04	0.3 ± 0.06	0.17 ± 0.04	0.34 ± 0.06	0.24 ± 0.04	0.15 ± 0.05
Elusimicrobia	*Elusimicrobia*	0.16 ± 0.06	0.31 ± 0.05	0.06 ± 0.03	0.33 ± 0.03	0.37 ± 0.07	0.05 ± 0.01
Nitrospirae	*Nitrospira*	0.07 ± 0.02	0.21 ± 0.1	0.28 ± 0.09	0.1 ± 0.03	0.11 ± 0.04	0.23 ± 0.06

Table S5.4 Mean relative abundance of fungal classes on experimental plots.

Phylum	Class	Non-biocide			Biocide		
		low	control	high	low	control	high
Ascomycota	*Sordariomycetes*	16.95 ± 3.06	16.07 ± 1.53	20.24 ± 2.35	16.24 ± 1.56	19.64 ± 2.38	25.53 ± 2.13
	Eurotiomycetes	19.32 ± 4.3	8.45 ± 2.56	8.95 ± 2.57	12.98 ± 2.08	9.5 ± 1.94	9.87 ± 2.39
	Leotiomycetes	10.25 ± 0.99	9.91 ± 0.7	13.94 ± 1.41	12.31 ± 0.99	10.76 ± 1.14	12.94 ± 0.39
	Dothideomycetes	3.53 ± 0.63	8.75 ± 2.33	19.61 ± 3.24	3.51 ± 0.83	7.5 ± 1.03	15.07 ± 1.47
	Geoglossomycetes	5.16 ± 1.92	4.59 ± 1.98	2.05 ± 0.73	3.38 ± 2.04	9.5 ± 3.16	1.45 ± 0.35
	Ascomycota_cls_Incertae_sedis	1.57 ± 0.23	1.79 ± 0.33	4.06 ± 0.82	1.29 ± 0.29	2.2 ± 0.54	4.17 ± 0.41
	Archaeorhizomycetes	3.25 ± 0.37	1.36 ± 0.62	0.12 ± 0.07	1.68 ± 0.74	1.57 ± 0.4	0.27 ± 0.09
	Pezizomycetes	0.42 ± 0.11	0.35 ± 0.06	0.73 ± 0.28	0.24 ± 0.06	0.35 ± 0.2	1.34 ± 0.43
	Lecanoromycetes	0.23 ± 0.05	0.12 ± 0.05	0 ± 0	0.47 ± 0.17	0.23 ± 0.13	0.15 ± 0.12
	Orbiliomycetes	0.12 ± 0.1	0.35 ± 0.13	0.25 ± 0.08	0.21 ± 0.05	0.13 ± 0.06	0.18 ± 0.07
Basidiomycota	*Agaricomycetes*	19.26 ± 5.89	29.87 ± 3.09	11.06 ± 5.37	24.13 ± 2.28	17.45 ± 4.13	6.76 ± 3.45
	Tremellomycetes	12.29 ± 0.74	10.92 ± 0.8	15.16 ± 2	14.69 ± 1.74	11.09 ± 0.77	16.12 ± 1.67
	Microbotryomycetes	0.35 ± 0.1	0.41 ± 0.1	0.3 ± 0.07	0.44 ± 0.03	0.48 ± 0.15	0.56 ± 0.24
	Spiculogloeomycetes	0.09 ± 0.05	0.12 ± 0.08	0.05 ± 0.02	0.03 ± 0.03	0.28 ± 0.12	0.04 ± 0.02
Glomeromycota	*Glomeromycetes*	0.16 ± 0.04	0.13 ± 0.05	0 ± 0	0.37 ± 0.09	0.51 ± 0.16	0.03 ± 0.02
Mortierellomycota	*Mortierellomycetes*	4.82 ± 1.16	4.41 ± 1.25	2.61 ± 0.92	5.1 ± 0.88	6.34 ± 1.2	3.82 ± 1.39
Rozellomycota	*Rozellomycota_cls_Incertae_sedis*	2.22 ± 0.33	2.4 ± 0.57	0.85 ± 0.25	2.93 ± 0.83	2.46 ± 0.29	1.68 ± 0.62

Table S6.1 Summary of basic sequencing statistics for bacterial and fungal community for single pant species mesocosms.

	Bacteria	Fungi
Number of reads per sample	48872 ± 1417	42332 ± 2401
Unique ASV per sample	551 ± 12	367 ± 14
Phylum identified (%)	99.91 ± 0.01	98.55 ± 0.13
Class identified (%)	99.6 ± 0.03	92.29 ± 0.42
Order identified (%)	97.04 ± 0.1	83.08 ± 0.59
Family identified (%)	75.63 ± 0.32	78.9 ± 0.54
Genus identified (%)	45.19 ± 0.54	73.63 ± 0.64
Species identified (%)	9.21 ± 0.28	58.39 ± 0.84

Mean values ± s.e.

Table S6.2 Proportion of total reads of specific ASVs for single plant species mesocosms.

Individual ASV total proportion (%)	Proportion of total reads greater than ASV proportion level (%)	
	bacteria	fungi
5	12	16
1	32	53
0.1	75	85
0.01	95	97
0.001	100	100
0.0001	100	100

Proportions of ASVs were calculated using rarefied data.

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