ASSESSING THE MINERAL NUTRIENT FLOW AND MICROBIAL ACTIVITY IN PASTURE SOILS

by

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May / 2020

A dissertation submitted to the University of Bristol in accordance with the requirements for award of the degree of Master of Science by Research in the Faculty of Science

Word count: 20930

Abstract

Soil is a mixture of various mineral and organic nutrients that serve as the primary nutrient base for plants and animals. The fate of these nutrients and their distributions in soil reflects the soil environmental quality. Microbial communities are particularly important for ecosystem dynamics as they are involved in biogeochemical cycling of micronutrients. However, their distribution through the soil profiles and size vary with the several physical properties such as depth of soil, soil pH and organic matter. Because microbial communities may be characterised by phospholipid fatty acid (PLFA) and glycerol dialkyl glycerol tetraether (GDGT) lipid signatures, the analysis of these lipids can provide information about the structure of microbial communities in soil.

This thesis reports the results of a study about the microbial community structure and micronutrient availability in soils of identical origin but with different soil physical properties. The main aims of this investigation were to: (i) correlate the changes in microbial communities with the distribution of micronutrients in soil profile, and (ii) assess the effect that grass root type has on the vertical distribution of micronutrients and microbial activity, and (iii) the effect of soil pH on nutrient availability and microbial community size and composition whilst incubated with sheep-manure.

Overall, the topsoil supported a larger microbial community which decreased with soil depth. However, the grass root type affects the microbial community and micronutrient distribution with deep-rooted grasses providing a distribution of nutrients through the soil profile. Exogenous manure addition to the soil affects the microbial community diversity by shifting the soil pH and large changes were observed in low pH soils because of the initial pH difference between soil and manure. Soil pH was observed to have a strong effect on the available micronutrients even in the control soils such that acidic soils are likely to have higher Cu, Fe, and Zn concentrations and lower Ca, K, Mg, and Mn concentrations, whilst the overall microbial community size does not change that specifically with soil pH.

Author’s Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's *Regulations and Code of Practice for Research Degree Programmes* and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED: ..... TUĞBA ISIK ..... DATE:..... 02.07.2020 .....

Acknowledgement

Firstly, I would like to thank my supervisors, Dr Ian D Bull and Dr Heather L Buss, for their instructive comments, confidence and providing me a suitable atmosphere for doing this research. Moreover, I would like to thank to my examiners, Prof Richard Evershed from University of Bristol and Dr Pete Maxfield from University of the West of England, for their improvements of my thesis with their valuable comments and corrections.

Many thanks to all members of Organic Geochemistry Unit for their help and guidance. I particularly thank Jerome Blewett and Dr Michaela Reay for their assistance in showing me the experimental procedures, and Dr Helen Whelton for her constant support when using the GC-MS. Outside the OGU, I would like to thank to Rothamsted Research and Mandy Kao for providing my samples, Dr Adam McAleer for his help using the ICP-OES, and Radim Sarlej for his help with inorganic extractions.

My MSc was funded by The Republic of Turkey Ministry of National Education YLSY Grant and I thank the Turkish Government for financially supporting me during this period.

Last, but not least, I would like to thank my family and my fiancé Ali. I would never have been able to finish my thesis without his continued support during this period.

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Abbreviations

BDS	Bligh-dyer solvent
brGDGT	Branched glycerol dialkyl glycerol tetraethers
C	Carbon
DCM	Dichloromethane
Defra	Department for Environment, Food and Rural Affairs
DMC	Dry matter content
F/B	Fungi-to-bacteria ratio
GC-MS	Gas chromatography – mass spectrometry
GDGT	Glycerol dialkyl glycerol tetraether
ICP-OES	Inductively coupled plasma – optical emission spectrometry
IPA	Isopropanol
IP	Isoprenoidal
IS	Internal standard
isoGDGT	Isoprenoidal glycerol dialkyl glycerol tetraether
LMWOA	Low molecular weight organic acids
NWFP	North Wyke Fam Platform
PB	Phosphate buffer
PLFA	Phospholipid fatty acid
TC	Total carbon
TLE	Total lipid extract
TN	Total nitrogen
TOC	Total organic carbon
TS	Total sulfur
WHC	Water holding capacity
% MC	Percentage moisture content

1. INTRODUCTION

1.1. The Importance of Soil Health and Soil Parameters

Soil is one of the essential parts of the Earth System, being an important interface between the reservoirs of the Earth with between 8-20 % of the terrestrial surface being grassland soil. It plays critical roles in terrestrial ecosystems in terms of nutrient cycling, water filtration, climate moderation and carbon sequestration, with grassland soils contributing more than 10% of the total biosphere C storage (Jones and Donnelly, 2004; Staddon, 2004). Ecological equilibrium, soil functionality and an ability to maintain a balanced ecosystem with high biodiversity are all related to soil health (Cardoso et al., 2013). However, substantial increases in human activities and intensive land-use have resulted in soil degradation and soil erosion leading to depletion in soil organic matter (SOM) and nutrient contents globally. Respiration and leaching also contribute to a decline in the soil C content and a decline in the soil nutrients has been recently observed in most agricultural soils (Ribeiro et al., 2010).

Microorganisms, nutrients, and SOM are three critical components of soil. The physical, chemical and, biological properties of terrestrial ecosystems depend on these components and their interactions have enormous impact on terrestrial processes. SOM is particularly important as a means of providing a source of nutrients for organisms and the capacity to hold water by binding the soil particles into aggregates (Wood et al., 2016). The total amount of SOM is influenced by soil properties and the quantity of annual inputs of plant material or animal excreta. SOM releases nutrients in a plant-available form until decomposition by providing a nutrient cycling system. To maintain this cycling, the rate of organic matter (OM) input must be equal to the rate of decomposition (Bot et al., 2005).

The microbial activity of soils and turnover rate of SOM is variable because of the chemical, physical and biological processes in different soils (Post et al., 2000; Jones and Donnelly, 2004). Soil texture, surface area, bulk density, porosity / pore size distribution and temperature are the primary physical determinants of the soil quality. It is demonstrated that the relationship between SOM and soil texture. According to literature findings, clay and silt protects C against the degradation by microbial communities in the soil (Hassink, 1997). The turnover rate of SOM depends on its protection in the soil (Dungait et al., 2012).The surface area of soil also affects turnover rates due to the reaction of minerals with SOM to form

organo-mineral complexes since higher surface area results in better protection of SOM (Lal, 2016).

The movement of microbial communities affected by pore structure of soils that the hierarchical pore structure facilitates the movement of these communities by resulting in an increase in SOM turnover rate (Six et al., 2004). In addition, the increase in temperature changes often stimulate SOM turnover rates in organic layers although mineral soils are not affected to a great extent (W.K. Lauenroth et al., 2013; Yang et al., 2017). The dark colour of soils is associated with the higher SOC content. Because the dark colours can absorb more heat than the lighter ones, the decomposition of SOM increases by increasing the soil temperature in organic layers (Schulze et al., 1993; Khvorostyanov et al., 2008). Together with physical parameters, there are some chemical parameters that affect turnover rates. Soil pH is one of the key parameters that influences the protection of SOM in soils because of its direct correlation with nutrient availability and microbial activity. For instance, the addition of organic materials increases both SOC content and pH of soil due to the 'liming effect' (McCarty et al., 1994). The alkaline pH of soils decreases chemical protection due to the lower adsorption of SOC by minerals (Mayer et al., 2001; Haynes, 2005). The stabilization of SOM is a function of the cation exchange capacity of soils and the presence of cations in the soil matrix because of the capability of mineral surfaces to adsorb SOM. It is showed that Ca cations protect SOM from mineralisation by reducing the solubility of organic C (Baldock et al., 2000). In the last years, there has been increasing interest about the contribution of soluble organic matter to the cycling and leaching of nutrients, which are inherently bound to organic substances in soil, such as N, P and S. Dissolved organic matter (DOM) is responsible for mobilising substantial amounts of these nutrients and controlling their transfer to aquatic systems (Kaiser et al., 2001).

OM input into soils can be achieved by manure or compost addition; both are organic sources of nutrients and enhance soil quality. The addition of these organic sources changes the biological aspects of soils in terms of the microbiological community. These communities support nutrient cycling, pathogen suppression and stabilization of soil aggregates (Mohammadi et al., 2011). Soil microorganisms are of great importance for plant nutrition because they degrade OM through the production of enzymes, and they enhance the enzymatic activity of soils (Mohammadi et al., 2011). The carbon cycle within the terrestrial ecosystem is dominated by the balance between photosynthesis and respiration and soil microbes facilitate the transformation of C and N between the environmental

compartments(Zhao et al., 2012). Thus, soil microbial biomass becomes an important indicator of soil health because of its contribution to the C cycle and the presence of microbial products in the soil.

In summary, the health of soil rests on ecological balance and the capacity of a soil to maintain the biodiversity below and above the surface. For the verification of soil health, some physical, chemical, and biological properties must be followed within a desired timescale. Whilst moisture, porosity, bulk density, aggregation and, soil texture are physical indicators, total C, N and P amounts, SOM, mineral nutrients and, cation exchange capacity are the chemical indicators for soil health. In addition, biological indicators such as soil enzymes, microbial biomass/population, and soil respiration can be used to make inferences about soil health (Cardoso et al., 2013).

1.2. Soil Types, Profiles and Horizons

Soil is the product of weathering process and can be categorised into six types with different characteristics, which are clay, sandy, silty, peaty, chalky and loamy (Kauranne, 1992). Clay soil is a heavy type of soil because of its rich nutrient content. They have a few pores and hard to cultivate. On the contrary to clay, sandy soils are easy to cultivate and lack of nutrients. Silty soils are well-drained soils and rich in nutrients. They retain moisture and in terms of cultivation, they are found between clay and sandy soils. Peaty soils are very rich in terms of organic nutrients due to their acidic nature, which improves the decomposition, but they lack nutrients. They have a dark colour and proper soils for plant growth. Conversely, chalky soils are alkaline and they are not suitable for plant growth because of plant unavailability of manganese and iron ions. Loamy soils are the perfect ones for plant growth because of their rich nutrients, being well drained and easy to cultivate and moisture retaining properties (Weil et al., 2017).

A soil profile is the vertical cross section of soil comprising different layers of soil whose physical, chemical, and biological characteristic differ from each other, these layers are called horizons. The differences arise from environmental parameters such as parent material, slope, vegetation, weathering, and climate. As a result of the breakdown of bedrock or deposition of geological materials by wind, water or ice, the accumulation of regolith occurs during the soil profile. Subsequently, regolith undergoes changes during the formation of soil from the parent material characterised by four different types of processes: transformations,

translocations, additions., and losses (Figure 1.1a). *Transformations* include the chemical of physical modification of soil constituents or the decomposition of plant roots to form SOM. *Translocation* is the lateral movement of inorganic and organic material, it has a major influence on soil formation. Introduction of exogenous materials to the soil profile *via* manure, fertilisers or accumulation of dust is considered an *addition*. Lastly, materials lost from the soil profile by leaching, erosion, or volatilisation, are classified as *losses*. Soil formation starts with the addition of litter and root residues to the surface layers of the parent material. This followed by the transformation of these litters by soil organisms into SOM, which increases the water holding capacity of the nascent soil and provides the required nutrients for fauna such as earthworms, ants, and termites to subsist. Thus, the transformations and translocations of OM start by releasing the mineral materials into soil (Weil and Brady, 2017).

Soil horizons are generally distinguished by obvious physical properties such as colour and texture, and they are defined by codes as shown in Figure 1.1b. However, all named soil horizons are not always found in every soil. For instance, the *E horizon* occurs rarely in soils developed under grassland (Weil and Brady, 2017). The capital letters refer to the master soil horizons and where sub-horizons also occur within master horizons they are designated lowercase letters following the capital master horizon letter. The *O horizon* refers to the organic matter layer comprising of plant and animal residuals. The upper part of this horizon is relatively undecomposed while the lower part may be strongly humified.

The *A horizon* forms from the mixture of organic and mineral fractions near to the soil surface and is generally referred to as topsoil. Individual soil particles come together to form granules and its darker colour differs from those of the original parent material. In addition, due to weathering some oxides and clay minerals are formed and this horizon loses clays and other minerals over time because of leaching. The *E horizon* is designated as the zone of maximum leaching of clay, iron and aluminium oxides and it usually found exhibits a lighter colour. However, the *E horizon* generally occurs in forest soils, which has high amounts of rainfall, and develops occasionally under the *O horizon*. The *B horizon* is considered a ‘zone of accumulation’ because leached materials from the *A* and *E horizons* accumulate within it. This horizon resides below *O, A or E horizons* and comprises less organic matter and more clay / inorganic material than the *A horizon.* However, the *B horizon* can become a part of topsoil in regions which possess shallow *A horizons*. Whilst the *B horizon* in humid regions comprises iron and aluminium oxides, calcium carbonate or calcium sulphate may

predominate in arid and semiarid regions. The colour of the *B horizon* is derived from these oxide and clay materials, which are the results of weathering. The *C horizon* consists of deposits and weathering of residual bedrock. This horizon is enriched with carbonates carried by leaching and it is generally considered the parent material of the soil. The *R horizon* denotes the layer of partly weathered or unweathered bedrock and occurs at the base of the soil profile (McDonald, 2009; Weil and Brady, 2017). When the *C horizon* has formed via weathering of the bedrock below, the *R horizon* is, in effect, the soil parent material.

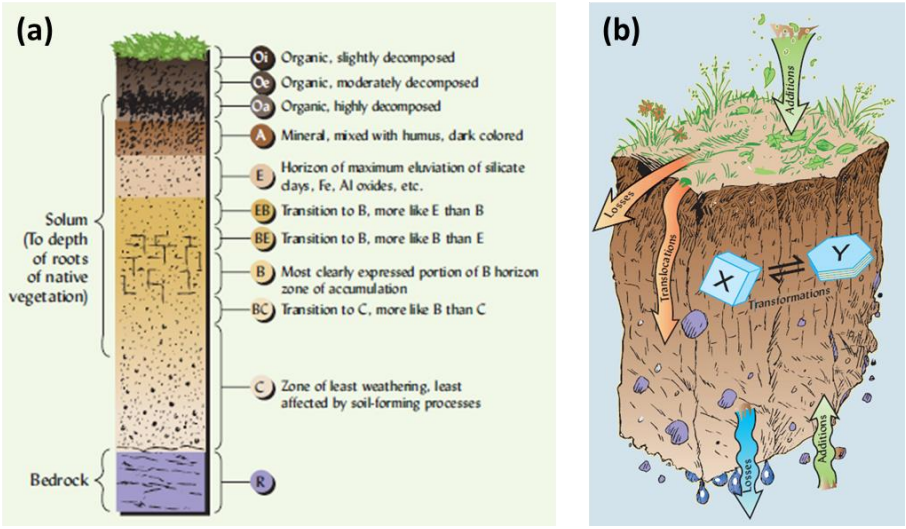

Figure 1.1 (a) Schematic illustration of driving factors for soil-profile development and (b) cross-section of a soil indicating soil horizons (Weil and Brady, 2017).

Most studies in the literature have focused on the analysis of topsoil, in the *A Horizon*, where the densities of some nutrients and microorganisms are highest. However, soil profiles are often many meters deep and large quantities of nutrients and microorganisms reside in subsurface horizons (Fierer et al., 2003). There are still some remaining questions about the general vertical distribution of SOC and mineral nutrients, the effect of vegetation type on SOC in deep soil layers and the major determinants of SOC content at different depths. Also, the chemistry and hydrology of soil changes at with depth because of the production of mineral nutrients via chemical weathering at the rock-soil interface. Different management practices and different land use types affect the vertical distribution of C, N and P nutrients in the soils (Chai et al., 2015). Climate and soil texture have been shown to be important determinants of the amount SOC present were the association of SOC with precipitation and temperature was observed closest in the top 20 cm of soil and decreased with depth (Jobbagy et al., 2000). However, vegetation has a major effect on the vertical distribution of SOC

(Jobbagy and Jackson, 2000). Zhao et al. showed the effect of vegetation cover and soil environment on the accumulation and distribution of SOC and SIC with depth. They claimed that low the C/N ratio of plant litter is favourable for SOC formation for three different vegetations (forest, shrub and grass) because high N content in the litter can stimulate microbial activity which results in faster decomposition of litter in SOM. According to vegetation type, decomposition of plant litter increased in the order of shrub > forest > grass correlating with the decrease in C/N ratio (Zhao et al., 2016). Jobbagy et al. hypothesized that vegetation type is the major determinant for the relative vertical distribution of SOC. Their results showed that the relative distribution of SOC in the first meter of soil was deepest in shrublands, intermediate in grasslands, and shallowest in forests (Jobbagy and Jackson, 2000). Furthermore, recent studies have demonstrated that vegetation type has a remarkable effect on the rooting patterns. While temperate forests have an average rooting depth of 3.7 m, desert vegetations reach a maximum rooting depth of 13.4 m to reach and absorb water where the deep roots gather and transmit the water to the top of the plant (Schulze et al., 1996). .

There are some mechanisms that affect the depth at which nutrient inputs occur such as weathering and atmospheric deposition. Leaching moves nutrients downwards and enhances the concentration of nutrients at depth. Conversely, biological cycling results in the movement of nutrients upwards due to the transportation of some nutrients aboveground and recycling on the soil surface by litterfall (Jobbagy et al., 2001).

1.3. Mineral Nutrients in Soils

Micronutrient deficiency is one of the most important causes of human morbidity and mortality. Human existence requires essential micronutrients (e.g. Fe, Zn, Cu, Mo, I, F, B, Se, Ni, Cr) and macronutrients (e.g. N, P, K, Ca, Mg, S, Cl) to meet metabolic demands; all these macro-/micronutrients must be supplied through soil (Lal, 2016). Micronutrients are the nutrients that the body needs in small amounts whilst macronutrients are required in large amounts. Today micronutrient deficiencies in various foods have significantly increased because of liming and leaching applications, loss of soil from erosion, as well as utilisation of high yield fertilisers instead of animal manures or plant residues (Gupta et al., 2008). Thus, the health of soil is important for the presence of nutrients with the obvious ramifications for human health. However, the total level of micronutrients is not a good indicator for their

availability to plants. Both the density and bioavailability of micronutrients are critical to achieving optimum nutritional status (Miller et al., 2013).

All plants require some specific elements to maintain their life cycle, these being: C, H, O, N, P, K, S, Ca, Mg, B, Cl, Cu, Fe, Mn, Mo, Ni and Zn. While some of these elements such as C, H and O are provided from air and water, plants derive the remaining 14 elements from soil, rain water or through additional amendments (Singh et al., 2015). These elements split into two groups according to their required amounts for plant growth as macronutrients (N, P, K, S, Ca, Mg) and micronutrients (B, Cu, Fe, Mn, Mo, Ni, Zn) (Mahler, 2004).

In order to improve the nutrient availability to crops, farmers use fertilisers to provide essential nutrients. In addition of fertilisers does not only affect the crop yield but also the physicochemical properties of the soil matrix (Saha et al., 2008). Fertilisation applications enhance soil microbial activity and biomass by increasing the SOM content (Welch, 2002). However, the status of micronutrients in soils is primarily affected by fertilisation because fertilizer application may change pH, calcium carbonate ($CaCO_3$) levels, physical structure and water holding capacity of soils. For example, changes in soil pH may result in a transformation of non-available micronutrients to an available form (Neilsen et al., 1986). Micronutrient fertilizers (Mg, K, P, Ca) affect the available forms of micronutrients in soils. Liming with $CaCO_3$ reduces soil acidity and increases Ca availability but it can reduce the uptake of certain micronutrients such as Zn, Cu, Fe, and Co. The formation of carbonate or phosphate can cause micronutrient cations to precipitate out of the soil and decreases their availability. This process is largely governed by pH by changing the release and desorption of adsorbed nutrients (Wei et al., 2006). Alternatively, gypsum or elemental sulfur amendments can increase available Fe, Mn, Zn, Cu, and Co by reducing the soil pH (Miller and Welch, 2013).

The physical structure of soils affects not only the protection of SOM but also the nutrient adsorption and desorption dynamics. Literature findings demonstrated the increase in phosphorous release with the decreasing aggregate size of soil matrix due to the limited accessibility of leaching solution to adsorption sites, i.e. lower surface area of larger aggregates (Wang et al., 2001).

Soil is the main source of trace elements for plants and transfer of trace elements between soil and plants is a vital part in cycling of nutrients. The mobility of trace elements depends on weathering processes and cation exchange capacity (CEC) of soil and the specific surface

area of soil correlates with CEC for trace elements (Kabata-Pendias, 2004). In addition, the condition of soil plays a crucial role on the availability of trace elements. While in acidic soils Cd and Zn are easily available to plants, in neutral or alkaline soils they become less available (Kabata-Pendias, 2010). Se is an important trace element for humans, animals, and plants. It is a component of selenoproteins as selenocysteine with some enzymatic activities such as redox function, which maintains membrane integrity (Rayman, 2000). Additionally, numerous studies have demonstrated the anticancer activity of Se compounds (Clark et al., 1996; Reid et al., 2002; Whanger, 2002). High levels of Se can be toxic and can cause deformities whilst its deficiency in soils results in heart diseases, hypothyroidism and weak immune system for human (Combs, 2000). Although Se is mainly found in insoluble elemental and selenide forms, the selenate (SeO_3^{2-}) and selenite (SeO_4^{2-}) forms are responsible for its activity in soils (Munier-Lamy et al., 2007; Shand et al., 2012). These forms of Se, especially selenite, are adsorbed on clay and oxide minerals, and accumulation of Se in soil may be related to the amounts of reactive surfaces (Shand et al., 2010). Iodine is another essential micronutrient for human health and its deficiency affects the production of thyroid hormones (thyroxine and triiodothyronine), which regulate the biochemical processes in metabolism (Zimmermann et al., 2012). Nearly 1.9 billion people worldwide are at risk of iodine deficiency disorders (Zimmermann et al., 2015). If iodine deficiency occurs during fetal development or pregnancy, the consequences can be stillbirths and myxedematous or neurologic cretinism (Bath et al., 2015). Iodine cycling is associated with the soil characteristics such as pH, presence of SOM and metal oxides. Soil pH acts on the initial adsorption of iodine ions and SOM influences the time-dependent sorption (Shetaya et al., 2012).

1.4. Analysis of mineral nutrients

The phytoavailability of metals in soils is a consideration but determination of the total soluble fraction of soil metal content is not enough to ascertain the availability of the nutrients to plants and risks the potential of soil contamination. There are a variety of abiotic and biotic parameters for phytoavailability such as adsorption onto and desorption from mineral surfaces, pH and precipitation (Menzies et al., 2007). Different extraction methods, analytical procedures and instruments are required to measure specific nutrients and forms of those nutrients. One-step extractions with solutions of chelating agents, mineral acids and neutral salts are frequently used due to their simplicity and ease of applicability.

Diethylenetriaminepentaacetic acid (DTPA) and ethylenediamine tetraacetic acid (EDTA) behave as chelating agents and solubilise the metals, which are in sorbed and bound phases (Ure, 1990). The mechanism based on the use of neutral salts, such as calcium chloride and sodium nitrate, is the displacement of cations with the ones located on mineral surfaces (Menzies, Donn, and Kopittke, 2007). However, these extractants are only useful under defined conditions and do not consider the soil – plant interactions in the rhizosphere zone (Feng et al., 2005). The rhizosphere zone is defined as a point for root–soil–microbe interactions and is a nutrient-rich environment for soil microbes. The roots of plants take water and nutrients from soil and return rhizodeposits including water-soluble exudates, dead fine roots, gases, and secretions of insoluble materials (Cheng et al., 2007). The microbes take part in root–soil interactions and soil–microbe interactions in the rhizosphere are mediated by roots (Berendsen et al., 2012; Zhang, Vivanco, et al., 2017).

Alternatively, acid leaching has become a widespread method in soil digestion procedures and aqua-regia is one of the most widely used acid leaching methods. This extraction method involves using a mixture of nitric acid and hydrochloric acid and refluxing the soil with this mixture. However, this method cannot be used to dissolve every metal ion from soils. While aqua-regia might recover Cd, Cu, Pb and Zn from soils, some metal ions, particularly Co, Cr, Cd do not dissolve at high yield (Sastre et al., 2002).

Mimicking real field conditions is an alternative way for the prediction of bioavailability of nutrients in soils (Wang et al., 2003; Mucha et al., 2010). Phytoextraction methods are gaining interest as a means to extract metals from soils in order to get reliable information about the bioavailability of nutrients because nutrients can be found in different plant-available forms in soils (Chen et al., 1996; Wang et al., 2003). Low molecular weight organic acids (LMWOAs) are used in many soil and plant processes. Root exudates are the primary source of organic acids and can occur in any soil horizon with root activity due to the loss of compounds from root or active exudation of organic compounds such as LMWOA (Mucha et al., 2010). These dissolved organic molecules, which arise from the breakdown of plant residues and exudation of plant roots, create complex structures by reacting with labile metal complexes in the soil solution. The nutrient content of extracted solutions after each of these processes can be determined by inductively coupled plasma optical emission spectroscopy (ICP-OES), inductively coupled plasma mass spectrometry (ICP-MS), ion chromatography (IC), and flame atomic absorption spectroscopy (FAAS), instruments widely used for the

determination of mineral nutrients in soil samples, although each has its own disadvantages (Table 1.1).

Table 1.1 Comparison of advantages and disadvantages of analytical techniques for nutrient analysis.

Method	Abbreviation	Advantages	Disadvantages	Ref
Flame Atomic Absorption Spectroscopy	FAAS	Accurate and precise	Expensive	(Taylor, 1999)
Inductively Coupled Plasma Optical Emission Spectroscopy	ICP-OES	High matrix tolerance, Easy to operate	Unable to measure low concentrations	(Spivey et al., 2016)
Inductively Coupled Plasma Mass Spectrometry	ICP-MS	Ability to measure low concentrations, Sensitive	Expensive, Spectral and non-spectral interferences	(Spivey and Neubauer, 2016)
Ion Chromatography	IC	High matrix tolerance, Predictable elution patterns	Limited to ionizable group, Inconsistency from column to column	(Barghouthi et al., 2012)

1.5. Organic Nutrients in Soils

Soil C pools consist of two distinct components: soil organic carbon (SOC) and soil inorganic carbon (SIC). The organic carbon storage capacity of soils is a key function for both climate regulation and other soil functions such as topography, parent material, organisms, depth of soil and land use (Jones and Donnelly, 2004; Wiesmeier et al., 2019). Land use is one of the most dynamic factors for SOC alteration in that grassland has generally higher SOC storage than forests and croplands although climate conditions can affect the overall storage capacity of any particular land-use regime (Guo et al., 2002; Rossel et al., 2014). The microbial

community in soil also affects SOC storage since it decomposes the OM (Schimel et al., 2003).

It is widely known that SOC sequestration takes place by the physical entrapment of aggregates. Formation of stable macroaggregates and organo-mineral complexes can protect SOC against microbial processes for years and maintenance of SOC is essential for soil structure, water retention, nutrient retention and gaseous emissions (Lal, 2016). These aggregates can be found in micro (< 2mm) or macro (≥ 4mm) forms, which are associated with long-term (≈ 40 - 70 days) and short-term (≈ 7 - 14 days) carbon storage, respectively (Bol et al., 2004). However, understanding the fate of C residues in soils is quite difficult because of the difficulty of differentiating between extraneuous OM amendments and pre-existing SOM (Dungait et al., 2010). To understand the C dynamics in the environment, bulk stable isotope determinations have been used but these methods can be used only for estimation due to the contribution of a variety of individual components to bulk $\delta^{13}C$ values. Alternatively, it is deminstrated that the use of a compound specific stable isotope ratio ($\delta^{13}C$ values) mass spectrometry (IRMS) method which enabled dung-derived OM and SOM to be differentiated and dung-derived C turnover determined (Dungait et al., 2010).

1.6. Components of SOM

SOM refers to all organic carbon containing substances in the soil that range from plant litters and microbial remains to highly polymerised products. SOM can be divided into two major parts which are non-humic and humic substances. The non-humic part comprises carbohydrates, proteins, amino acids, alcohols, aldehydes, ketones, lignin, vitamins, enzymes, lipids, and alkaloids whilst the, humic part consists of polydispersed, acidic, and amorphous substances with high molecular mass. Because polysaccharides and lignin are the major organic components found in plant litter, they are commonly used as predictors to understand the decomposition dynamics of the plant litter. Plant litter is the primary source of organic matter to soils and can be divided into different classes such as storage materials (intracellular and structural components in membranes) and extracellular or cell wall components.

Proteins, starch and chlorophyll are intracellular storage materials. Proteinaceous C consists of long chains of various amino acids and may also exist as shorter chain length polypeptides, whilst the subunits of starch comprise of lower molecular mass polymers such amylose and amylopectin, all of which are built from the monomer glucose (Kogel-Knabner, 2002). The

relative abundances of different amino acids provide information on the origin and stability of nitrogen-containing compounds (Poirier et al., 2005). Polysaccharides, lignin, tannins, lipids, cutin and suberin are all components that are found in plant cell walls.

Cellulose is also the most abundant plant cell wall polysaccharide and after deposition on soil, a high percentage of cellulose can remain for up to five years (Paustian et al., 1992). The non-cellulosic polysaccharides such as hemicelluloses have different compositions of sugar monomers, which are bound together with a range of different glycosidic linkages, and overall have a lower degree of polymerization than cellulose (Dungait et al., 2009).

Although lipids constitute a minor amount of SOM, many of them are highly recalcitrant and important soil biomarkers such as PLFAs (Poirier et al., 2005). Lipids are loosely defined as organic substances that are insoluble in water but can be extracted by using non-polar solvents. They are found both in plants and microorganisms, and the level of them usually decreases with increasing the depth of soil. Soil lipids are resistant to biodegradation relative to other small molecular components of SOM, e.g. amino acids and monosaccharides, although they can be mineralized eventually. The levels of lipids in soils results directly from processes such as addition of plants, microbial synthesis, and degradation. Moreover, their hydrophobic properties have a net positive effect on soil aggregation and aggregate stability although they also promote some negative effects such as low water retention. Lipids in soil originate predominantly from plants and microorganisms, whereas soil animals have a more minor contribution. Table 1.2 represents the occurrence of various lipid classes in plants and microorganisms (Kogel-Knabner, 2002; Bollag, 2017).

1.7. Microbial biomass profile of soils

The composition and activity of soil microbial biomass largely determines biogeochemical cycling and the turnover processes of OM; it can be a rapid indicator of soil quality Several methods can be used to estimate the amount of microbial biomass in soil but few of them can differentiate between different groups of microorganisms. Biomass of different organism groups can be estimated by measuring specific substances and there are two common methods for the examination of microbial populations: using ribosomal RNA and PLFA analysis (Frostegard et al., 1996).

Table 1.2 Occurrence (×) of various lipid classes in plants and microorganisms. (Kogel-Knabner, 2002)

Lipid class	Plants	Microorganisms
N-alkanes	×	×
Branched alkanes	×	×
Olefines	×	×
Cyclic alkanes	×	
Monoketones	×	×
β - diketones	×	
Secondary alcohols	×	×
Alcandioles	×	
Free fatty acids	×	
Primary alcohol esters	×	×
Triesters	×	
Primary alcohols	×	×
Aldehydes	×	
Terpenoids	×	

1.8. Phospholipid fatty acids (PLFAs)

Phospholipids are essential membrane components of all living cells and are not found in storage products or dead cells. Whilst straight-chain fatty acids generally denote eukaryotes, branched-chain analogues are indicators for Gram-positive and sulfate-reduced Gram-negative bacteria (Haack et al., 1994). Moreover, Gram negative bacteria contain hydroxy acids in the lipid portion of lipopolysaccharides in their cell walls and methyl branching on the tenth carbon atom is specific for actinomycetes (Kroppenstedt, 1992). Branched-chain, cyclopropane and β-OH fatty acids are unique to bacteria among the other organisms. PLFA analysis can provide a quantitative description of the microbial community and its composition; determined chain length, saturation, and branching. PLFA analysis can be applied to characterise and quantify microbial biomass, to provide insights into the functional status of the microbial community and as biomarkers of community structure. In the literature good correlations have been found between the concentration of PLFAs and microbial

biomass. In addition, Zak et al. demonstrated that plant diversity affects the microbial community soil due to the differences in biochemical composition of different plant species (Zak et al., 2003).

The extraction of PLFAs from soil samples is generally achieved by using the Bligh-Dyer extraction method, which isolates the total lipid fractions from biological matrices based on a triple solvent system (Bligh et al., 1959). Post-extraction, the extract is fractionated into simple lipid, glycolipid, and phospholipid fractions. The latter fraction is isolated and then subjected to mild alkaline methanolysis. The resulting fatty acid methyl esters (FAMEs) are quantitatively analysed by GC-MS with the resultant chromatogram providing the relative abundance of each PLFA present in the cell membranes of the soil microbial community (Willers et al., 2015). This information about PLFA distributions, i.e. varying chain length, saturation and branching, can then be used as a 'fingerprint' of microbial community (Steer et al., 2000). A calculation of soil microbial biomass, comprising bacteria and fungi may also be made using the total concentration of PLFAs (Baath et al., 2003). Specific PLFAs are indicative of different taxonomic groups for PLFAs where the 14:0, 15:0, 16:0, 17:0, 16:1ω5c, 16:1ω7c, 16:1ω9c, 17:1ω8c, 18:1ω5c, 18:1ω7c, a15:0, a17:0, cy17:0, cy19:0ω8c, i14:0, i15:0, i16:0, i17:0 and i19:0 components are bacterial biomarkers. Whilst the 18:2ω6,9, 18:1ω9 and 18:3ω6 components are used as a measure of fungal-specific biomarker (Frostegard and Baath, 1996). Some specific branched PLFAs, such as 10Me-16:0, 10Me-17:0 and 10Me-18:0, are used to identify actinomycetes. For the gram-positive and gram-negative bacteria biomarkers 14:0, 17:0, 18:0, i14:0, i15:0, i16:0, i17:0, a15:0, a17:0 and cy17:0, cy19:0ω8c, 16:1ω5c, 16:1ω9c, 16:1ω7c, 17:1ω8c, 18:1ω5c, 18:1ω7c are taken into account, respectively (Zhang et al., 2015). The representative structures of different PLFAs are given in Figure 1.2.

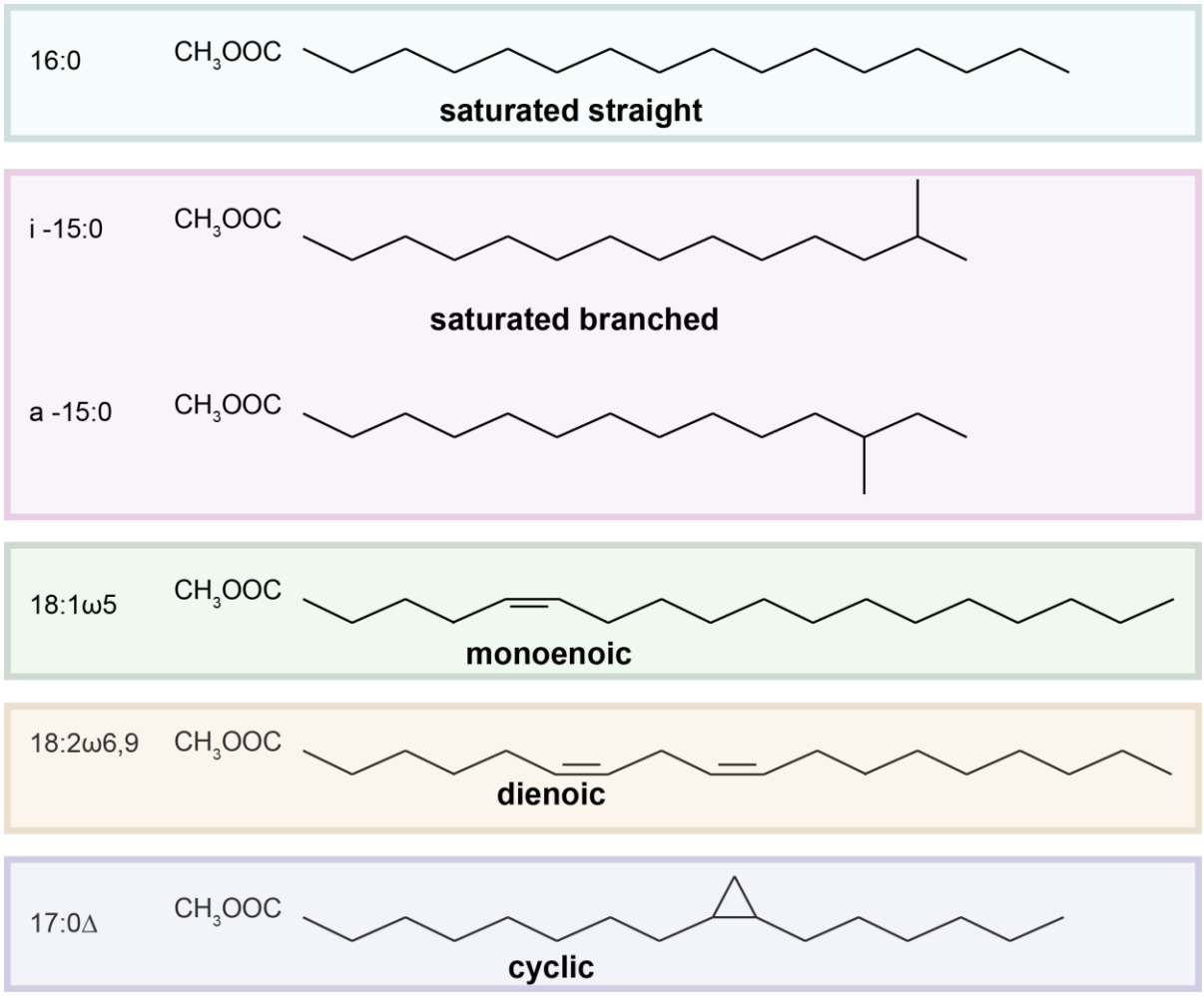

Figure 1.2 The representative structures of saturated straight-chain, saturated branched-chain (*iso* and *anteiso*), monoenoic, dienoic, and cyclic fatty acid methyl esters.

1.9. Glycerol dialkyl glycerol tetraether lipids (GDGTs)

Glycerol dialkyl glycerol tetraether (GDGT) lipids, synthesized in the membranes of Archaea and some bacteria, are a class of lipids with high molecular weights relative to most other lipids. Archaea are the second domain of prokaryotes that can be distinguished from bacteria. Whilst phopholipids typically comprise straight-chain fatty acids bound, *via* ester bonds, to a glycerol, phosphorylated with a polar head group, Archaea can synthesize membrane lipids in with an isoprenoid structure, rather than straight chains, termed isoprenoid GDGT's (isoGDGTs). isoGDGTs consists of two head-to-head C_{40} isoprenoid chains with a varying number of cyclopentane and cyclohexane rings, which are connected by ether bonds to two terminal glycerol groups (Figure 1.3a) (Tierney, 2017). Bacterial membranes are in bilayer form and behave as a barrier to water and ions because of the hydrophobic alkyl chains. Alternatively, Archaea form monolayer lipid membranes that provide more stable ether bonds

rather than the common ester bonds found in bilayer structures. This difference in membrane stability confers the ability to live in harsh extremophilic conditions (Huguet et al., 2006; Huguet et al., 2012). They have been used to define the archaeal community structure based on the currently known lipid distributions of archaeal cultures (Blaga et al., 2009; Schouten et al., 2013). For instance, Crenarchaeol is one of the unique isoGDGTs, it contains a cyclohexyl ring and is considered a biomarker for aerobic ammonia-oxidising Thaumarchaeota (Damste et al., 2002). GDGT-0 is dominant in methanogenic archaea, whereas GDGT-1 / -2 / -3 are synthesized by both thermophilic and mesophilic crenarchaea (Dirghangi et al., 2013).

When the alkyl chains comprising the core structure of GDGTs are branched instead of isoprenoid derived, they classified as branched GDGTs (brGDGTs). brGDGTs were first discovered in peat deposits and are primarily observed to occur in soils (Damste et al., 2000). Their structure is similar to that of isoGDGTs but they possess branched C_{30} alkyl chains with 4-6 methyl groups (Figure 1.3b) (Tierney, 2017). Their presence in soils has enabled the construction of useful environmental proxies such as the cyclisation ratio of branched tetraethers (CBT) and methylation index of branched tetraethers (MBT). CBT quantifies the relative abundance of cyclopentyl rings and is related to soil pH, whilst MBT expresses the degree of methylation and is related to the mean annual temperature (MAAT) and soil pH (Weijers et al., 2007; Schouten et al., 2008; Fawcett et al., 2011). They were suggested to be produced by anaerobic bacteria because of their greater occurrence in deeper and anoxic parts of peat bogs (Weijers et al., 2006). GDGTs in living organisms have polar head groups and are referred as intact GDGTs (I-GDGTs) but the polar head groups are enzymatically cleaved after cell death leaving core GDGTs (C-GDGTs) (White et al., 1979; Huguet, Martens-Habbena, et al., 2010). GDGTs can be detected either with (I-GDGT) or without (C-GDGT) polar head groups using high performance liquid chromatography-mass spectrometry (HPLC-MS), and a wide range of GDGT proxies can be identified. The relative abundance of C-GDGTs has been used as in proxies such as the TEX_{86} temperature proxy (Schouten et al., 2002) and BIT soil carbon proxy (Hopmans et al., 2004). After the separation of I-GDGTs and C-GDGTs using silica gel column chromatography, C-GDGTs can be directly analysed by HPLC-MS coupled with an atmospheric pressure chemical ionisation (APCI) interface. I-GDGTs can be analysed using the same instrument post acid hydrolysis of the lipid fraction (Schouten, Hopmans, and Damste, 2013).

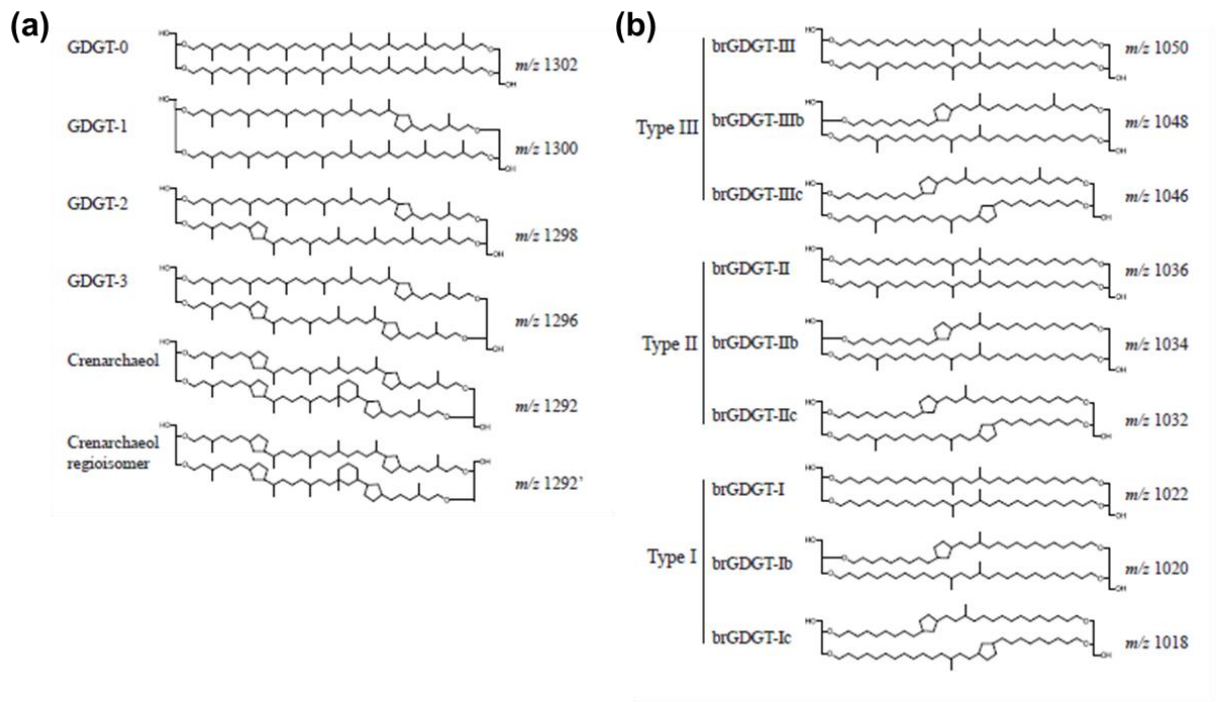

Figure 1.3 Core structures of (a) isoGDGTs and (b) brGDGTs with mass-to-charge ratios (*m/z*) (Tierney, 2017).

1.10. Analysis of organic nutrients

The analysis of total organic carbon (TOC) is achieved by TOC analyses that determines the CO_2 formed when organic carbon is oxidised, and inorganic carbon is acidified. Dissolved organic carbon (DOC) is the fraction of TOC defined as that which can pass through a filter having ranges between 0.22 to 0.7 µm. The remaining fraction on the filter is termed particulate organic carbon (POC). Chromatography is an essential tool for the analysis of the organic components of soil samples and mass spectrometry (MS) coupled chromatography instruments has been extensively used for the analysis of organic compounds in soil. It characterises compounds through a combination of chromatographic retention time, molecular mass, and molecular structure (Hu et al., 2017). Table 1.3 makes a brief comparison between these methods in terms of their advantages and disadvantages.

Table 1.3 Comparison of advantages and disadvantages of analytical techniques for nutrient analysis

Method	Abbreviation	Advantages	Disadvantages
Gas chromatography - mass spectrometry	GC-MS (Garcia et al., 2008)	High reproducibility Ease of use	Long derivatisation steps Unable to thermally labile compounds
Liquid chromatography – mass spectrometry	LC-MS (Garcia et al., 2008)	No need for derivatisation Able to quantify the semi-volatile or non-volatile samples	Columns sensitive to packaging Sample must be soluble
Capillary electrophoresis – mass spectrometry	CE-MS (Whitman et al., 1998)	No need for derivatisation	Narrow application due to migration time fluctuations

1.11. Mechanisms behind the bacteria and fungi mediated soil nutrient bioavailability

The availability of micronutrients in the rhizosphere soil is controlled by plant properties and interactions of plant roots with microorganisms. The soil microorganisms obtain the required nutrients from soil environment to survive and the ability of soil to hold these nutrients is explained by its anion or cation exchange capacity. The physical characteristics of soil, such as the amount of clay and organic matter, determine anion and cation exchange capacity of soil that is directly related with the plant nutrient availability (Magdoff et al., 2004). Figure 1.4 demonstrates the mechanisms between microbial communities and nutrients in soils.

The soil structure is influenced both by mineral nutrients and presence of microbial communities as well as their exudates in soil. Microbial communities, bacteria and fungi, improve the soil structure by formation of pores, which contain numerous active sites for biological and mineral processes, and soil aggregates that are important in terms of water infiltration, aeration and proper soil structure (Degens, 1997; Rashid et al., 2016). However, fungi and bacteria have different mechanisms for the aggregation of soil particles. Fungal microorganisms release extracellular surface polysaccharides to form aggregates whilst bacteria release exopolysaccharides, which form organo-mineral complexes by binding soil particles into aggregates (Gupta et al., 2015). It is known that the rhizosphere soil has 10- to

100-fold higher microbial biomass than the bulk soil because of the steady exudation of organic compounds from the plant roots (Hartel, 2005).

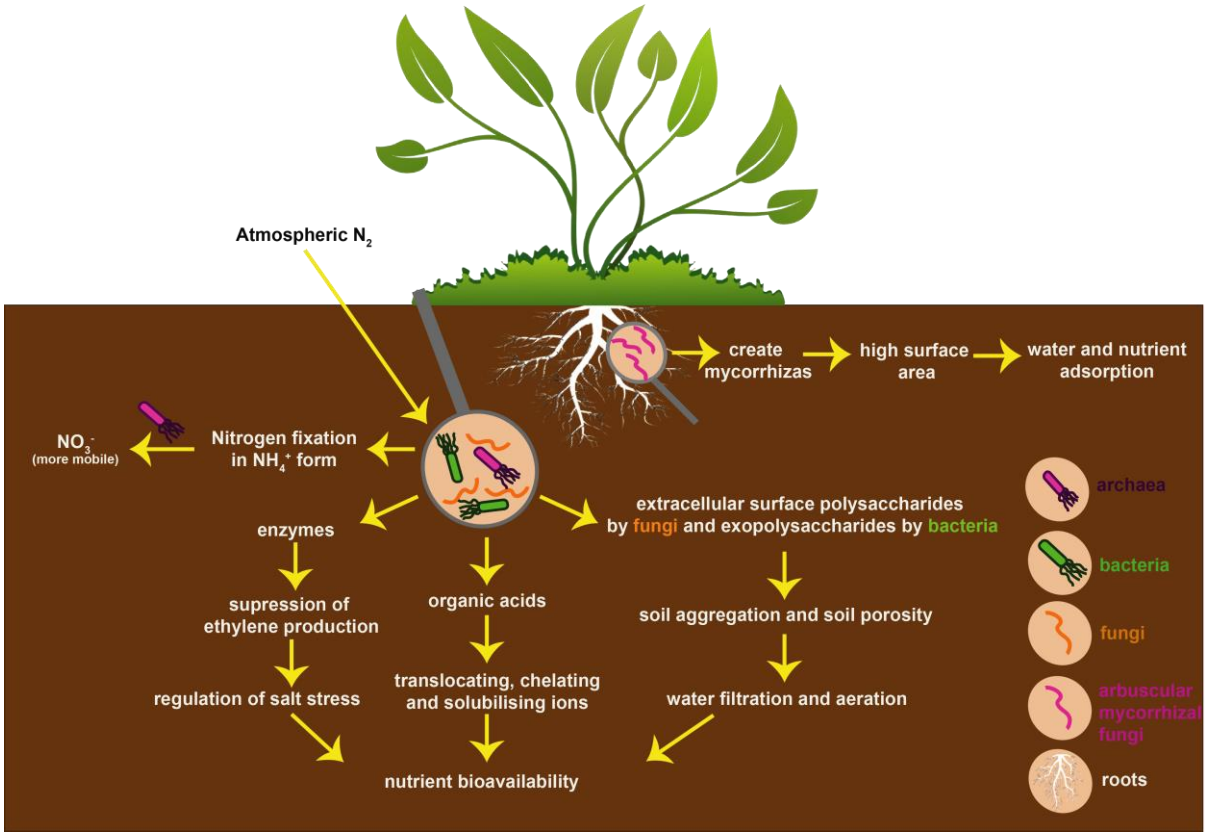

Figure 1.4 The schematic diagram showing the role of microbial communities in root interactions and nutrient availability (Landeweert et al., 2001; Rashid et al., 2016).

Microbes take role for the mineralization, translocation and mobilization of soil nutrients like P, K, and Fe as well as the nitrogen fixation that Arbuscular mycorrhizal fungi contributes the up to 20% of total N demand of grasslands (Owen et al., 2015). Various types of bacteria and fungi produce organic acids, which facilitate the plant uptake of nutrients from rhizosphere by increasing their mobility. For example, bacteria produces siderophores, which are specific compounds to chelate and solubilize Fe, whilst fungi translocate Fe from mineral to organic soil horizon for decomposition and mineralization (Rashid et al., 2016). The arbuscular mycorrhizal fungi are a group of fungal communities and inhabit the roots of plants by creating symbiotic associations called as mycorrhizas. Mycorrhizas affect the nutrient and water adsorption of plants by increasing the surface area of plant root and influences the plant growth (Bonfante, 2001).

The exudation of organic acids through the roots alter the plant nutrient availability by weathering mechanisms (Landeweert et al., 2001). Weathering occurs either by chemically, by transformation of rock-forming minerals into dissolved substances with the effect of water and acids, or by biologically that is mediated by microorganisms. The carbon-rich root exudates facilitate the microorganisms in soils and accelerate weathering of minerals that results in enhanced availability of essential plant nutrients (Landeweert et al., 2001). One of the most well-known weathering processes is dissolution that rocks with magnesium carbonate or calcium carbonate are dissolved by acidic solutions by releasing Mg or Ca ions into the soil. Hydrolysis takes place when minerals interact with acids by producing soluble salts, whilst oxidation breaks the minerals by oxygen and water by improving Fe-rich compounds (Viers et al., 2007). The plant released low molecular weight organic acids behave as chelators for most of the ions in soils. Oxalate, citrate, and malate are the strongest chelators for Al^{3+} and Fe^{3+} ions (Jones, 1998), whilst oxalic acid forms complexes with K, Ca, Mg, Mn, Zn, Cu, Al, and Fe (Gadd, 1999). Moreover, the mycorrhizal fungi species produces oxalic acid and solubilizes calcium phosphates by mobilizing K, Ca, and ammonium species in mineral layers (Paris et al., 1995).

Moreover, salt stress (a huge increase in sodium content) creates a nutritional imbalance that prevents the plant growth by decreasing the available macronutrients (N, P, K, Ca, Mg and S) in soils. Under these circumstances, an efficient root system is required to supply the required nutrient to the plant. However, salt stress increases the ethylene production in plants. Ethylene is an important plant hormone but excess amounts inhibit the root growth (Burd et al., 1998). Rhizobacteria binds the roots of plants, deactivates the ethylene-forming enzyme and enhance the plant growth by reducing the salt stress and increasing plant uptake of nutrients (Nadeem et al., 2009).

1.12. Motivation

Soil is an organic carbon mediated system that provides the environment required for the continuity of life. It comprises a diverse microbial community including archaea, fungi, and bacteria that have distinct effects on the health of plants and animals in the soil ecosystem (Fierer, 2017). Microbial communities play a central role in vital processes in soils such as nutrient flow, carbon sequestration and fertility, as well as transforming organic matter into bioavailable forms for other organisms. Therefore, microbial communities are a key

component of ecosystem dynamics and drive biogeochemical cycles (Falkowski et al., 2008; Gougoulias et al., 2014). However, the abundance of soil microbial communities can vary considerably with the physical properties of a soil. The nitrogen availability and soil organic carbon content of soils has a profound effect on the bacterial community structure (Peacock et al., 2001; Cederlund et al., 2014). Fierer et al. determined the vertical distribution of microbial biomass and specific microbial populations in two soils of deep horizons (up to 2 m). Phospholipid fatty acid (PLFA) composition signatures indicated that the composition of microbial communities changed significantly with the soil depth and the number of PLFAs detected in the soil samples decreased from the surface down to 2 m depth. The vertical distribution of these microbial groups is attributed to the decline in carbon availability with soil depth (Fierer, Schimel, and Holden, 2003). Moreover, fertilization has an indirect effect on the microbial community by altering soil pH. When soils with a broad range of pH values were investigated, the alteration in the composition of bacterial and archaeal communities was clearly observed, i.e. alkaline conditions were found to be favourable for bacteria whilst acidic conditions promoted fungal growth (Hartman et al., 2008; Baker et al., 2009; Rousk et al., 2009; Griffiths et al., 2011).

Phospholipid fatty acids (PLFAs) are membrane-derived lipids the distributions of which can provide a broad ‘fingerprint’ of bacteria and eukarya microbial domains in soils. Similarly, glycerol dialkyl glycerol tetraether (GDGT) lipids are another class of membrane lipids synthesized by a wide range of archaea and some specific bacteria (Schouten, Hopmans, and Damste, 2013). Thus, consideration of their concentrations and composition can provide information about biogeochemical cycles (Naeher et al., 2014; Zheng et al., 2015). In conjunction with the microbial communities, micronutrients are essential components for soil health. Since soil is the main source of trace elements for plants, transfer of trace elements between soil and plants is a vital part in flow of nutrients. Microbial communities improve the soil health by breaking down organic matter to make nutrients available to plants, therefore they are responsible for driving the various cycles of macro- and micronutrients (Sahu et al., 2017). If the role of microbial communities in nutrient flows (such as Fe, S, P, and N) is to be taken into account (Fierer, 2017), comprehensive information about nutrient dynamics needs to be determined. Through this thesis, a fundamental information about the vertical distribution of nutrients and microbial communities in soil and the effect of pH on nutrient availability and the microbial community in sheep manure amended soils will be given.

1.13. Approaches and Aims

The need to follow the soil health in terms of physical, chemical, and biological properties is vital to maintain the biodiversity (Black et al., 2003; Wall et al., 2015). PLFAs are of particular interest as these represent the diversity and size of microbial communities in soils. Soil depth, grass type, soil pH, and manure amendment are some of these properties which affect the biodiversity in soils (Wiesmeier et al., 2019). Microbial communities carry out fundamental processes such as enzyme activities and any activity in these communities has the potential to alter the bioavailability of nutrients (Allison et al., 2007). Thus, understanding the effect of soil depth, grass type, soil pH and manure amendment on the relationship between microbial communities and micronutrients is essential to enhance the plant bioavailability of micronutrients.

The specific aims and hypotheses addressed in this work are:

i. Assessing the changes in microbial communities and micronutrients through soils chosen from three different depths and comparing the effect of two different grass type on these changes [**A1**].

ii. Assessing the effect of soil pH on the diversity of microbial communities and the plant availability of micronutrients in pasture soils [**A2**].

iii. Observing the effect of sheep-manure amendment on the diversity of microbial communities and the plant availability of micronutrients in soils from different pHs [**A3**].

iv. The effect of microbial communities on the soil structure and its indirect effect on micronutrient availability will be investigated [**A4**].

v. The rooting type will change the soil structure in terms of porosity and the vertical leaching of nutrients would be encouraged that results a change in microbial community structure and micronutrient availability [**H1**].

vi. The presence of microbial communities on roots will create an interface and affect the nutrient uptake by the roots [**H2**].

vii. The deep rooting grasses will facilitate the mobility of nutrients into deeper regions that enables the required sources for microbial communities [**H3**].

viii. The root exudates have the chelating ability for metal ions and deep-rooting grasses will affect the plant uptake of micronutrients through roots [**H4].**

ix. The input of manure to soil will affect the microbial community structure in soils directly by supplying nutrients and indirectly by changing soil pH [**H5**].

x. The change in soil pH will affect the solubility and mobility of micronutrients [**H6**].

xi. Different bacterial communities will prefer different soil pH to grow and this will affect the fungal to bacteria ratio in soils [**H7**].

1.14. Scope of Thesis

The first part (*Chapter 1*) of thesis comprises a background introduction to the research, which describes the importance of soil health, soil parameters, and the analyses for organic compounds and mineral nutrients. The details about the experimental methods and instrumental analysis are given in *Chapter 2*. The third part of the thesis (*Chapter 3*) deals with the vertical distribution of microbial communities and mineral nutrients and assess the effect of two different grass types with different root lengths. In *Chapter 4*, the soil profile is assessed, in terms of microbial community diversity and micronutrient availability, in soils with differing pH, whilst incubated with sheep-manure. Three time-points are chosen for the incubation, which are t=0, t=5 week and t=10 week. However, the soils from t=10 time-point were not analysed in terms of PLFAs because of the Covid-19 pandemic and lockdown. In addition to that, GDGTs were assessed in terms of soil pH not for t=5 and t=10 week manure amendments. The last part of thesis (*Chapter 5*) is a conclusive chapter that summarises the purpose and results, as well as giving suggestions for future work.

2. MATERIALS AND METHODS

2.1. General

Through all experiments, HPLC grade solvents (Rathburn Chemicals, UK) and analytical grade chemicals were used. All glassware was furnaced at 450 °C for 4 h before use. Analytical blanks were prepared with each batch of samples to monitor for any source(s) of contamination. All experiments were carried out in triplicates and standard deviations were calculated from triplicate results.

2.2. Materials

Details about the collection and preparation of soils are given in the relevant parts of Chapter 3 and Chapter 4.

2.3. Analytical protocols - organic geochemistry

2.3.1. Total lipid extract

The total lipid extract (TLE) was obtained using a Bligh Dyer based extraction method (White et al., 1979). In a separating funnel, phosphate buffer (PB) solution was prepared by dissolving monobasic potassium phosphate (KH_2PO_4, Sigma-Aldrich) in 500 mL double distilled water to give 0.05 M solution. The pH was adjusted to 7.2–7.4 by adding sodium hydroxide pellets (NaOH, Fisher Chemical). For the removal of contaminants, the total mixture was extracted with 50 mL DCM three times. For the preparation of Bligh Dyer Solvent (BDS), methanol (MeOH), dichloromethane (DCM) and PB were combined in a 2:1:0.8 (*v/v*) ratio. The dried soil samples (1 or 2 grams) were weighed in 50 mL glass centrifuge tubes and 15 mL of BDS was added in each tube. After 10 seconds vortex mixing, the tubes were sonicated for 15 min and centrifuged for 10 min at 3000 rpm. The supernatant was transferred to a 100 mL glass centrifuge tube. These steps - the addition of BDS, vortex mixing, sonication, and centrifugation – were repeated four times in total to maximise extraction of lipid from the soil. Following this, 16 mL of PB and 16 mL of DCM were added to the centrifuge tubes containing the supernatant solutions such that the solvents were

present at a ratio of 1:1:0.9 (MeOH:DCM:PB *v/v*) the solution was then centrifuged for 5 min at 2500 rpm. Subsequently, the DCM phase was transferred into a 100 mL round bottom flask using a pasteur pipette and these steps were repeated four times. After collecting all of the DCM phase the DCM was removed by rotary evaporation (Figure 2.1).

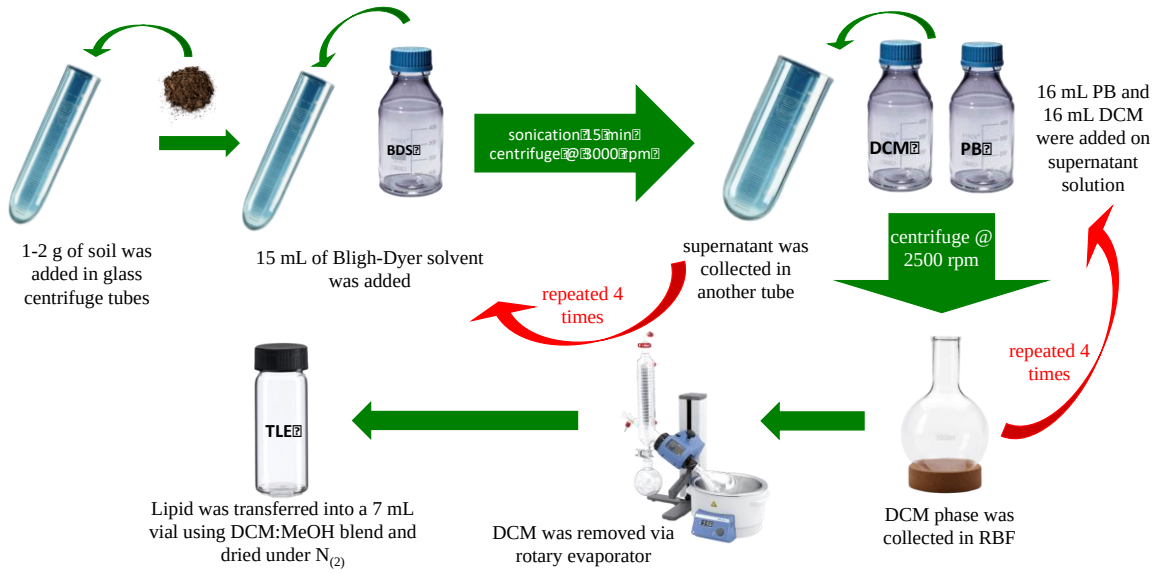

Figure 2.1 Schematic representation of the Bligh-Dyer method for total lipid extraction (TLE).

Once all DCM had evaporated, two aliquots of DCM:MeOH (2:1 *v/v*) were added in to the round bottom flask whilst gently shaking to dissolve all extract on the internal glass surface. The solution was transferred to a 7 mL glass vial. The flask was washed four times with DCM:MeOH (2:1 *v/v*) and all resulting solution collected in the same vial and split into two equal aliquots: one for glycerol dialkyl glycerol tetraether (GDGT) analysis and one for phospholipid fatty acid (PLFA) analysis. Solvent in both vials was evaporated under a gentle stream of $N_{2(g)}$.

2.3.2. GDGT extraction

Separation of core (C) and intact polar (IP) GDGT fractions was achieved by silica column chromatography (Figure 2.2a). A small piece of glass wool was coiled and placed in Pasteur pipette to obstruct the flow of silica particles. Then, silica gel (60 Angstrom pore size, for column chromatography, Fisher Chemical) were added to the pipette and the column was washed 3 times with Hex:EtAc (1:2 *v/v*) mixture. In a sample 7 mL vial, 2–3 droplets Hex:EtAc (1:2 *v/v*) were added and the lipid extract was dissolved. After all washing solvent

had passed through the column, the dissolved sample was added on top and nearly 8 mL of Hex:EtAc (1:2 v/v) mixture was added slowly as the mobile phase. The C-GDGT fraction eluting in the Hex:EtAc solution was collected in a vial and solvent was evaporated with gentle stream of $N_{2(g)}$. After all of the Hex:EtAc solution passed through the column, 10 mL of MeOH was added slowly as the second mobile phase for elution of the IP-GDGT fraction. Then, the collected fraction was split in to 2 aliquots: one for IPL-derived core lipids and another one is for the determination of separation efficiency of C-GDGT and IP-GDGT fractions. The aliquots were dried under a gentle stream of $N_{2(g)}$.

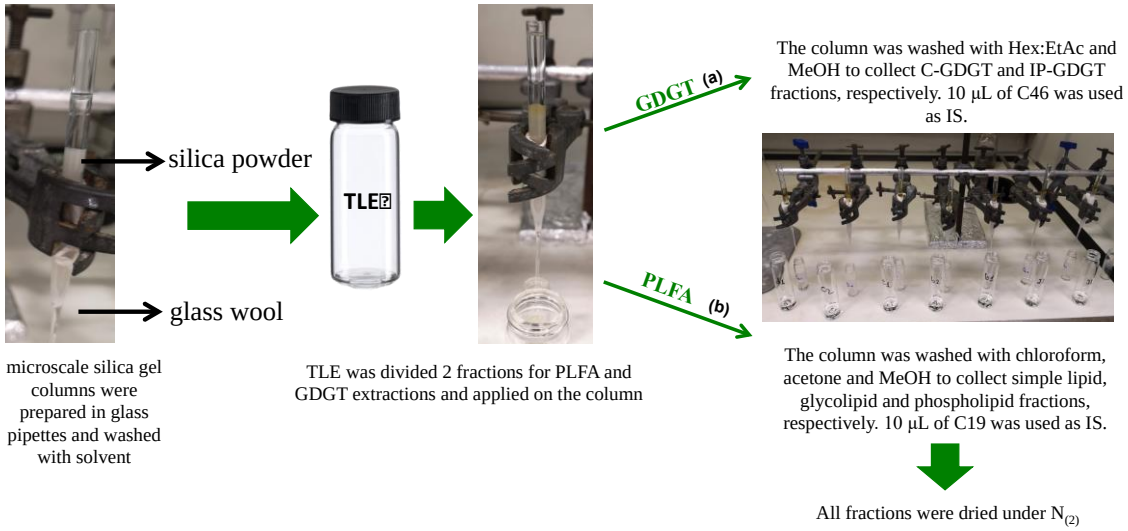

Figure 2.2 Schematic representation of silica column chromatography for **(a)** GDGT and **(b)** PLFA separation.

For the cleavage of polar head groups of the IP-GDGT fraction, acid hydrolysis was performed. Into the IP-GDGT fraction vial, 3 mL of 2M methanolic HCl (simple addition of concentrated HCl to methanol) was added and the solution was heated at 70 °C for 3 hours. Subsequently, the solutions were allowed to cool and 3 mL of DDW was added followed by addition of 2M potassium hydroxide (KOH, Fisher Chemical) solution in MeOH drop-by-drop. The pH was monitored with pH paper and adjusted to between 4 and 5. 5 mL of DCM was then added and the solution shaken. The DCM phase was transferred into a clean glass vial and this liquid–liquid extraction step repeated four times. The extracted IPL-derived GDGT fraction in DCM was evaporated under a gentle stream of $N_{2(g)}$ (Figure 2.3).

The GDGT fractions, both the isoprenoidal and branched GDGTs, were dissolved in Hex:IPA (99:1, *v/v*) and passed through 0.45 µm polytetrafluoroethylene (PTFE) filters. Samples were than dried under a gentle stream of $N_{2(g)}$ and transferred to LC-MS vials by dissolving in 90

µL Hex:IPA (99:1, *v/v*). To enable quantitative analysis, 10 µL (24.04 ng mL^{-1}) of a C_{46} GDGT internal standard (Huguet et al., 2009) was added prior to analysis.

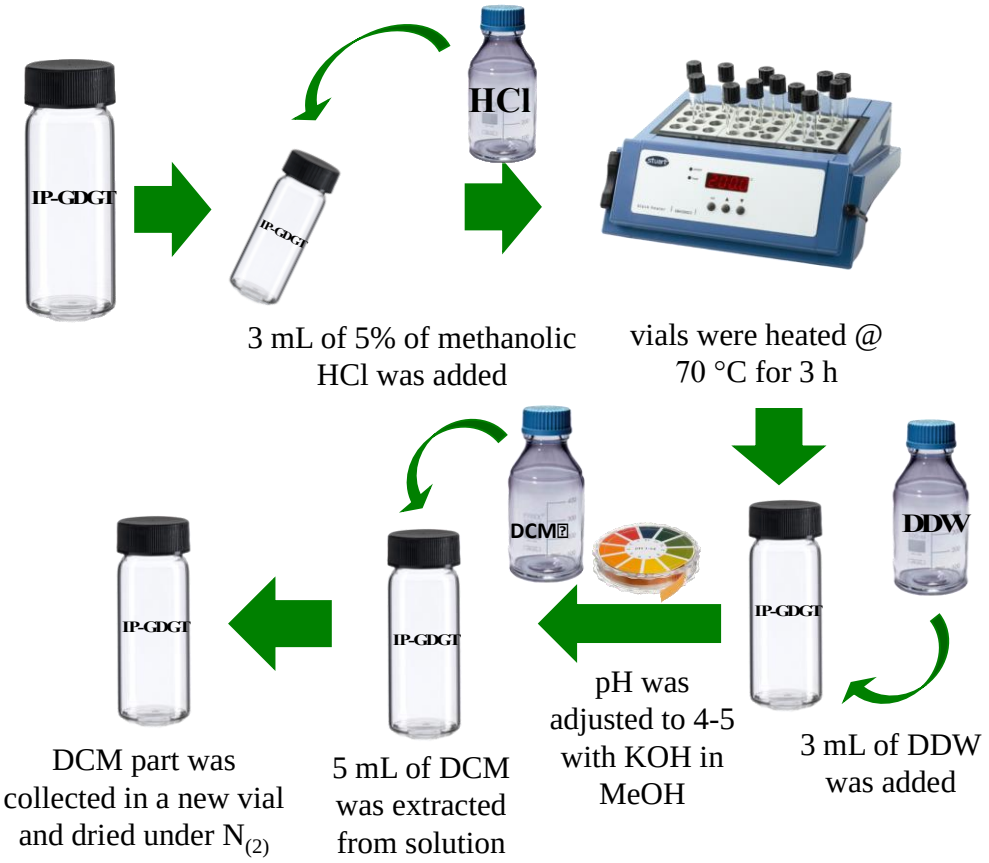

Figure 2.3 Schematic representation of acid hydrolysis for IP-GDGT fraction.

2.3.3. PLFA isolation

Separation of simple lipid (SL), glycolipid (GL) and phospholipid (PL) fatty acid fractions was achieved by silica column chromatography. Silica columns were prepared as for the GDGT extraction above (Figure 2.2b). The column was conditioned with chloroform by eluting it with 4 column bed volumes of solvent. The TLE was dissolved in nearly 0.5 mL of chloroform and the TLE solution applied to the column. A SL fraction was eluted with 5 mL of chloroform into a 7 mL vial. Then, 10 mL of acetone was passed through the column and the GL fraction was eluted into a 28 mL vial. Finally, the PL fraction was eluted with 5 mL methanol into a 7 mL vial. Solvent was removed from all collected fractions under a gentle stream of $N_{2(g)}$. To enable the quantification of PL fractions, 10 µL of internal standard C_{19}

alkane solution (0.1 mg · mL^{-1} in hexane) was added in vials and blown down under a gentle stream of $N_{2(g)}$.

Prior to the analysis of lipid fractions by gas chromatography, acid catalysed methylation (transmethylation) was performed (Figure 2.4). Anhydrous methanol was chilled in an ice bath and acetyl chloride (puriss. p.a., ≥99.0%) was added dropwise in methanol to achieve 5% anhydrous HCl in methanol. The solution was left to cool and sealed with PTFE tape. The methylated PL fraction was then dissolved in 5 mL of the 5% anhydrous HCl in methanol solution and heated at 50 °C for 2 hours. After cooling to room temperature, 5 mL of a saturated sodium chloride (NaCl, Fisher Chemical) solution was added and mixed well using vortex followed by the addition of 1 mL of hexane. A liquid–liquid extraction was performed by removing the organic layer into a clean vial following vigorous shaking. The addition of hexane and liquid–liquid extraction part was repeated 3 times. In order to remove any residual water, sodium sulphate (Na_2SO_4, Anhydrous, 99+%, Extra Pure, Fisher Chemical) columns were prepared and the hexane supernatant passed through these columns eluting with hexane. This part was repeated 3 times and extracted hexane was, combined and removed under a gentle stream of $N_{2(g)}$.

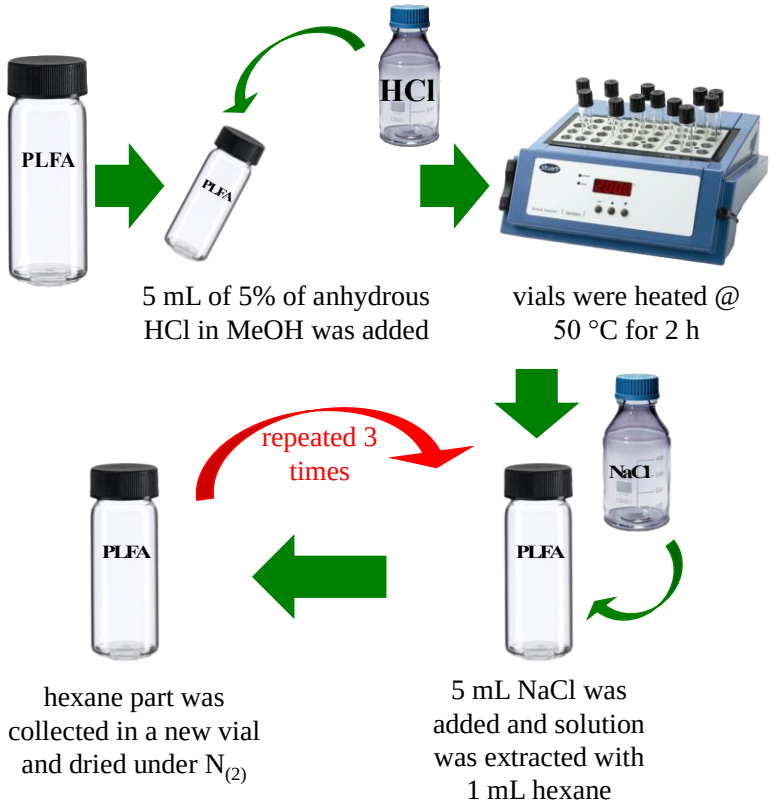

Figure 2.4 Schematic representation of transmethylation for PLFA's.

2.4. Instrumental analysis of organic compounds

2.4.1. HPLC-MS

All LC-MS analyses were performed on high performance liquid chromatography / atmospheric pressure chemical ionisation – mass spectrometry (HPLC/APCI-MS) using Thermo ACCELA LC-MS Thermo-Quantum Access MS and separation was achieved on an Alltech Prevail cyano column (150 mm × 2.1 mm; 3 μm i.d.). Samples were analysed following the procedure by Schouten et al (Schouten et al., 2007). The injection volume was 15 μl and the elution gradient was 99% hexane and 1% isopropanol, isocratically for the first 5 min, followed by a linear gradient to 1.8% isopropanol over 45 min with a constant flow rate of 0.2 ml min^{-1}. Analyses were performed using selective ion monitoring (SIM) mod to increase sensitivity and reproducibility; protonated molecular ion ($M+H^+$) GDGT peaks were integrated (Table2.1) (Zheng et al., 2015).

Table 2.1 $[M+H]^+$ *m/z* of isoprenoidal and branched GDGTs analysed by HPLC/APCI-MS in SIM mode.

GDGT structure	$[M+H]^+$ *m/z*
GDGT-0	1302
GDGT-1	1300
GDGT-2	1298
GDGT-3	1296
GDGT-4	1294
Crenarcheaol and Cren. isomer	1292
brGDGT-Ia	1022
brGDGT-Ib	1020
brGDGT-Ic	1018
brGDGT-IIa and IIa’	1036
brGDGT-IIb and IIb’	1034
brGDGT-IIc and IIc’	1032
brGDGT-IIIa and IIIa’	1050

2.4.2. GC-MS

All GC-MS analyses were performed on a ThermoScientific ISQ7000 series GC-MS. The TLE fractions were introduced manually in spitless mode onto a polar column (60 m x 0.32 mm i.d., 0.1 µm film thickness, Agilent, VF-23ms). The MS was operated in electron ionization (EI) mode operating 70 eV with a GC interface temperature of 250 °C and a source temperature of 250 °C. The emission current was 150 µA and spectra were acquired in full-scan mode (*m/z* 50-650) with a scan time of 0.2 sec. The temperature program was as follows: initial temperature was held 50 °C for 1 min followed by an increase to 100 °C at a rate of 10 °C min^{-1}. Then, the temperature was increased to 250 °C with a rate of 4 °C · min^{-1} and held 15 min. The acquisition and analysis of MS data was carried out using Xcalibur software (version 3.0, ThermoScientific). The identification of compounds was achieved by comparison of GC-MS retention times to a Bacterial Acid Methyl Ester standard (BAME Mix, solution, 10 mg/mL total concentration in methyl caproate, Sigma-Aldrich), published mass spectra and computer databases.

The sum of PLFAs i14:0, i15:0, a15:0, i16:0, i17:0 and i18:0 was chosen to describe Gram-positive bacteria (excluding actinomycetes), and fatty acids with methyl group on the 10th carbon atom were selected to quantify the actinomycetes. The sum of cyclopropyl fatty acids, cy17:0 and cy19:0, and monoenoic fatty acid 16:1ω9 was used to quantify of Gram-negative bacteria. The sum of *cis*- and *trans*- isomers of monoenoic 18:1ω9 and dienoic 18:2ω9,12 was used to quantify the fungal biomass (Thoms et al., 2010; Willers, van Rensburg, and Claassens, 2015). Total PLFA amount was described as the sum of all Gram-positive and Gram-negative bacteria, actinomycetes and fungal biomass.

2.5. Analytical protocols - inorganic geochemistry

2.5.1. Aqua-regia digestion

The aqua-regia method is commonly used for the wet extraction of total metals in soils and it is safer to use when compared with other extraction methods such as hydrofluoric acid. The method was modified (Radojevic et al., 1999) using the BCR residual method, which is detailed later (Arain et al., 2008). Aqua-regia solution was prepared by mixing the concentrated HCl with 1M HNO_3 in a 3:1 (v/v) ratio. Nearly 0.5 g of each soil sample was weighed in 100 mL glass beakers. 8 mL of aqua regia was then added to soils, with swirling

to wet the sample in the beaker, and left in the fume-hood overnight. Subsequently, the beakers were placed on a heater set at 100–110 ℃ and the solvent was evaporated for nearly 1 hour without boiling the solution. After the solvent in the beaker was removed and a slurry was formed (drying the soil was avoided), the beakers were cooled to room temperature. The soils were filtered through Whatman no. 542 filter paper after adding 0.5–1.0 mL of 1M HNO_3 to the beakers and solutions were collected in 25 mL glass volumetric flasks. The solutions were diluted to 25 mL with 1M HNO_3 and stored in plastic tubes for ICP-OES analysis (Figure 2.5).

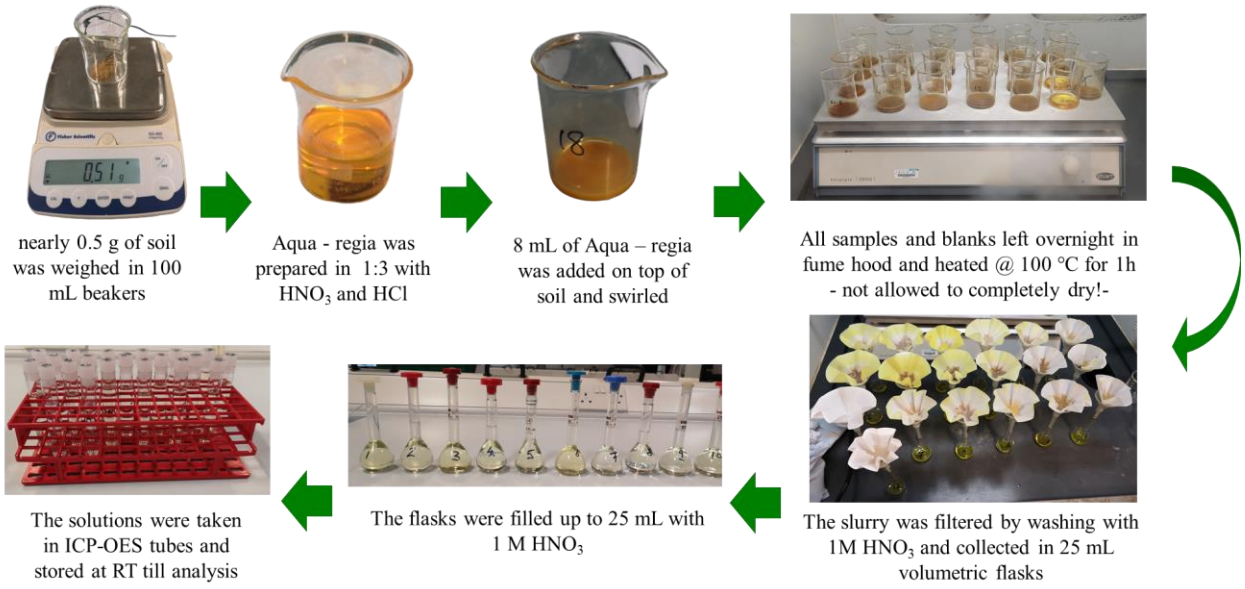

Figure 2.5 Schematic representation of Aqua-regia digestion method.

2.5.2. Low molecular weight organic acids (LMWOA) extraction

LMWOAs mimics the leaching driven by plant roots and the products of microbial activities (Fox et al., 1990). Acetate extraction of soils (BCR method) was used for the leaching of exchangeable ions. Nearly 1.0 g of soil was weighed in 50 mL PP centrifuge tubes and mixed with 30 mL of 0.11 M acetic acid (diluted from glacial acetic acid, Extra Pure, Fisher Chemical). After mixing the solution with a vortex mixer for ≈ 10 s, the tubes were placed on an end-over-end shaker overnight (≈ 16 h) at room temperature. Subsequently, the tubes were centrifuged for 20 min at 3000 rpm and the supernatant transferred to a 50 mL beaker. The acetic acid was evaporated at ≈ 80–90 ˚C on a heater until only a little amount of solvent remained in the beaker. Afterwards, 15 mL of 1M HNO_3 was poured into the beaker and the solution was filtered through Whatman no. 542 filter paper into 25 mL volumetric flasks. The

flasks were topped up with 1M HNO_3, shaken and the solution was transferred to clean PP centrifuge tubes for storage (Figure 2.6).

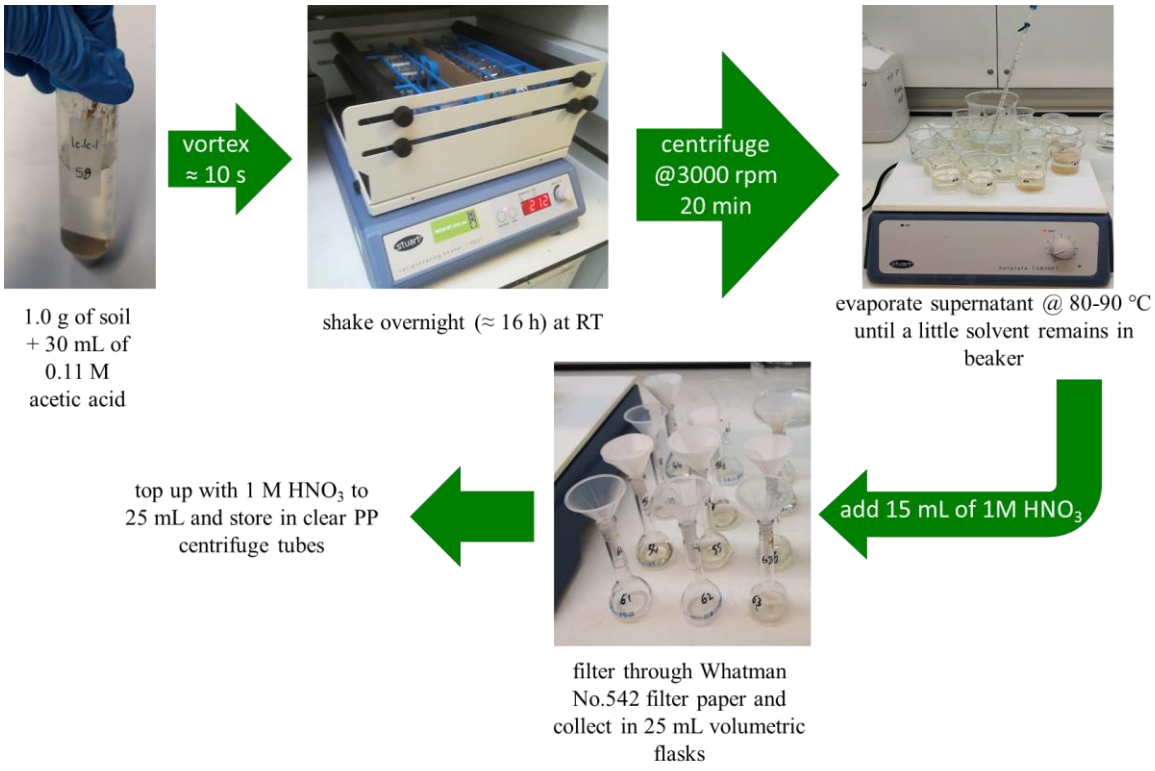

Figure 2.6 Schematic representation of acetic extraction (LMWOA extraction).

2.6. Instrumental analysis of inorganic nutrients

2.6.1. Inductively coupled plasma – optical emission spectrometry (ICP-OES)

The concentrations of all elements were determined by inductively coupled plasma – optical emission spectrometry (ICP-OES, Agilent 710). Before starting the analyses, calibration graphs were created for the quantitative determination of elements. A semi-quantitative solution was prepared by taking nearly 0.5 mL of each sample in a plastic tube. This solution was analysed in ICP-OES and the concentration range of each element was recorded. 5 different concentrations were determined for the calibration graph of each element. After the calculation of the required volumes, the calibration standards were prepared in 50 mL volumetric flasks by dilution with 1% (*v/v*) HNO_3. All samples and standard solutions with blank controls were analysed by ICP-OES using a correction to eliminate possible errors arising from instrument drift.

2.6.2. Elemental analysis (EA)

Total carbon (TC), total nitrogen (TN) and total sulfur (TS) analyses were performed using an Elementar vario PYRO cube. Samples were weighed in tin capsules (5 mg for manure and 10 mg for soils) and analysed *via* EA, which is capable of analysing C/N/S *via* catalytic combustion/reduction (1150˚C).

2.7. Data processing

The integration of peaks for both GDGTs and PLFAs were acquired by ThermoScientific Xcalibur software (version 3.0). Quantification of GDGTs was achieved through the comparison of integrated peak areas with that of an internal standard (C_{46} GDGT). The similar approach was used for the quantification of PLFAs where the integrated peak areas were compared with the peak area of a C_{19} internal standard (nonadecane). The concentrations were calculated using the following Equation 2.1:

$$C_{compound} = \frac{\left(\frac{A_{compound} \times C_{IS}}{A_{IS}}\right)}{m_{soil}} \quad 2.1$$

Where:

$C_{compound}$ = concentration of interested GDGTs or PLFAs (μg g^{-1})

$A_{compound}$ = peak area of interested GDGTs or PLFAs

C_{IS} = concentration of internal standard

A_{IS} = peak area of internal standard

m_{soil} = mass of extracted soil (g)

The interpretation of ICP-OES results was performed following the School of Earth Sciences laboratory protocol (A. McAleer, personal communication). The instrument provided concentration data by comparing the sample signals with the signals of calibration solutions, which were prepared at 5 different concentrations (details are given in Appendix I and II). The blanks were then subtracted from sample data and all data were corrected with the drift

sample's data to eliminate any deviation originating from the instrument. The concentrations were calculated using the following Equation 2.2:

$$C_{nutrient} = \frac{\left(\frac{C_{solution} \times V_{solution}}{1000}\right)}{m_{soil}} \quad 2.2$$

Where:

$C_{nutrient}$ = concentration of interested nutrient ($mg \cdot g^{-1}$)

$C_{solution}$ = concentration of analysed solution in ppm ($mg \cdot L^{-1}$)

$V_{solution}$ = volume of solution

m_{soil} = mass of extracted soil (g)

One-way ANOVA and two-way-ANOVA statistical analyses were applied in MATLAB to determine the significant differences in observations throughout the work. The significance level was set at $P \leq 0.05$ for all statistical analyses.

3. VERTICAL DISTRIBUTION OF NUTRIENTS AND THE ROLE OF SOIL STRESS ON MICROBIAL ACTIVITY

3.1. Aims and objectives

Subsurface microbes play an important role in soil formation, ecosystem biogeochemistry and the maintenance of soil quality. The vertical distribution of microbial biomass and specific microbial populations in deep soils profiles have been studied in literature (Fierer, Schimel, and Holden, 2003). PLFA signatures indicated that the composition of microbial communities changed significantly with soil depth and the number of different PLFAs detected in soil samples decreased from the surface down to 2 m depth. Furthermore, it was demonstrated that the ratios of cyclopropyl/monoenoic precursors and total saturated/total monosaturated fatty acids increased with soil depth suggesting that the microbes in deeper soils are more carbon limited than surface ones. Conversely, the presence of gram-positive bacteria increases in proportion with greater soil depth, whilst the abundance of gram-negative bacteria decreases. The vertical distribution of these microbial groups is attributed to the decline in carbon availability with soil depth (Fierer, Schimel, and Holden, 2003).

This chapter considers an investigation where the vertical distribution of microbial communities and mineral nutrients were assessed in soil profiles under two different grass types with different root lengths. Figure 3.1 shows the schematic representation of two different grasses.

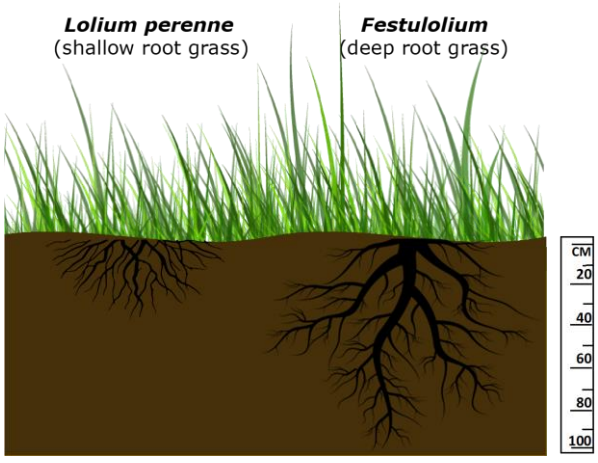

Figure 3.1 The representative image of *Lolium perenne* and *Festulolium*.

To assess the effect of two different grass types soils beneath conventional grass with 30 cm root depth and deep root grass with 1 m root depth were collected from the North Wyke Farm Platform (NWFP) at Rothamsted Research) and were subjected to extraction and analysis as outlined in Chapter 2 (Figure 3.2). The micronutrient concentrations were analysed with inductively coupled plasma optical emission spectroscopy (ICP-OES) and microbial communities were investigated by analysing their PLFA and GDGT distributions.

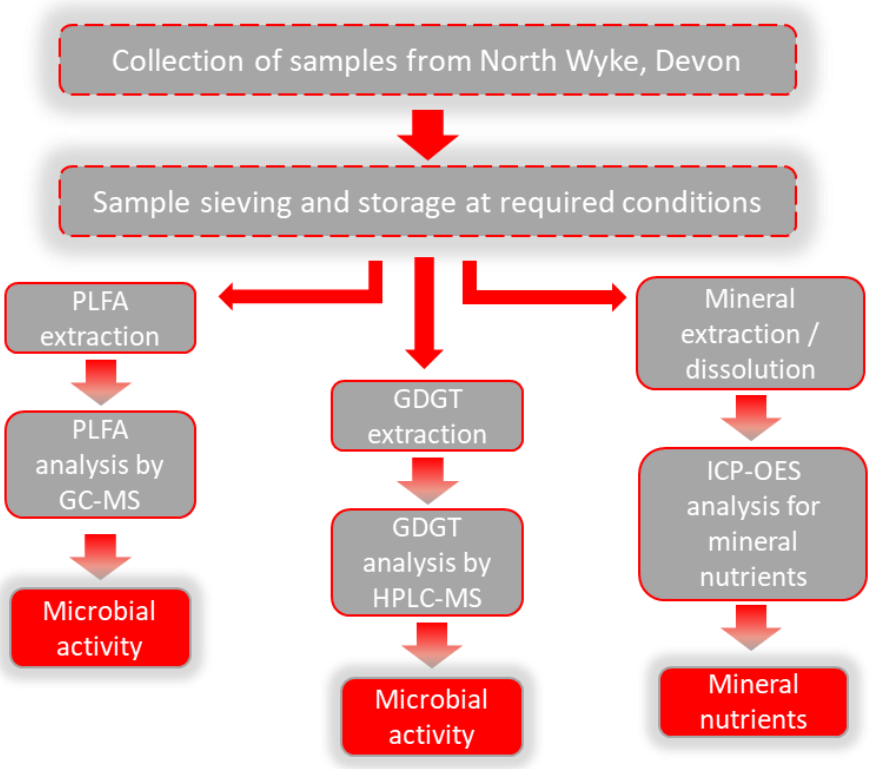

Figure 3.2 Flowchart showing the experimental steps.

3.2.Key aspects for materials and methods

A total of 18 samples were analysed (including analytical blanks) that comprised soils from under both *Lolium perenne* (British ryegrass, has a conventional rooting depth of about 30 cm) and *Festulolium* (hybrid of *Lolium perenne* and *Festuca*, grows roots to about 1 m depth) grown in adjacent, hydrologically isolated fields that are grazed by sheep. The samples were collected *via* auger from the North Wyke Farm Platform (NWFP), Rothamsted Research and supplied (dried overnight in a 100˚C oven and sieved) by Dr. Heather Buss (Figure 3.3). NWFP is a large-scale research facility to investigate the flow of nutrients from soil to food by aiming a sustainable farming approach. The platform is established with one of the

hypotheses of Rothamsted Research and researches are carried out to identify the optimum land management methods for the transfer of essential nutrients from soil to food by contributing a cleaner environment. The red farmlet on the map has sward improvement and reseeded regularly about every 4 years. This farmlet is associated with improved animal performance or environmental resilience (e.g. deep-rooting grasses). The green farmlet on the map has permanent pasture with the use of artificial fertilizers. Both the Red and Green farmlets are fertilized with nitrogenous fertilizer (Orr et al., 2016). Approximately 1 g of each soil was extracted by Bligh-Dyer extraction as described in Section 2.3 and 0.5 g of soil was extracted by aqua-regia digestion as described in Section 2.5.

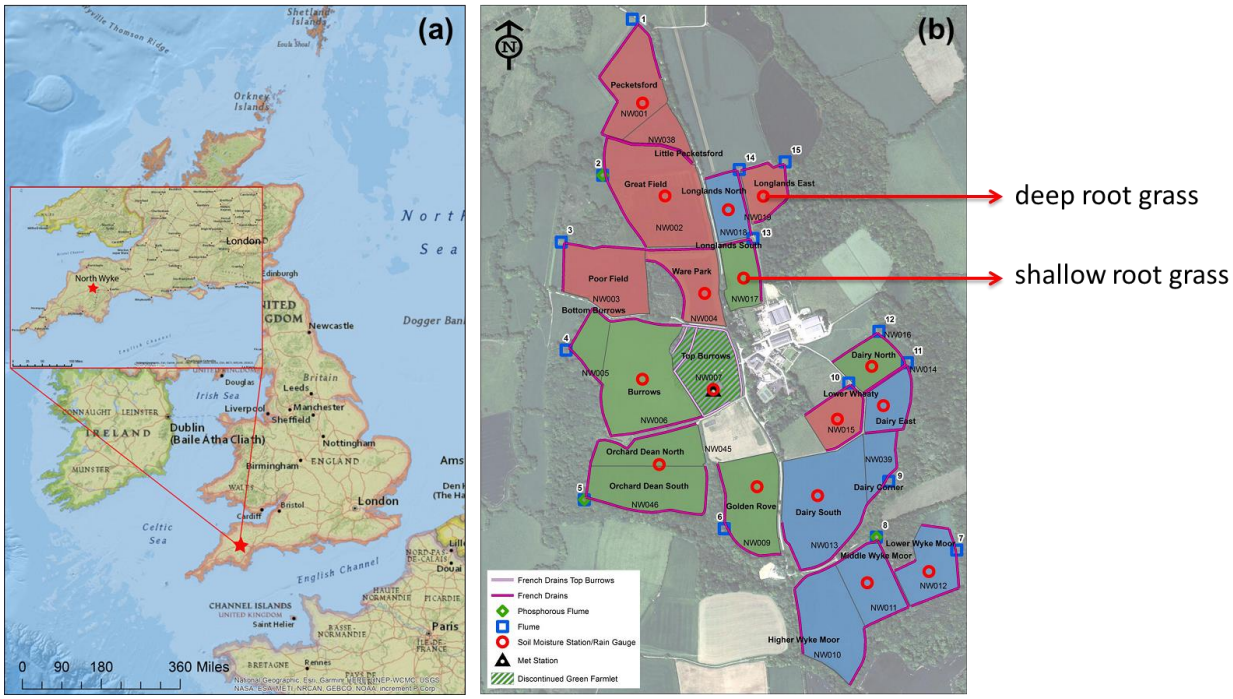

Figure 3.3 (a) The maps showing the location of North Wyke Farm Platform, Rothamsted Research sampling site that is denoted with a red star and maps were created using ArcGIS® software by Esri (ArcGIS® and ArcMap™ are the intellectual property of Esri and are used herein under license. Copyright © Esri. All rights reserved. For more information about Esri® software, please visit www.esri.com). **(b)** The site map of North Wyke Farm Platform showing the chosen fields for deep root and shallow root grasses (http://resources.rothamsted.ac.uk/farm-platform-national-capability/farm-platform-map. Access date: 04.06.2020).

3.3.Results

3.3.1. Glycerol dialkyl glycerol tetraether (GDGT) lipids

Isoprenoidal (isoGDGT) and branched (brGDGT) GDGTs are the main membrane constituents of cultured hyperthermophilic archaea and eubacteria, respectively (Schouten et al., 2000). Both isoGDGT fractions from intact polar lipids and brGDGT fractions from core lipids were found at all depths for both shallow and deep root grass soils. Figure 3.4 shows the HPLC-APCI-MS chromatograms, which reveal the presence of isoGDGTs with depth for different root types. Whilst topsoil (10cm) results show evidence for isoGDGTs in both shallow and deep root samples, there is a dramatically decreasing trend in the 50 and 90 cm soils for both grass types. Figure 3.4 shows the change in amounts of isoGDGTs with depth. When the concentration of lipids per gram dry soil is compared for shallow and deep root grasses, topsoil exhibits a higher level of isoGDGTs for shallow root grasses then the deep root ones. However, the deep root grass soils from 90 cm depth exhibit higher concentrations of isoGDGTs than the shallow root ones (ANOVA, P=0.017).

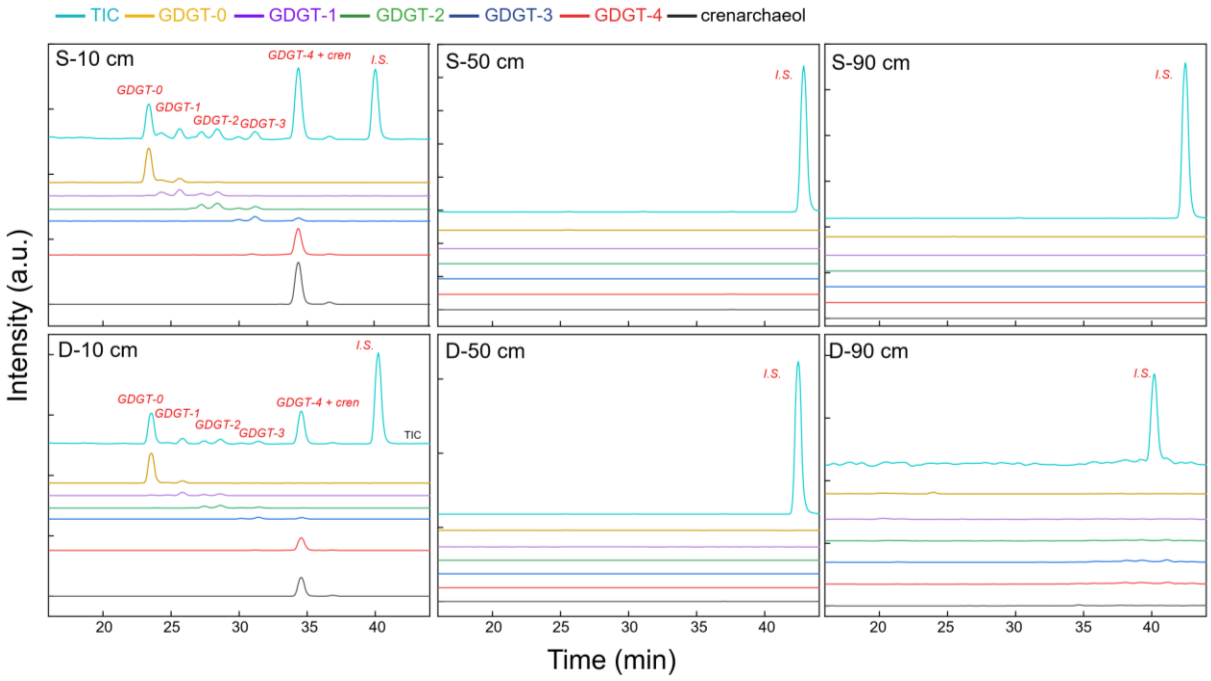

Figure 3.4 The representative HPLC-APCI-MS partial chromatograms of isoGDGT fractions of soils from shallow (S) and deep root (D) grasses from different depths).

The amounts of brGDGTs follow the same decreasing trend with soil depth as observed for the isoGDGTs, although the overall amounts are about 7-20 times higher than those of isoGDGTs (Figure 3.5). For each soil depth and grass type, brGDGTs comprise more than

85% of the total pool of GDGTs. The topsoil for both grass types has considerably higher brGDGT levels than the soils collected from 50 and 90 cm. However, the brGDGT concentration in deep root grass soils collected from 90 cm is slightly higher than in the shallow root one (ANOVA, P=0.003) while the difference between brGDGT soils at 50 cm is not significant (ANOVA, P=0.425).

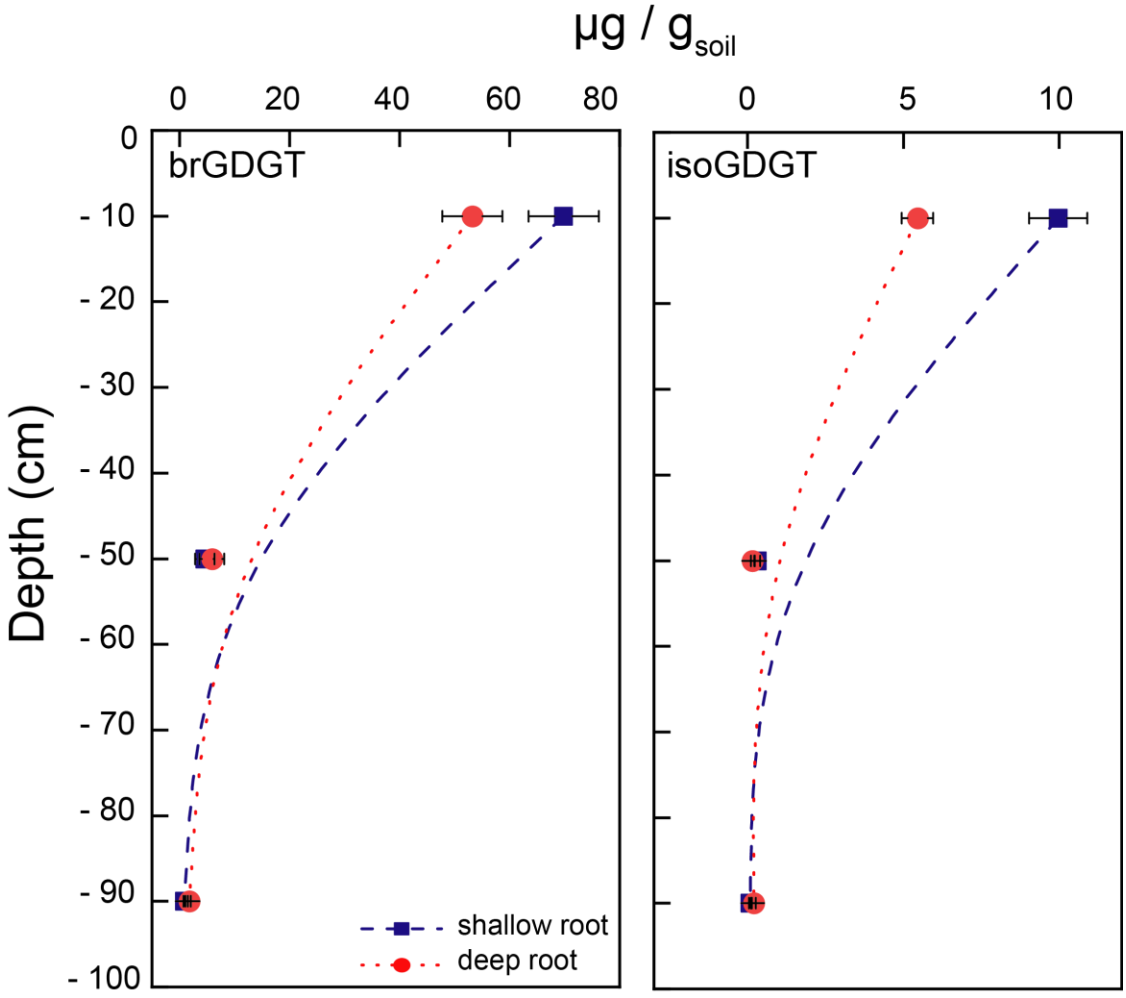

Figure 3.5 The change in amounts of different isoGDGTs and brGDGTs with depth (ANOVA, P<0.04 for all results except for brGDGT at 50 cm P=0.425).

The HPLC-APCI-MS chromatograms in Figure 3.6 denote the presence of brGDGTs even in the deep root grass soils (D-90) whilst the shallow root grass soil (S-90) exhibits smaller peaks. Moreover, there is a clear difference between the S-90 and D-90 chromatograms that show higher amounts of brGDGTs in the D-90 soil than the S-90 soil.

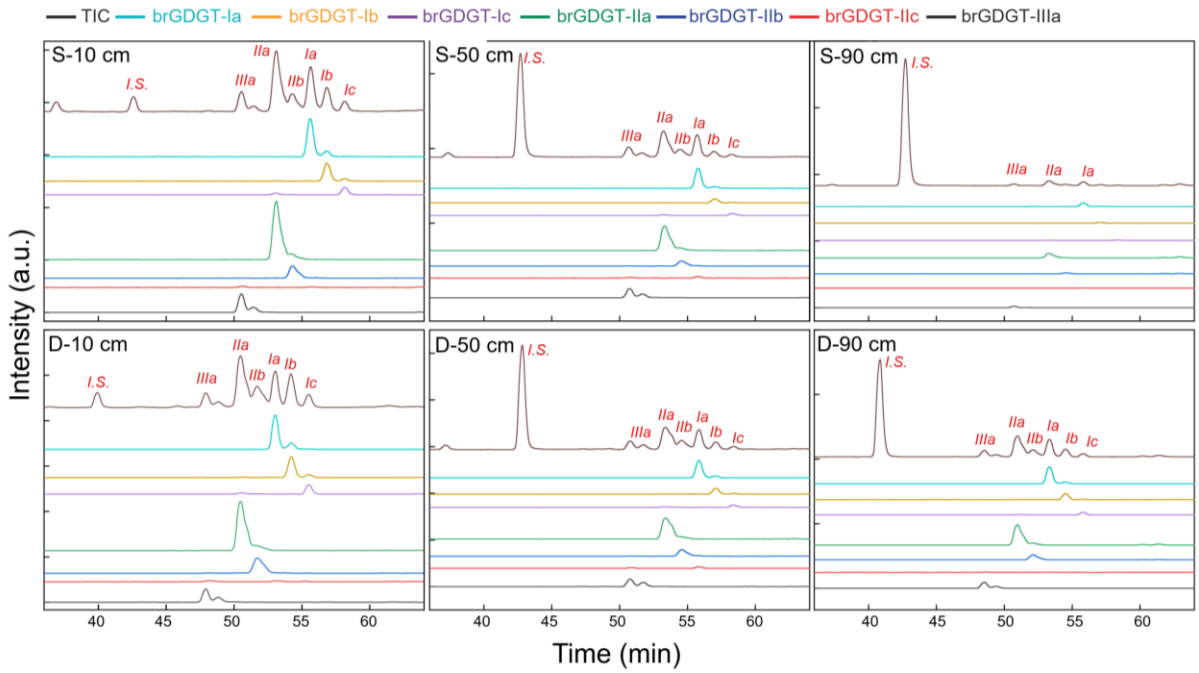

Figure 3.6 The representative HPLC-APCI-MS partial chromatograms of brGDGT fractions of soils from shallow (S) and deep root (D) grasses from different depths.

Crenarchaeol is a specific biomarker that is found ubiquitously in soils and peats, and contains a cyclohexane moiety in addition to the cyclopentane moieties; it is synthesized by mesophilic Crenarchaeota (Schouten et al., 2000; Pitcher et al., 2009). Nearly 50% of isoGDGTs in the topsoil (10cm) come from crenarchaeol residues whilst this percentage is around 20 % for the subsoils (50 and 90 cm).

3.3.2. Phospholipid fatty acids (PLFAs)

Phospholipid fatty acids (PLFAs) are a key part of cellular membranes from the domains of Bacteria and Eukarya, and their analysis enables the size of microbial communities in soils to be calculated (Zhang et al., 2015). Figure 3.7 shows the GC-MS chromatograms, which indicate the presence of PLFAs with depth for the different root types. The intensities of peaks for the topsoil (10cm) are higher than the subsoils (50 and 90 cm) and they are much higher than that of the internal standard (IS), whilst the peaks for subsoils exhibit lower intensities. Moreover, the diversities of PLFAs are wider in the topsoil than the subsoils, this is clearly seen in the chromatograms of soils S-90 and C-90.

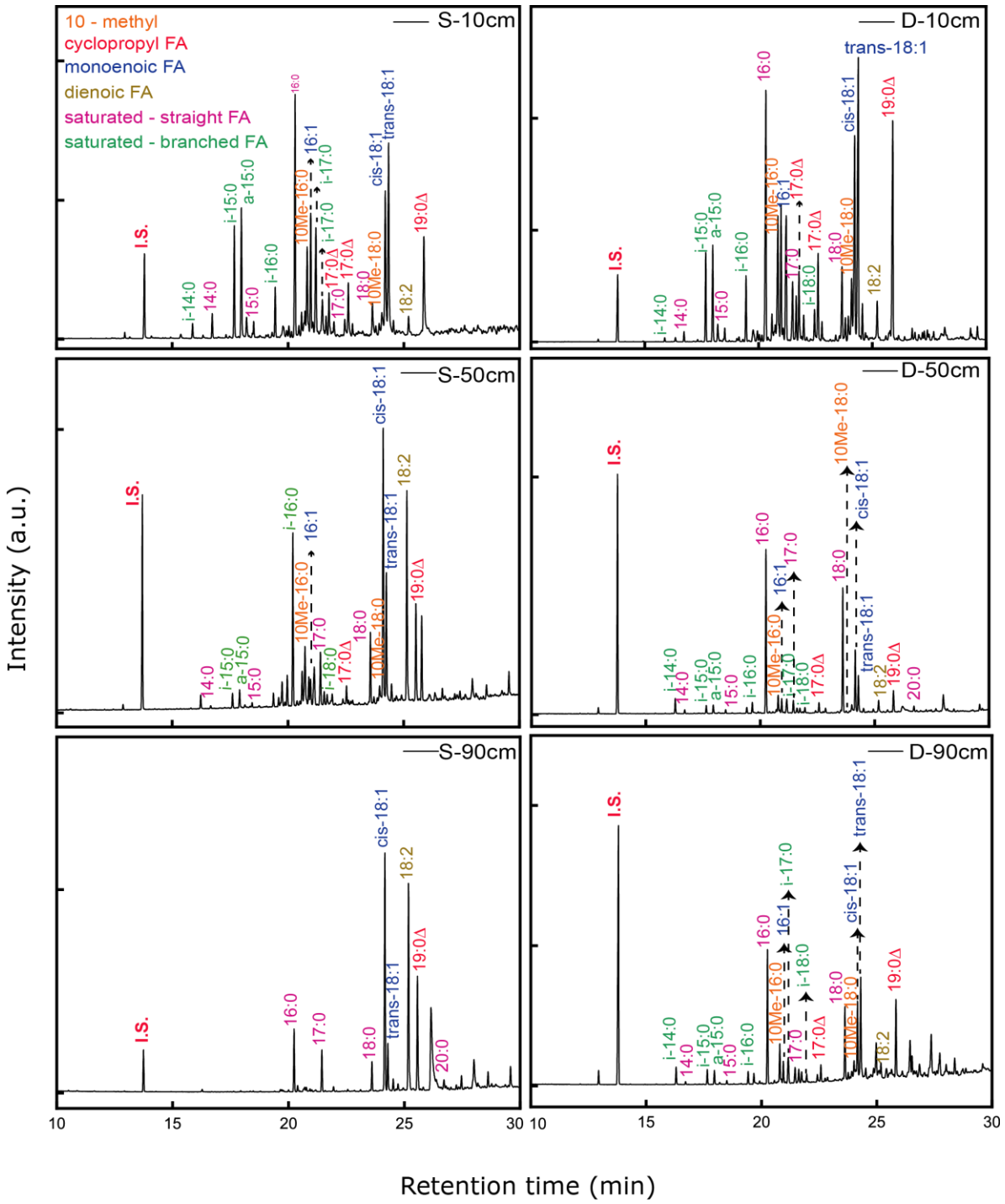

Figure 3.7 The representative GC-MS partial chromatograms of PLFA fractions of soils from shallow (S) and deep root (D) grasses from different depths.

Figure 3.8 demonstrates the change in the total amount of PLFAs in each soil depth for different root types. The total amount of PLFAs dramatically decreased with soil depth for both root types. Although there is a significant difference between shallow root and deep root grass for topsoil and 50 cm subsoil (ANOVA, P=0.0017), the amount of PLFAs at 90cm depth is nearly same for both grass types (ANOVA, P=0.103).

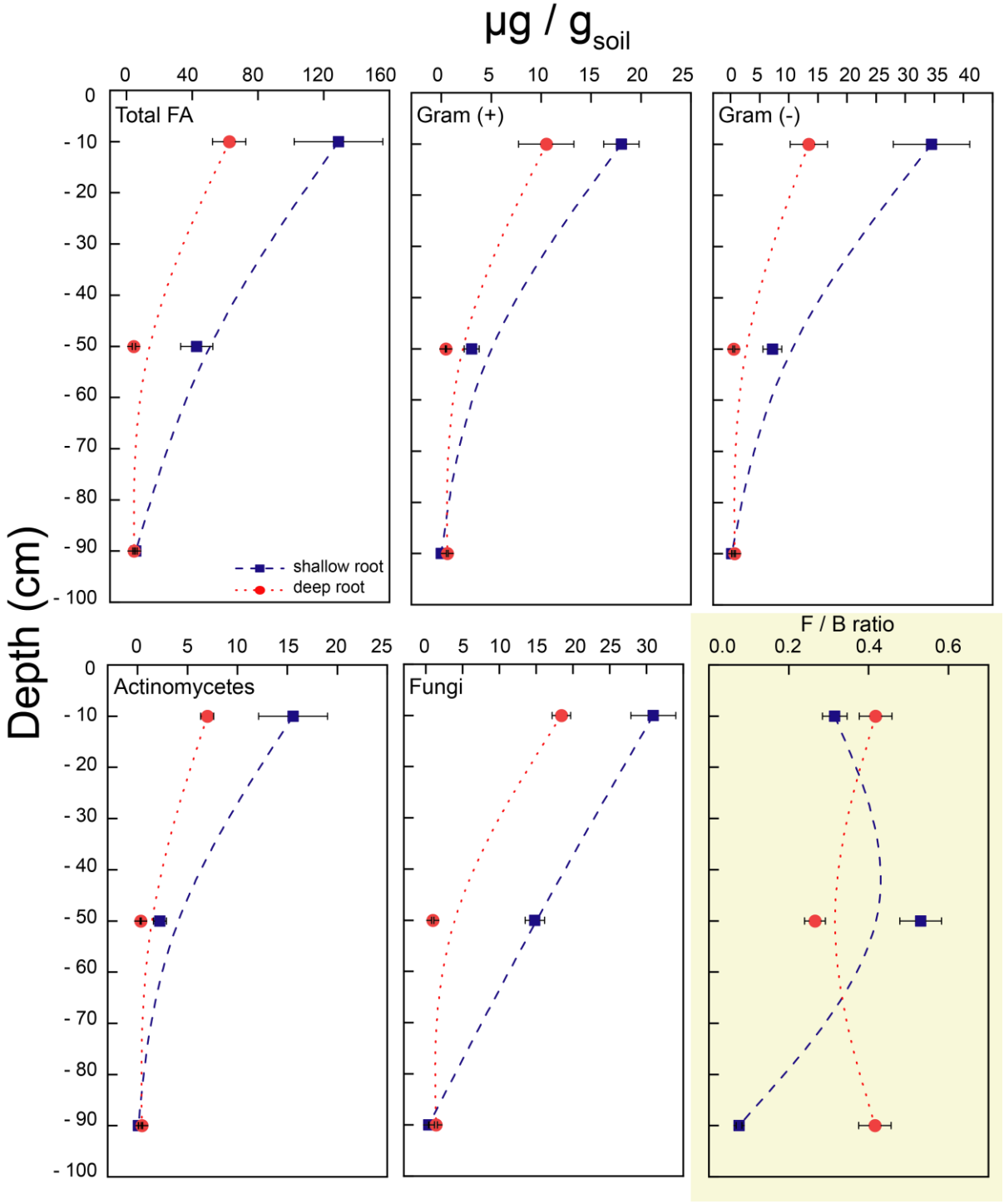

Figure 3.8 Concentration change of total FA, Gram-positive, Gram-negative, actinomycetes, fungi and fungal-to-bacteria ratio with soil depth (ANOVA, $P<0.02$ for all results except 90 cm total FA data).

The change in amounts of Gram positive, Gram negative and actinomycete biomarkers in shallow root and deep root grasses with depth is given in Figure 3.8. Whilst Gram positive bacteria derived PLFAs were found in subsoils (90 cm) for deep root grasses, none were

detected in the equivalent shallow root soil although the amount observed in the topsoil for the shallow root grasses is higher. The amounts of Gram-negative bacteria and actinomycetes in the shallow root grass subsoil (90cm) are again slightly higher (ANOVA, P=0.0014 and 0.0083 for Gram-negative and actinomycetes, respectively)than the deep root subsoil (90cm) but in this case they are found in small quantities in the deep root grass soils as well. The amount of PLFAs in shallow root grass soils, for Gram +/- bacteria and actinomycetes, decreases with soil depth. There is a different trend for deep root grass soils where the PLFA amounts for all three microbial classes, decrease from 10 to 50 cm, they slightly increase in 90 cm ones for the deep root grass soil.

The gradual decrease of fungal biomass with soil depth for both grass types is given in Figure 3.8. Fungal biomass decreases with soil depth for the shallow root grass soils and the lowest amount of biomarkers are observed at 90 cm depth. For the shallow root grass soil, the fungal to bacteria ratio (F/B) increases from 10 to 50 cm, while this ratio decreases for deep root grass soils. However, there is an opposite trend from 50 to 90 cm depth where the F/B ratio decreases for shallow root grass soils whilst increasing for the deep root soils. The F/B ratio in the deep root grass soils are considerably higher (ANOVA, $P<0.05$ for all results) than shallow root ones.

3.3.3. Mineral nutrients

The concentration change of selected nutrients in soils is given in Figure 3.9. Ca, Mg and Mn show a decreasing trend with soil depth for both grass types. Deep root grass soils exhibit a higher concentration of Ca independent of soil depth (ANOVA, P=0.0454 for 10 cm, P=0.196 for 50 cm and P=0.0234 for 90 cm) whilst Mn concentration is higher at 10 and 50 cm depth (ANOVA, $P<0.04$ for both) and the difference at 90 cm depth is not significant (ANOVA, P=0.1404). The shallow root grass topsoil has a higher Mg concentration than the deep soil one (ANOVA, P=0.0148) but there is a sharp decrease at 50 cm depth followed by a little increase at 90 cm depth whilst Mg concentration in the deep root grass soil decreases. Cu shows a different trend for both grass types where 10 and 90 cm soils have nearly similar amounts while the Cu concentration at 50 cm depth is higher than both those observed at 10 and 90 cm depth. The deep root grass soil has a decreasing trend in K concentration with depth but the shallow root soil exhibits a changing trend that the lowest K concentration was observed at 50 cm depth (ANOVA, $P\leq0.05$).

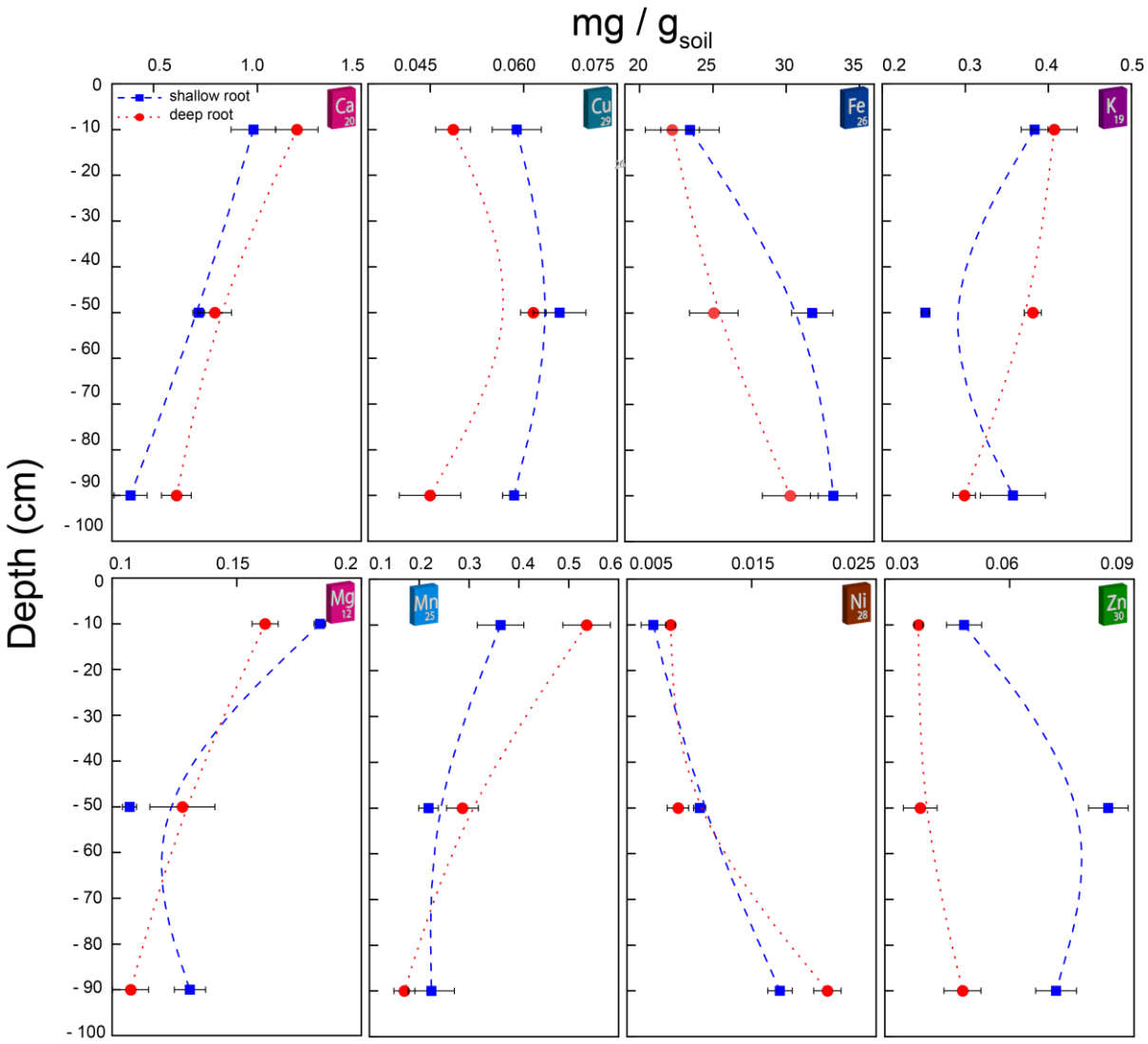

Figure 3.9 Distribution of plant essential micronutrients (Ca, Cu, Fe, K, Mg, Mn, Ni, Zn) with depth (details about significance levels are given in Appendix).

In contrast to the aforementioned macronutrients, Fe, Ni, and Zn showed increased with soil depth for both grass types (Figure 3.9). Fe concentration at all depths is higher in the shallow root grass soils than the deep root ones. A similar increasing trend in Ni concentration is observed for both grass types but at 90 cm depth, deep root grass soil has a slightly (ANOVA, $P<0.07$) higher concentration than the shallow root soil. The Zn concentration in deep root grass soils changes in a similar manner to the Fe concentration in that there is a gradual increase with depth. However, the highest Zn concentration for the shallow root grass soil is observed at 50 cm depth whilst the soils from 10 and 90 cm depth have lower concentrations.

Figure 3.10 shows the distribution of some heavy metals in shallow and deep root grass soils with depth. Al, As and Cr concentrations in both soil types decrease with soil depth. Although the topsoil of the deep root grass soil exhibits a higher concentration of Al and Cr than the shallow root grass soil, the concentration of both in the shallow root grass soil is higher at 90 cm depth. The concentration of Cd seems to be unaffected by depth of soil, but it should be considered that Cd concentration in these soils is a bit low to be detected by ICP-OES.

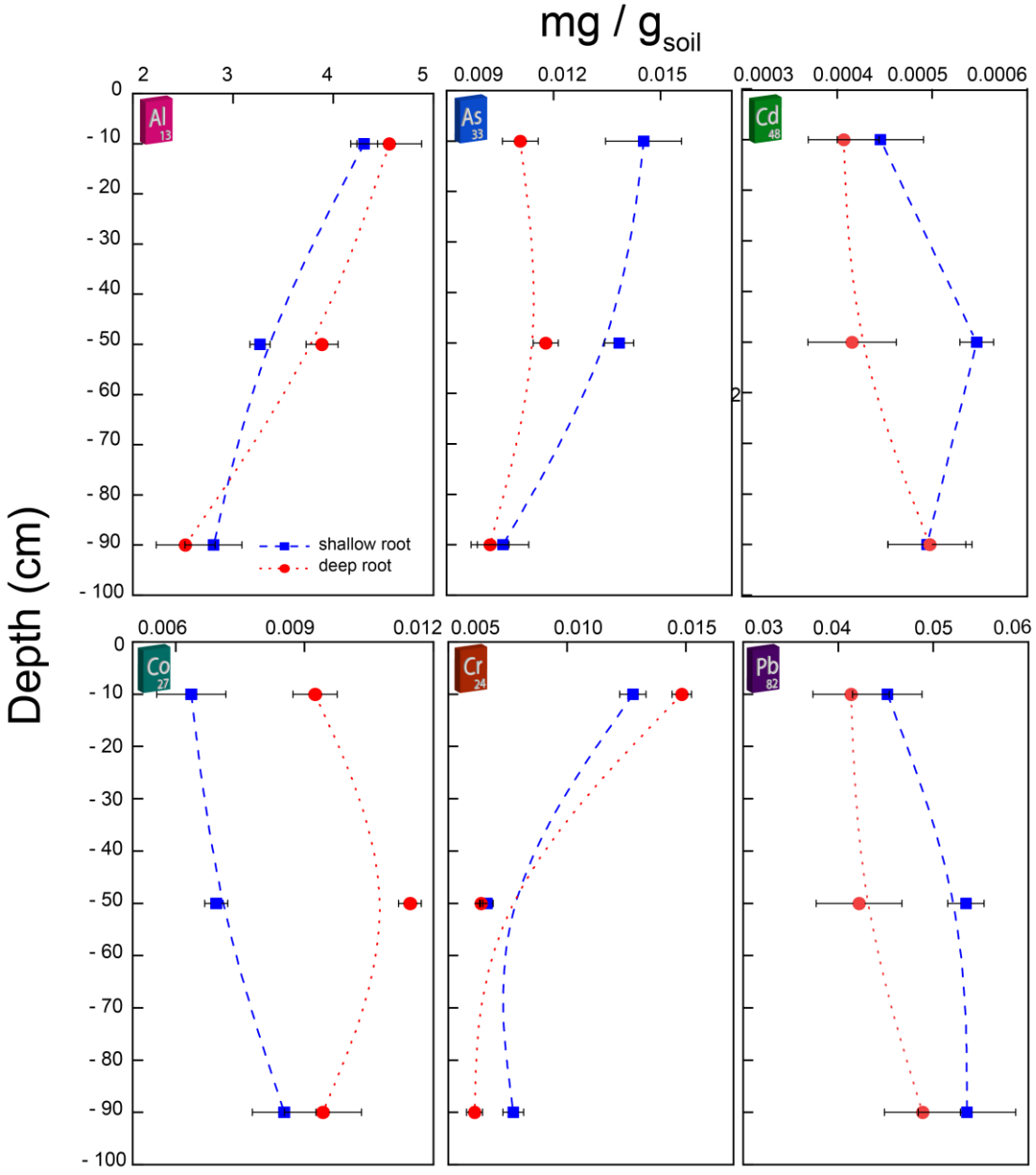

Figure 3. 10 Distribution of heavy metals Al, As, Cd, Co, Cr and Pb with depth (details about significance levels are given in the Appendix).

The concentration of Co and Pb shows an increasing trend with soil depth as seen in Figure 3.10. Whilst there is an increase with soil depth in the Pb concentration in both soils, the shallow root grass soils exhibit higher Pb concentrations than the deep root grass soils. Conversely, the shallow root grass soils exhibit lower Co concentrations which increase slightly with depth whilst the deep root grass soil from 50 cm depth has a higher Co concentration (ANOVA, $P<0.0001$) than observed for the 10 and 90 cm soils.

3.4. Discussion

The changes in microbial communities and micronutrients through three different soil depth have been compared for two different grass types **[A1]**. The root length is important for the mobility of nutrients into deeper regions because the improved porosity in soil encourages the leaching of nutrients through soil profile as well as the chelating ability of root exudates for metal ions improved the nutrient uptake from the deeper regions through the roots as outlined in §1.13 **[H1]**, **[H3]** and **[H4]**. Moreover, the fungal communities on roots create an interface between soil and the nutrient uptake is improved due to the increased surface area that verifies the hypothesis **[H2]** given in §1.13.

It was found that there were significant differences in the amounts of most biomarkers and micronutrients between the topsoil and subsoils (50 or 90 cm). The topsoil offers better nutrient resources for microorganisms (Li et al., 2017) such that total GDGTs, PLFAs and the diversity of both were observed highest in the first 10 cm. The decline in carbon resources with soil depth is a reason for the decrease in microbial community size, which is carbon limited (the total organic carbon results can be found in Appendix Table A3.2 which was supplied by Dr Heather Buss) (Fierer, Schimel, and Holden, 2003).

In terms of PLFA and GDGT concentrations, shallow root grass soils have higher biomarker concentrations than deep root ones for the topsoil (10 cm). The reason for this trend might be that the deep root grasses enable the mobility of microorganisms through deeper parts of soil (Gocke et al., 2017). Moreover, there is a concentration increase for all microbial communities in deep root grass soils from 50 to 90 cm although their concentration is lower than in the topsoil. It was shown that that deep root grass soils have always higher concentration of microbial communities than the shallow root ones at 90 cm depth independent from the topsoil concentration. GDGTs have been analysed to see whether there is any extremophile in deeper regions or not. Extremophiles are able to live in harsh

conditions such as high pH, high temperature, or high pressure. brGDGT concentrations comprise more than 85% of total GDGTs for all grass types and soil depths. The reason for the high levels of brGDGTs might be that they are not derived from living cells (core lipids) and generally come from fossilized membrane lipids as mentioned in literature (Weijers et al., 2009; Huguet, Fosse, et al., 2010). Additionally, the presence of GDGT constituents in deeper regions verifies the extremophiles, like GDGTs, thrive in extreme environments and harsh conditions. These results establish the criteria for **[H3]** that deep root grasses facilitate the mobility of nutrients and provide proper environment for microbial communities.

The availability of nutrients is associated with parent material and several parameters such as soil properties, plant properties and interaction between the roots and microbial communities (Jones, Hodge, et al., 2004). In literature, the concentrations of micronutrients are generally higher in the topsoil and show a decreasing trend with soil depth (Gupta, Kening, and Siyuan, 2008). Both GDGT and PLFA concentrations given in Figures 3.5 and 3.8 are highest in topsoils and decreases with soil depth because the required nutrient source (C, N, O) for microbial communities is getting lower in deeper regions. Although the lower PLFA and GDGT concentrations of deep rooting grass soils in topsoil, their concentrations are higher than shallow root ones at 90 cm depth. Because deep rooting increases the porosity of soil and encourages the vertical leaching of elements as mentioned in §1.11, the nutrients can be supplied for microbial communities in deep regions as mentioned in **[H1]**.

Fungi to bacteria ratio in Figure 3.8 is higher in deep rooting grasses at 90 cm depth because of the resistivity of fungal communities in harsh conditions (Schlatter et al., 2018). Moreover, arbuscular mycorrhizal fungi (AM fungi) lives on the roots of plant and increases the surface area of roots by creating mycorrhizas, which are responsible for water adsorption and nutrient uptake. The presence of AM fungi might be the reason of higher metal ion concentrations (such as Ni, Ca) in deep rooting grass soils (Figures 3.9 and 3.10).

Rhizodeposition, which is the release of organic compounds from plant roots, affects the distribution of microbial communities in the soil profile and is associated with nutrient uptake (Dennis et al., 2010). The root exudates have chelating ability for specific ions in soils. Fe concentration in both shallow and deep root grasses increases with soil depth and this might be explained by the chelator production ability of roots to chelate Fe cations. Mn plays a vital role in plants and its dynamics are also similar to Fe and it is abundant in acidic and topsoils (Dotaniya et al., 2015). Mn concentration in both grass types decreases with soil depth with

the topsoil having higher concentrations than the subsoils (50 and 90 cm). Because the pH of all soils is around 6, the decreasing trend of Mn is understandable (the pH data can be found in Appendix Table A3.1 supplied by Dr Heather Buss). Zn concentration in both grass types increases with soil depth because plant roots make it possible to release Zn chelators which enhance the mobility of Zn cations (Cakmak et al., 1996). Thus, **[H4]** is supplied by these findings.

Moreover, the carbon rich root exudates facilitate the microbial communities in deeper regions and these microorganisms accelerate the weathering of minerals by proving our hypothesis **[H2]**. Weathering reactions create porosity, form soil and help to release essential micronutrients (Frings et al., 2019). Chemical weathering occurs by the interaction of minerals with water or organic acids produced by microorganisms as mentioned in §1.11. The presence of microbial communities in deeper regions, especially for deep rooting grass soils, contributes the weathering processes in these regions by organic acid production as well as the water supply by deep roots. This contribution of microbial communities results in a slightly increase in the concentrations of Fe, K, Ni and Zn ions in Figure 3.9.

Because of anthropogenic activities, hazardous heavy metals contaminate soils, atmosphere and aquatic environments worldwide and the distribution of these elements is needed to clarify the contamination level in soils (Fujikawa et al., 2000). The adverse effect of heavy metals on soil microbial communities has been mentioned in various studies and the predominance of Gram negative to Gram positive bacteria has been found in metal-contaminated soils (Doelman, 1985; Duxbury, 1985; Frostegard et al., 1993). The higher concentration of Gram-negative bacteria in topsoil (Figure 3.8) may arise from high Al, As, and Cr concentrations in the topsoil (Figure 3.10). Moreover, fungi are less sensitive to heavy metals than bacteria (Doelman, 1985) and the results show that fungal biomass, under both grass types, is higher than Gram-positive and actinomycetes (Figures 3.8).

3.5. Conclusion

This study provides an insight into the distribution of microbial communities and micronutrients through soils chosen from three different depth, and also the effect of two different grass types was investigated. The results states that the highest diversity of both PLFAs and GDGTs were observed in topsoils whilst there is a decrease in the microbial community size with depth due to the limited nutrient sources in deeper regions. Moreover,

deep root grasses provide higher microbial community size at 90 cm depth than the shallow root grasses that is to say deep roots enable the mobility of microbes and nutrients through soil profile. Also, the presence of roots and microbial communities in soil profile improves the soil structure by forming pores and aggregates that results in, leaching and vertical distribution of nutrients through soil profile. Moreover, the activity of microbial communities is improved by root exudates and consequently, the weathering of minerals is accelerated in deeper regions. The chelating ability of root exudates increased the Fe, K, Ni and Zn concentrations at 90 cm depth. However, further research is required to gain a deeper understanding about the relation between nutrient dynamics and microorganism patterns through the soil profile.

4. EFFECT OF PH AND MANURE ADDITION ON NUTRIENT AVAILABILITY AND MICROBIAL ACTIVITY

4.1. Aims and objectives

Addition of mineral fertilisers, manure and compost to the soil microbial communities generally results in an increase in microbial biomass due to increases in the supply of nutrients and/or carbon to the soil. In addition, fertilisation has an indirect effect by altering soil pH. Zhang et al. noted that application of fertilisers indirectly affects the soil bacterial diversity by changing the soil pH more than the effect of direct input of nutrients (Zhang, Shen, et al., 2017). For example, it is known that soil acidification decreases the bacterial diversity after nitrogen fertilisation applications (Zeng et al., 2016).

Research conducted on soils from the Hoosfield and Park Grass experiments at Rothamsted Research, Harpenden, UK demonstrated that the pH of soil is the main parameter that controls microbial activity (Pietri et al., 2008; Rousk, Brookes, and Baath, 2009; Rousk et al., 2010; Zhalnina et al., 2015). It has been shown by the Rothamsted experiments that bacterial growth is favoured in neutral or slightly alkaline conditions while acid pH is optimal for fungal growth (Rousk, Brookes, and Baath, 2009). Moreover, Pietri et al. tested the effect of pH changes or substrate input on the soil microbial community and, from the results of PLFA analysis, concluded that populations of bacteria and fungi in soils are affected by both soil pH and substrate input (Pietri et al., 2009). For example, liming raises the pH of soil by increasing the availability of C and N to the microorganisms. An increase in total fungi biomass was reported with reduced microbial biomass in soils with increased pH (Fritze, 1991).

The aim of this Chapter (which was mentioned in § 1.13) is to assess the microbial community and micronutrient content, in soils of identical origin with differing pH, whilst incubating with and exogenous input of sheep-manure and investigating leaching nutrients from manure to soil. Micronutrients are essential for both animals and human but most plants are deficient in terms of micronutrients. Soil pH has a significant effect on the preservation of soil biomass. The microbial communities fundamentally affect soil chemistry; hence any such activity has the potential to alter the bioavailability of nutrients. In addition, micronutrients are removed from soil as plants are harvested or grazed, but manure can return a fraction of

the removed nutrients. Thus, understanding the effect of pH on the relationship between microbial communities and micronutrients and getting fundamental information about what happens when soil is amended by a nutrient source is essential to optimising the plant bioavailability of micronutrients. Figure 4.1 shows the steps for this part of the study.

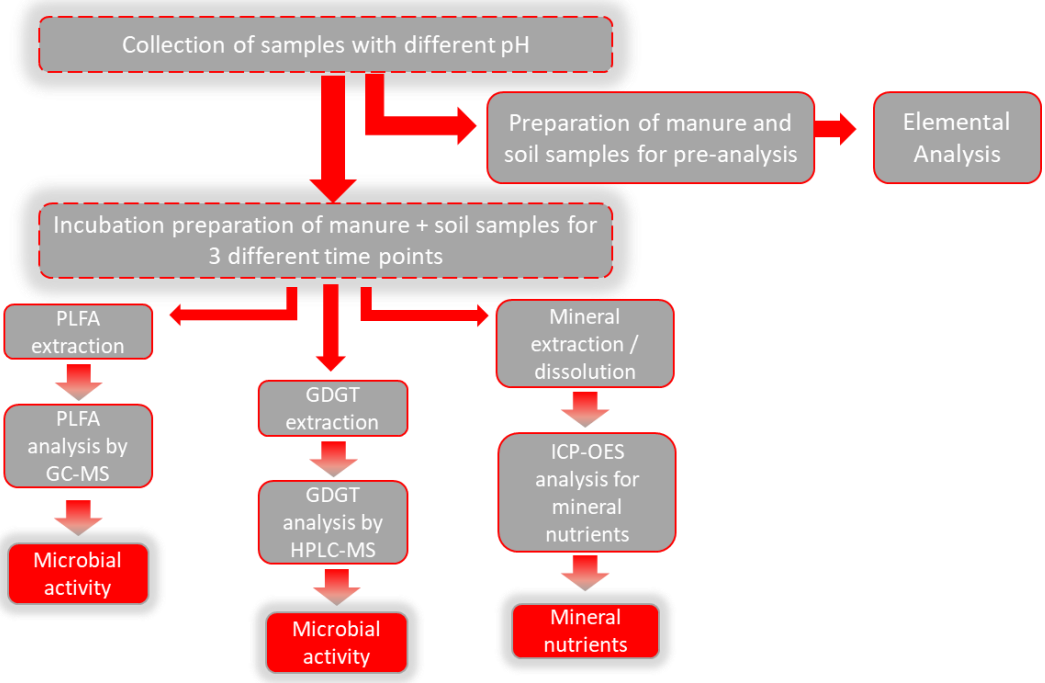

Figure 4.1 Flowchart showing the experimental steps.

4.2.Key aspects for materials and methods

The soils were collected from the Park Grass Experiment at Rothamsted Research, Harpenden, UK, and a total of 60 samples were analysed. Park Grass is the world's oldest permanent grassland experiment and includes treatments initiated in 1856 including controls and various combinations of P, K, Mg, Na, with N application. For this investigation, plot 9 in receipt of P, K, Na, Mg and N as either ammonium sulphate or sodium nitrate was selected. It has 4 sub-plots, which were divided in 1965 to extend the pH range on each treatment (Macdonald et al., 2018). Four sub-plots (9/1a, 9/1b, 9/1c, 9/1d) having same fertilisation type, but different pH values (7.1, 6.4, 5.2, 4.1) were been selected and from each plot nearly 1 kg of soils were collected. An auger was used to collect samples from 23 cm depth and cores for each plot were collected in an aluminium can. The soils were then wrapped with furnaced aluminium foil and retained in plastic bags. Figure 4.2 shows the

location of sampling site and the photographs of selected plots. After storing the samples in fridge overnight, all visible vegetation was removed from the soils that were then spread on aluminium foil and left to air dry (Figure 4.3a).

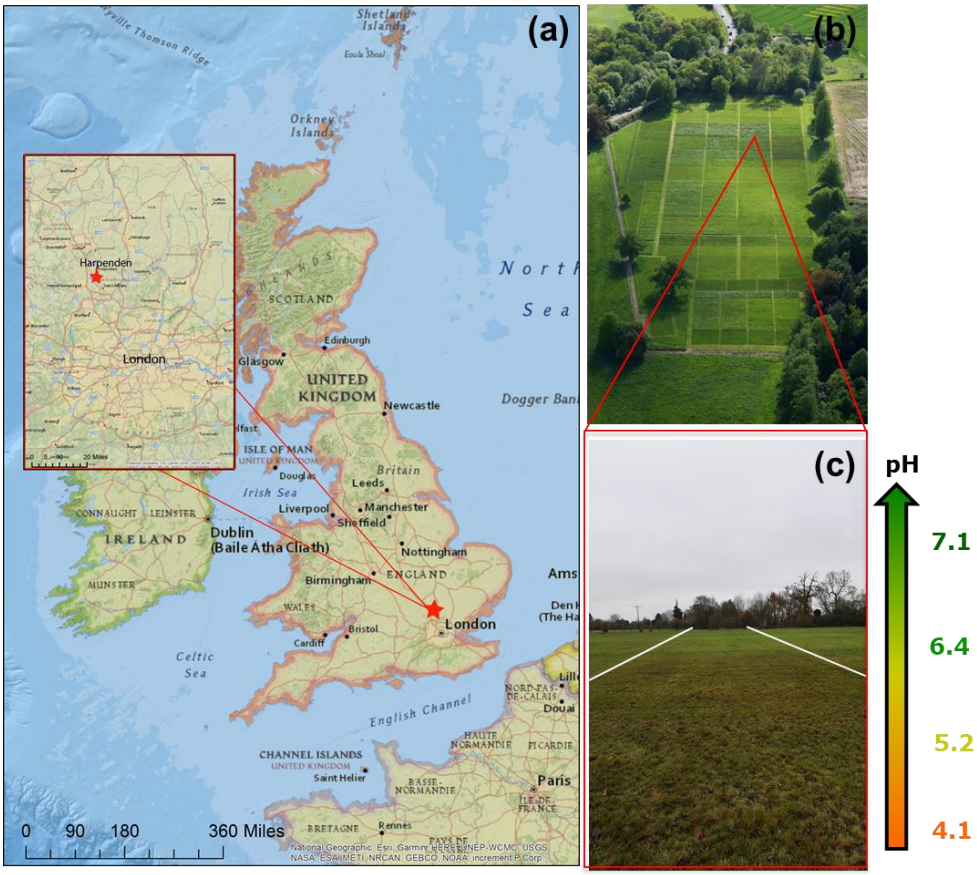

Figure 4.2 (a) The maps showing the location of Park Grass, Rothamsted Research sampling site that is denoted with a red star and maps were created using ArcGIS® software by Esri (ArcGIS® and ArcMap™ are the intellectual property of Esri and are used herein under license. Copyright © Esri. All rights reserved. For more information about Esri® software, please visit www.esri.com). **(b)** The photograph of Park Grass in Rothamsted Research, Harpenden (the electronic Rothamsted Archive. www.era.rothamsted.ac.uk/Park Access date: 02.12.2019), and **(c)** the appearance of Plot 9/1.

Once dry, soils were sieved through a 2 mm grating to remove rocks, roots, and big aggregates (Figure 4.3b). The sieved soils were placed into plastic bags, tied with rubber bands and a piece of paper on top of bag to prevent soil respiration. All soils were stored in a fridge at -20˚C. The residual pieces (> 2 mm) were covered with aluminium foils and stored at fridge**.**

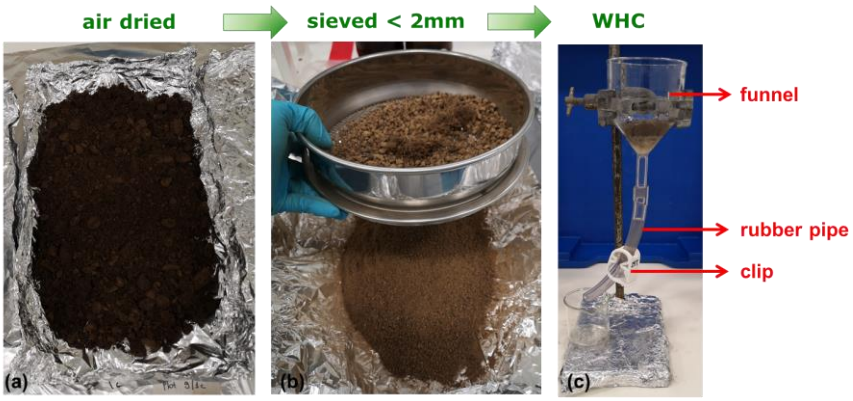

Figure 4.3 Photographs of soils **(a)** air drying, **(b)** sieving by 2 mm sieve and **(c)** WHC measurement.

The dry matter content (DMC) was calculated by weighing three replicates of 10 g of the soil, in pre-weighed glass petri dishes, from each plot. The soils were dried in an oven at 105 ˚C for 24 h and the final weight of petri dishes were determined. Percentage DM and percentage moisture contents (MC) were calculated by using Equations 4.1 and 4.2:

$$\%\, DMC\ = \left(\frac{\text{dry wright of soil}}{\text{fresh weight of soil}}\right) \times 100 \qquad 4.1$$

$$\%\, MC\ = 100 - \%\ \text{DMC} \qquad 4.2$$

Soil water holding capacity (WHC) was determined volumetrically by using a funnel with a rubber pipe and clip so that the pipe could be sealed shut (Figure 4.3c). The funnel was plugged with 0.3 g of glass wool and the WHC of glass wool + funnel was calculated in triplicate. After shutting the clip, 10 mL of water was added and left for 30 min. Then, the clip was removed, water was drained by waiting 30 min and the volume of drained water was measured. The same procedure was applied by adding 10 g of soil on the top of glass wool. All measurements were carried out in triplicate for each plot. The WHC of each plot was determined by using Equations 4.3, 4.4 and 4.5:

$$DDW\ retained\ by\ soil\ (A) = 10 - (\text{DDW retained by wool} + \text{DDW colleced}) \qquad 4.3$$

$$WHC\ (ml\ per\ 100\ g\ of\ fresh\ soil; B) = (10\ \times \text{A}) + MC \qquad 4.4$$

$$WHC\ (ml\ per\ 100\ g\ of\ oven\ dried\ soil) = (\frac{B}{\%\ DMC}) \quad 4.5$$

Where MC is the volume of water in fresh soil calculated by multiplying %MC by the mass of soil (in this case 100 g). Table 4.1 shows the calculated results for DMC, MC and WHC in soils from the different pH plots.

Table 4.1 Calculated results of dry matter content (DMC), moisture content (MC) and water holding capacity (WHC) of soils.

pH	% DMC	% MC	WHC
7.1	71.89 ± 0.63	28.11 ± 0.63	66.89 ± 5.49
6.4	72.50 ± 0.63	27.50 ± 0.63	67.77 ± 9.08
5.2	70.24 ± 0.88	29.76 ± 0.88	75.63 ± 6.18
4.1	77.31 ± 1.19	22.69 ± 1.19	53.12 ± 5.98

Before starting the incubations, total carbon (C), nitrogen (N) and sulfur (S) contents of the soils were determined by elemental analyser. In addition, the application rate of sheep manure was calculated by using the N content in sheep manure. Table 4.2 summarizes the initial C, N and S content of soils and manure.

The soil incubations were carried out as described in Reay (2019) (Reay, 2019). Incubation tubes were prepared by plugging the bottom of end with a piece of glass wool to support the soil whilst preventing aerobic conditions and nearly 20 g of dry soil was added to the tubes which were then covered with pierced aluminium foil to reduce any evaporation (Figure 4.4). A control for each time-period was also prepared, all soils were prepared in triplicate. Before starting the incubation with manure, a pre-incubation was carried out to reduce any priming effect, defined as 'the increase in soil organic matter (SOM) decomposition rate after fresh organic matter input to soil', and to activate existing microbial communities (Fontaine et al., 2003; Yue et al., 2016).

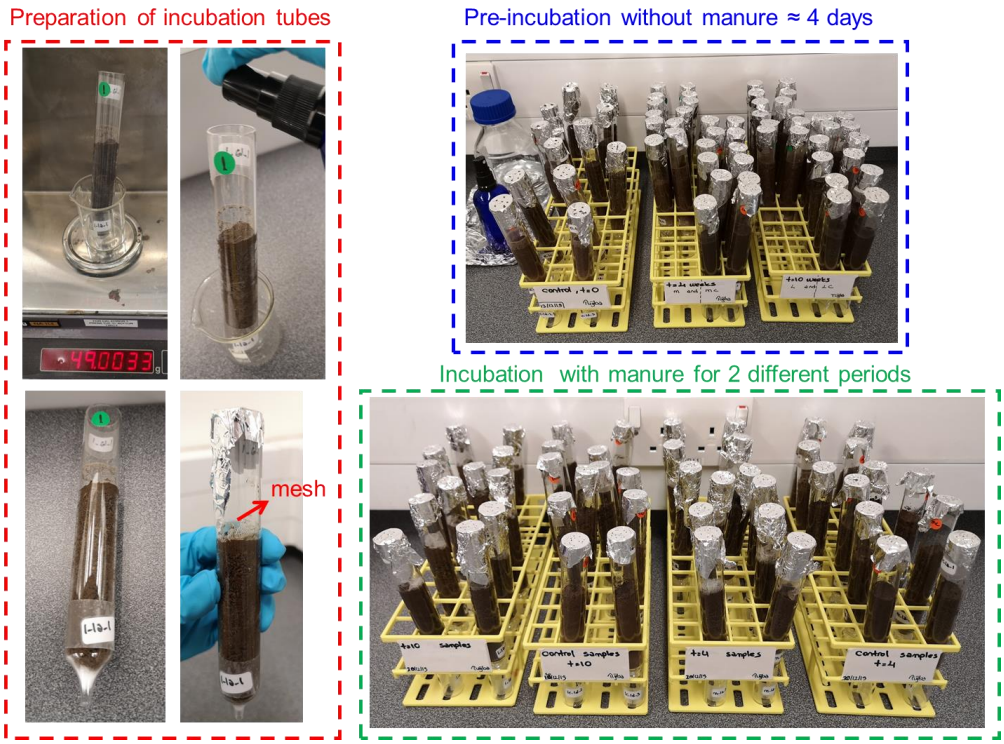

Figure 4.4 The photographs showing the preparation of incubation tubes by separating the manure and soil with a plastic mesh, set-up for pre-incubation and incubation with manure for t=5 and t=10 weeks.

The soils were wetted to 50% of their WHC and left for 5 days in a temperature controlled dark room at 20 ˚C. After pre-incubation, sheep manure was added on the top of the incubation tubes (for t=5 weeks and t=10 weeks) with 3.5% application rate (0.7 g homogenised sheep manure for 20 g soil) and a plastic mesh was placed between sheep-manure and soil to make easier the removal of manure after incubation. The control soils for t=5 weeks and t=10 weeks tubes were left without adding any manure.

Table 4.2 Measurements of carbon, nitrogen, sulfur and carbon to nitrogen ratio (C/N) in soils from different pH and sheep manure.

pH	N [%]	C [%]	S [%]	C/N
7.1	0.44 ± 0.01	4.94 ± 0.16	0.02 ± 0.01	11.29 ± 0.03
6.4	0.34 ± 0.01	4.08 ± 0.15	0.04 ± 0.01	12.14 ± 0.14
5.2	0.41 ± 0.01	5.46 ± 0.28	0.06 ± 0.01	13.36 ± 0.25
4.1	0.33 ± 0.10	4.27 ± 0.10	0.12 ± 0.01	13.14 ± 0.29
sheep manure	2.25 ± 0.10	42.44 ± 1.02	0.56 ± 0.01	18.89 ± 0.35

The application rate of manure was calculated as follows: The dry matter in faeces was known to be 22% and according to the EA result, nitrogen amount in dry manure was 2.25 ± 0.1 % (w/w). In that case, the nitrogen amount in fresh sheep manure (without drying) was calculated as 0.49 %. According to Defra, there is 100 kg-N $\cdot ha^{-1}$ is needed for hay and our aim should be to supply nearly 50 – 60% of this requirement (DEFRA, 2010). The available N from manure is known as 5-10% after application and the requirement for N application was calculated as nearly 110 t $\cdot$ ha^{-1}. By assuming 1 ha of soil (20 cm depth and 1.6 g $\cdot$ cm^3) is nearly 3200 t $\cdot$ ha^{-1}, the required application rate was determined as 3.5 %.

Before starting the incubation, the soils were re-wetted to 50% of WHC and then left in the same incubation room for 5 or 10 weeks. Throughout the incubation period, the soil was maintained at 50% WHC by weighing the tubes every 3-4 days and re-wetting with DDW.

After 5-day pre-incubation, the control soils for t = 0 (first time-period of study) were separated. The incubation was halted by immersion of tubes in liquid N_2 just after pre-incubation for t = 0 soils, and after 5 and 10 weeks for other soils to prevent further N transformations. The soils were freeze-dried and ground to homogenise them (Figure 4.5). All soils were stored in freezer at –20 ˚C.

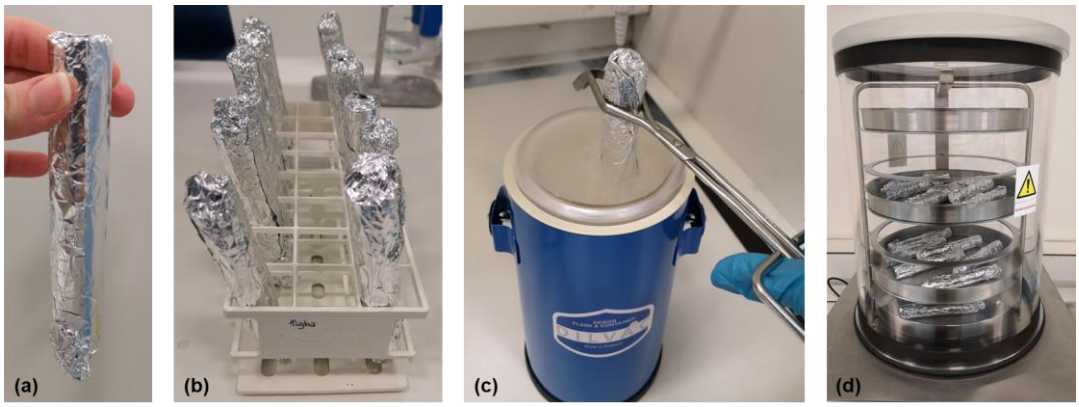

Figure 4.5 (a-c) Stopping the incubation by immersing the tubes in liquid nitrogen and **(d)** freeze-drying by lyophilisation.

4.3. Results

4.3.1. Bulk soil percentage TC, TN and TS

Bulk %TC, %TN and %TS for soils, with different pHs, across the time course with manure application are shown in Figure 4.6. The effect of manure addition is generally not significant

such that significance levels are generally above 0.05 (Table A4.1). For the acidic soils (pHs 5.2 and 4.1), there is an increasing trend in %TC with time while for neutral soil, pH 7.1, the %TC decreases even below the initial level at t=0. For the pH 6.4 and pH 4.1 soils, there is a significant decrease of %TC with manure addition (ANOVA, $P<0.04$). For control soils with pHs of 6.4 and 4.1, there is a decrease after 5-weeks incubation followed by an increase above t=0 levels (Figure 4.6a). For pH 5.2, there is a constant increase with time for both control soils and those amended with manure.

The change in %TN infers the operation of different pathways. The %TN of soils at pH 7.1 shows a decreasing trend with manure addition. Additionally, control soils at the same pH have exhibit a decreasing trend in nitrogen (Figure 4.6b). Manure application to the pH 6.4, 5.2 and 4.1 soils results in an increase in %TN. The %TN in the control soils at pH 6.4 stays the same with time whilst there is a constant increase in the control soils at pH 5.2. For the control soils at pH 4.1 a drop in first 5 weeks below the t=0 level followed by an increase back to t=0 after 10 weeks is observed. The percentage carbon-to-nitrogen (C/N) ratios for all soils provided given in Table A4.4.

In terms of %TS results, there is a constant decrease with time in soils from pH 7.1. Whilst the control soil has nearly the same %TS after 5 weeks, there is a significant decrease in corresponding manure amended soil (ANOVA, $P=0.0006$). Extending the incubation time up to 10 weeks does not have any effect on %TS for soils at pH 7.1 (Figure 4.6c). %TS for pH 6.4 soils does not change remarkably except for the control soil after 10 weeks of incubation. For the pH 5.2 soils, there is a slight increase after 5 weeks incubation for both control and manure amended soils which is followed by a sharp decrease at 10 weeks. Manure application for the pH 4.1 soils increases the %TS at 5-weeks (ANOVA, $P=0.0448$) but decreases %TS level at 10 weeks.

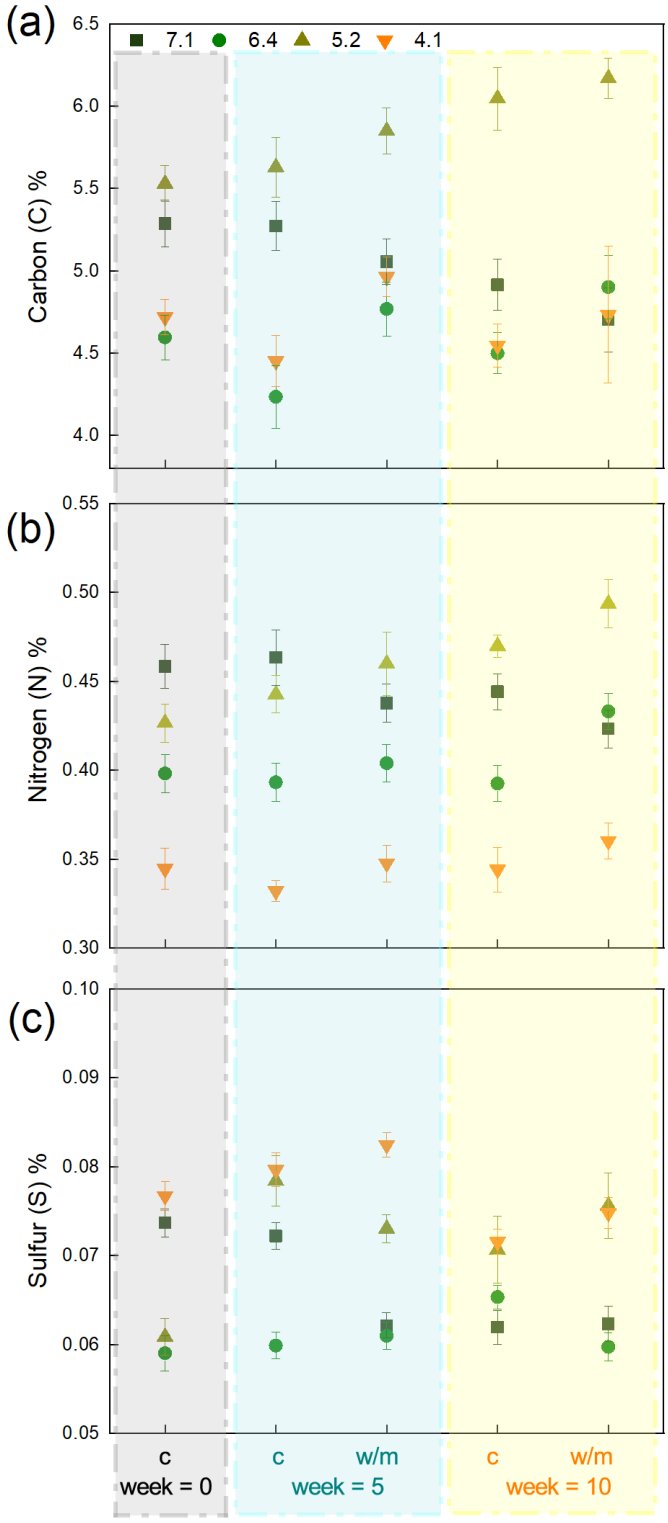

Figure 4.6 Percentage **(a)** total C, **(b)** total N, and **(c)** total S for soils from pH 7.1 (■), 6.4 (●), 5.2 (▲) and 4.1 (▼) for different time periods (see Appendix 2 for details about significance levels). Control and manure applied soils were denoted as 'c' and 'w/m', respectively.

4.3.2. Phospholipid fatty acids (PLFAs)

Figure 4.7 shows the change of total PLFA concentration in the control and 5-week incubation soils with and without manure application. The GC-MS chromatograms of all replicates for the control and 5-week incubated soils are provided in Appendix 2. Control soils exhibit a significantly higher amounts of PLFA compared to the soils incubated for 5 weeks except for the soil with at pH 4.1 (ANOVA, $P<0.00005$). The total amount of PLFAs for pH 7.1, 6,4 and 5.2 soils are about the same whilst the soil at pH 4.1 has lower total amounts of PLFAs. Moreover, it is clear that the effect of manure application does not have any significant (ANOVA, $P>0.05$) influence on PLFA concentrations for nearly neutral soils (pH 7.1 and 6.4) at t=5 weeks. However, PLFA amounts decrease for the 5-week manure application for acidic soils (pH 5.2 and 4.1) significantly (ANOVA, $P<0.001$) relative to the unmanured soils.

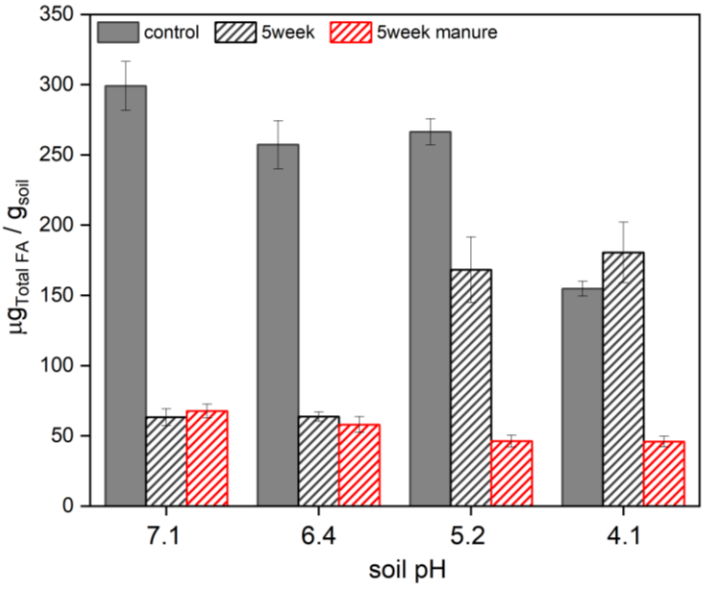

Figure 4.7 Concentration change of PLFAs with pH and manure application with time (see Appendix 2 for details about significance levels).

Gram-positive and Gram-negative bacteria concentrations in the different pH soils and the effect of manure application on these concentrations are given in Figure 4.8. Control soils have higher amounts of PLFA for the pH 7.1, 6.4 and 5.2 soils than the those from pH 4.1. The effect of manure application is not significant for pH 7.1 and 6.4 (ANOVA, $P>0.05$), whilst there are significant changes in PLFA concentrations after 5 weeks of manure incubation for pH 5.2 and 4.1 (ANOVA, $P<0.0001$). The Gram-positive bacteria increase

after 5 weeks of incubation even in the control soils and a similar increase is seen in for the Gram-negative bacteria in the soil at 5.2.

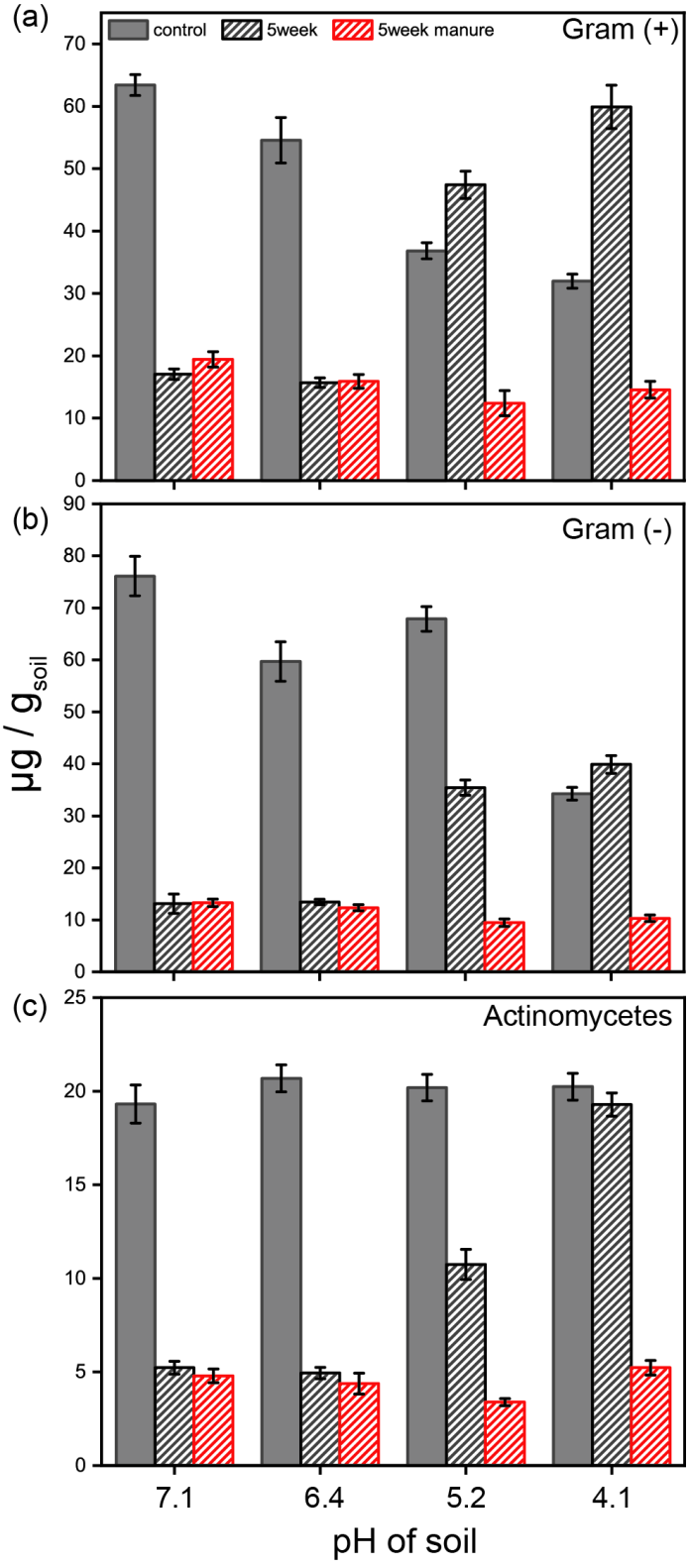

Figure 4.8 Change in PLFA levels for Gram-positive, Gram-negative and actinomycetes with pH and manure application with time (see Appendix 2 for details about significance levels).

The amount of actinomycetes is similar to that in the control soils across the pH range before incubation (Figure 4.8). However, they decline dramatically in the pH 7.1 and 6.4 soils after the 5 week incubation and the manure application appears to have no effect on actinomycete concentration ($P>0.05$). For pH 5.2 and 4.1, the amount actinomycetes decreases after 5 weeks and manure amendment enhances this still further (ANOVA, $P<0.0005$). Figure 4.9 shows how the fungal biomass changes with pH and over five weeks. The fungal biomass is high in pH 7.1, 6.4 and 5.2 soils while the lowest concentration is observed at pH 4.1. Incubation of pH 7.1 soil with manure does not have any significant effect on fungal biomass (ANOVA, $P>0.05$) and for pH 6.4, there is a slight decrease with manure application (ANOVA, $P=0.0260$). Fungal biomass decreases dramatically in the pH 5.2 and 4.1 soils after 5 weeks of incubation with manure.

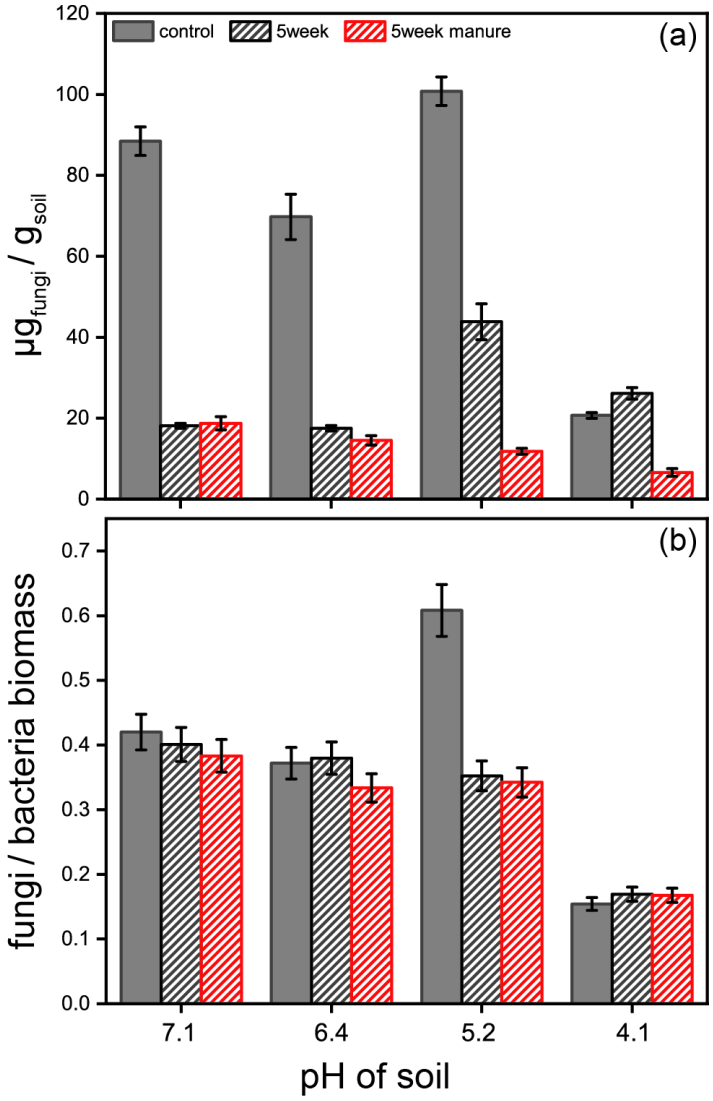

Figure 4.9 Concentration change of fungi and fungal-to-bacteria ratio with pH and manure application with time (see Appendix 2 for details about significance levels).

The fungal to bacteria biomass ratio (F/B) ranges are similar for 7.1, 6.4 and 4.1 pH plots in that there is not any significant change even after 5-week incubation (Figure 4.9). For the soil at pH 5.2, the highest F/B is observed for control soil and after the 5 week incubation period, that ratio is decreased; and manure application does not produce a significantly different result (ANOVA, $P>0.05$).

4.3.3. Glycerol dialkyl glycerol tetraether lipids (GDGTs)

The changes in GDGT concentrations with soil pH are given in Appendix (Figure A4.4). isoGDGTs and brGDGTs show different trends with soil pH. From pH 7.1 to 6.4, isoGDGTs show an increasing trend and the highest isoGDGT amount is observed at pH 6.4. The isoGDGT concentration then decreases with decreasing pH. Conversely, the lowest brGDGTs concentrations are observed at pH 6.4 and the concentration significantly increases the in pH 5.2 and 4.1 soils (ANOVA, $P<0.04$).

4.3.4. Mineral nutrients

The concentration of selected nutrients shows different trends for each element with changing pH and incubation time. Figures 4.10 and 4.11 show the change in Ca, Cu, Fe, K, Mg, Mn, Ni and Zn concentrations with pH and the effect of incubation with manure at different time points. Red bars indicate the results for soils, which were amended with sheep manure whilst grey bars represent the results for the control soils. Ca concentration decreases sharply with pH in that the neutral soil has the highest Ca amount. When the effect of manure addition is considered by comparing control and manure applied soils, there is not significant difference except for the pH 4.1 and 7.1 soils (ANOVA, $P<0.02$). Moreover, the incubation period has a remarkable effect on Ca concentration in that the 10-week incubation increased the amount of Ca compared to the 5 week incubation at pH 7.1 (ANOVA, $P=0.0004$).

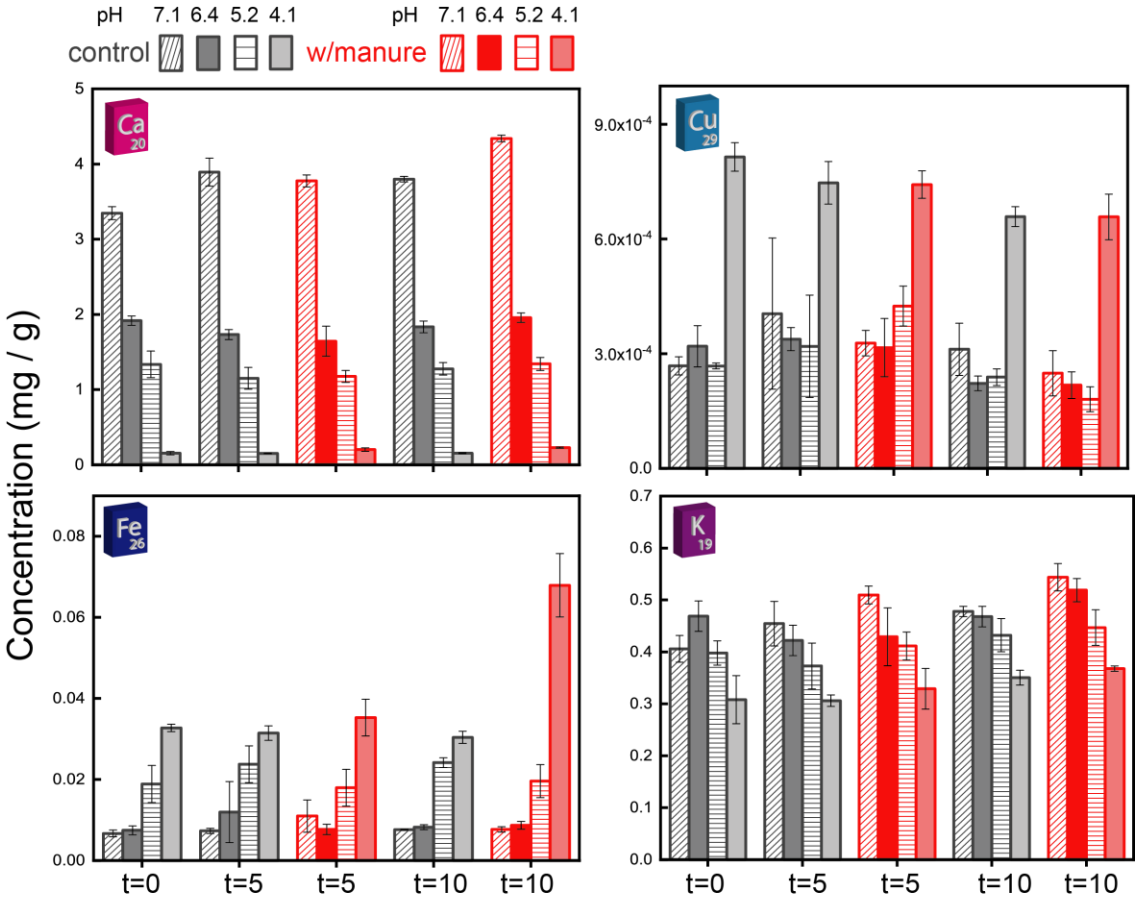

Figure 4.10 The concentration of plant essential micronutrients (Ca, Cu, Fe, K) with depth (see Appendix 2 for details about significance levels).

Conversely to Ca, the concentration of Cu increases with decreasing pH in that the highest Cu concentration was observed at pH 4.1. The manure addition does not make any significant effect to Cu concentrations in that both control and manure applied soils, for all pH plots, have similar amounts of Cu (ANOVA, P>0.05). In terms of the effect of incubation period, the Cu concentration in soils at pH 5.2 decreases with time (ANOVA, P=0.0015). The concentration of Fe gradually increases with decreasing pH as seen in Figure 4.10. Whilst control soils have nearly the same amount of Fe and there is not any significant change between the control and manure applied soils for all pH plots, Fe concentration rises sharply after at 10 week incubation point for pH 4.1 soils (ANOVA, P=0.0007). Furthermore, the incubation period is effective for pH 4.1 soils in that there is a significant difference between 5 week and 10 week incubation points (ANOVA, P=0.0023). For K concentration, the decrease in soil pH results in a decline in K. There is a significant difference in the amount of K between control and the 10 week incubated soils from the pH 7.1 and 6.4 plots (ANOVA,

P=0.0154 and 0.0411, respectively), whilst other soils do not have any noteworthy change. There is not a large effect of incubation time on the amounts of K in soils for all pH plots.

The trend of change in Mg concentration with pH is similar to Ca concentrations in that the lowest Mg amounts are observed at pH 4.1 (Figure 4.11). For the 5 week incubation soils, there are no significant changes between the control and manure amended soils except for the pH 4.1 plot in that manure has an additive effect on the amount of Mg. However, the difference between the control and the 10 week incubated soils is significant for all pH plots where manure application has a remarkable effect on the amount of Mg in soils after 10-weeks of incubation (ANOVA, P<0.02).

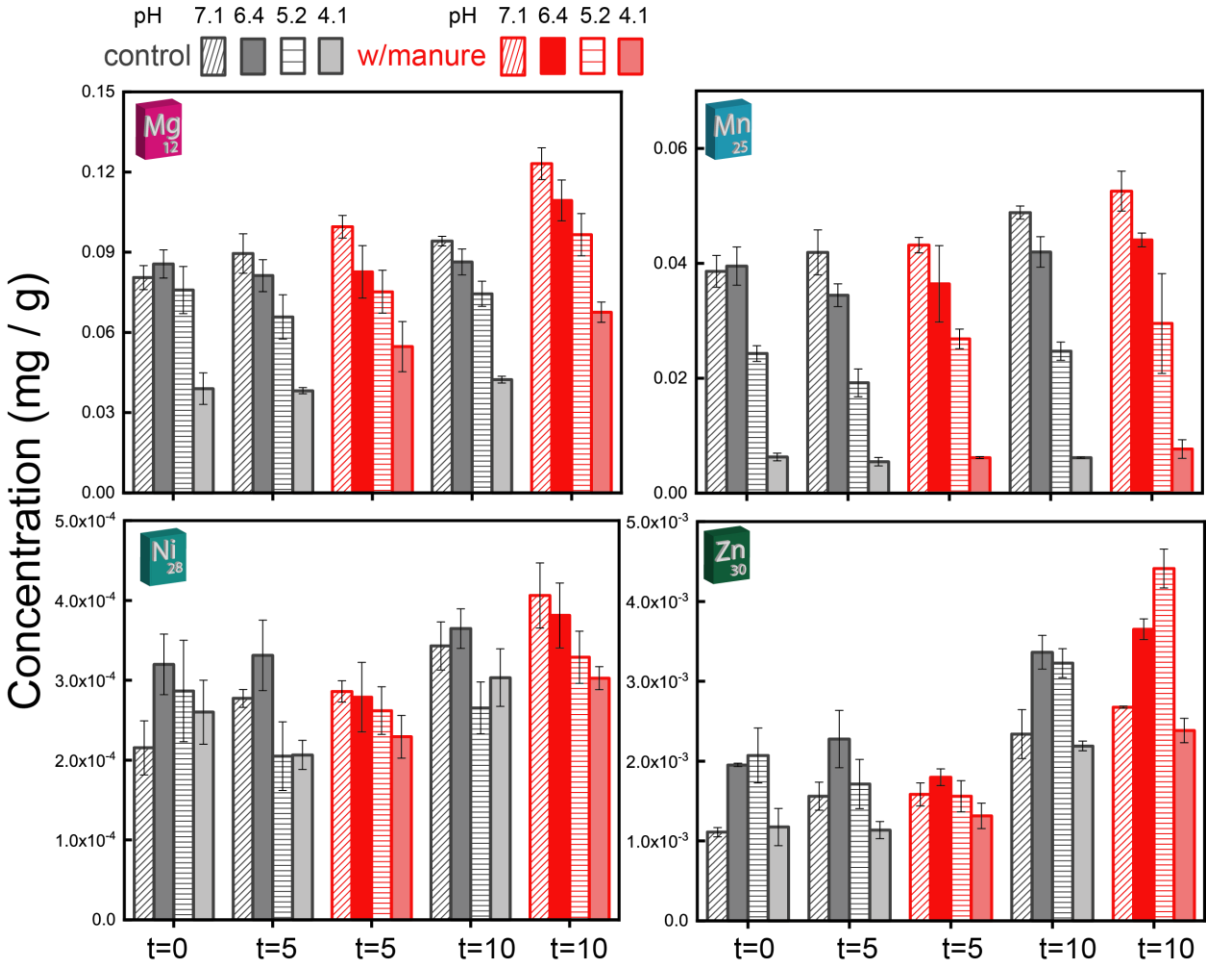

Figure 4.11 The concentration of plant essential micronutrients (Mg, Mn, Ni. Zn) with depth (see Appendix 2 for details about significance levels).

The effect of incubation time on Mg concentration is significant for the pH 7.1 and 6.4 soils, increasing incubation period enhances the leaching of Mg ions through the soil (ANOVA, P=0.0050 and 0.0206, respectively). The concentration of Mn decreases with acidity with a

sharp decrease observed after pH 6.4. However, the effect of incubation with manure does not have any significant effect on levels on Mn in that the concentration change in the control and manure amended soils is not significant for all pH plots (ANOVA, $P>0.05$). The effect of incubation time is clear for the pH 7.1 soils with an observed enhancement between the 5 and 10 week points (ANOVA, $P=0.012$).

Zn concentration in the different pH soils follows a different trend whereby Zn concentration firstly increases and then decreases after pH 5.2. The highest Zn amounts were observed at around pH 6.4 and 5.2 for all soils (Figure 4.11). The effect of incubation time on Zn amounts is significant for the pH 7.1, 6.4 and 4.1 pH plots with the longer incubation period improves amount of Zn (ANOVA, $P<0.0005$). However, the effect of manure is not significant with little observable difference between the control and manure amended soils (ANOVA, $P>0.05$).

4.4. Discussion

Leaching compounds from manure to the soil directly affects the microbial communities in soils by supplying nutrients, but it is indirectly effective by altering soil pH as given in §1.13 [**H5**]. Therefore, the addition of manure to soils is expected to increase the available nutrients and microbial activities. The effect of sheep-manure amendment in soils from different pH was observed in terms of microbial community diversity and plant availability of micronutrients [**A3**]. The plant availability of micronutrients is decided by using LMWOSs to mimic the real conditions in plant-soil interactions. The alterations in soil pH affect the solubility and mobility of micronutrients as well as the presence of different microbial communities through the soil profile as outlined in §1.13 [**H6**] and [**H7**]. Moreover, it is shown that the microbial communities effect the availability of micronutrients by changing the soil structure in different ways [**A4**].

Organic compounds in soils are influenced by soil pH because pH regulates the nutrient bioavailability and OM turnover in soils (Zhou et al., 2019). According to elemental analysis results given in Figure 4.6, soil pH affects the TC, TN, and TS percentages in soils. The TC% of soils from pH 5.2 plot shows a constant increase both with time and manure application. The reason of increase after manure application is the nutrients supplied from sheep-manure. However, the increase in control soils with time might have another mechanism that the presence of extracellular enzymes is responsible about this process. When soil becomes dry,

microbial activity slows down and biomass synthesis reduces. The addition of water into soils during the incubation period re-activates the enzymes in dry soil (Sardans et al., 2010). The soils from pH 5.2 have a high moisture content (%MC) (Table 4.1) and drying of these soils for EA analysis deactivated the enzymes that results in a reduced TC%. During the soil incubation, adjusting the water levels activates the enzymes again and the increase in TC% was observed (Figure 4.6a). Conversely, TC% in soils from pH 7.1 decreases with time and manure addition. The decrease in TC% can be explained by soil respiration, which is CO_2 production by biological active soil organisms. When manure begins to breakdown and increases the biomass production, the respiration rate increases in short term but the positive impact is observed after long term applications (USDA, 2020). Moreover, soil respiration is more effective at high soil pH (Rousk, Brookes, and Baath, 2009) and also calcium carbonate content in high pH soils favours the carbon mineralisation, which is the CO_2 production by the contribution of inorganic carbon (Stevenson et al., 2006). These results are consistent with literature that soil pH is negatively correlated with TC and TN percentages in soils because of the organic matter accumulation in low pH soils (Zhou et al., 2019). Additionally, the carbon loss via respiration and carbon gain via nutrient leaching from manure seem balanced for pH 6.4 and 4.1 soils that they have a nearly steady TC% through incubation period.

The addition of manure on pH 6.4, 5.2 and 4.1 plots improved TN% with time because of the leaching nitrogen from the sheep-manure. However, addition of nitrogen to soil may have different effects on soil respiration that nitrogen input increases soil respiration due to the excess increase in microbial activities (Burton et al., 2002). Our results showed that at pH 7.1, the TN% decreases with time and manure addition (Figure 4.6b).

pH affects all chemical, physical and biological processes in soils therefore, it is effective on the microbial community (Pietri and Brookes, 2008; Rousk et al., 2010). Recent studies suggest that soil pH is one of the main predictors of the microbial diversity in soils and that the highest bacterial diversity is often found in neutral soils (Fierer et al., 2006; Lauber et al., 2009; Zhalnina et al., 2015). Control soils (t=0) have significantly higher total amounts of PLFAs compared to the soils sampled after 5 weeks of incubation (Figure 4.7). The reason of this difference might be the instability of soils even after the 5 day pre-incubation period (Steen et al., 2016; Razanamalala et al., 2018).

The reason for the low PLFA amounts at pH 4.1 might arise from the peat formation on the top layer (nearly first 4 cm) of the pH 4.1 plot; experiments for this plot were conducted on the soil layer from 4 to 23 cm. Peat forms when the plant materials are partially decomposed in acidic conditions (Efretuei, 2016). Amblés et al. attributed the accumulation of fatty acids in acidic soils due to the lack of iron hydroxide or specific clay minerals (Ambles et al., 1989). Since the majority of the organic matter content is found in peat and the peat is not considered in this study, the lower PLFA concentration is understandable.

Actinomycetes are a sub-group of Gram-positive bacteria and they are widely distributed in various environments. Although they are known as neutrophils (preferring neutral conditions to grow), some of these bacteria grow in acidic conditions (Poomthongdee et al., 2015). Actinomycete concentrations after 5 weeks incubation of the control soils at pH 4.1 and 5.2 are significantly higher than the other pH plots but manure amendment decreases their concentrations significantly (Figure 4.8). The acidophilic actinomycetes might be the reason of this high concentration difference (Poomthongdee, Duangmal, and Pathom-aree, 2015).

It is uncertain whether it is nutrient supply or pH is the strongest determinant of the final microbial community structure in soils. Zhang et al. also stated that fertilizer applications shape the microbial community by changing pH rather than the direct input of nutrients in soil (Zhang, Shen, et al., 2017). Sheep-manure is a rich material in terms of essential nutrients, which are required for plant growth. It contains N, P, and K as well as the micronutrients such as Ca, Cu, Fe, Mg, Mn, Mo, S and Zn that they originated from the feed that the sheep has eaten. These nutrients are found in a mixture of inorganic and organic forms that inorganic ones are water soluble and plants can uptake them right away whist organic forms became available after degradation of manure by microbial communities (Parvage et al., 2015; Schoenian, 2019). Thus, the leaching of inorganic – water soluble – compounds in sheep manure is expected after the incubation experiments. Moreover, sheep-manure is generally alkaline (around pH 8-12) and does not make any significant change to the pH of nearly neutral soils (pH 7.1 and 6.4). However, acidic soils (pH 5.2 and 4.1) are affected by the addition of alkaline manure with dramatic changes before and after the incubation period (Figures 4.7 and 4.8). Throughout this study, manure application did not show any influence on the pH 7.1 and 6.4 plots for all PLFA concentrations, whilst significant differences were observed in pH 5.2 and 4.1 plots. Thus, these results prove **[H5]**.

Rousk et al. showed that neutral and alkaline conditions favour bacterial growth while fungal communities prefer acidic conditions (around pH 4.5) (Rousk, Brookes, and Baath, 2009). According to this literature finding, the high F/B ratio at pH 5.2 for control soils comes from the fungal communities' preference for acidic soils (Figure 4.9). Because the manure application shifts pH to the alkaline region, the F/B ratio is therefore decreased at the 5 week incubation point that establishes **[H7]**.

Soil pH affects the availability of nutrients indirectly by changing the activity of microbial communities, which are responsible for organic matter decomposition. Therefore, the availability of nutrients is affected due to the breakdown of organic matter, which releases the nutrients into the soil in the form of available by plants. Nutrients are usually available around pH 6–7 range and for this reason it is the most convenient medium for the growth of plants (Center, 1998; Norton, 2013; Sullivan et al., 2017).

According to the reports of Rothamsted Research, in 1965, the plot 9 were divided into four subplots that sub-plots 'a' and 'b' have been limed previously that refer to pH 7.1 and 6.4, respectively. Other sub-plots 'c' and 'd'(refer to pH 5.2 and 4.1, respectively) have not been limed. Now, the plots for pH 5.2, 6.4 and 7.1 receive different amounts of chalk when it is necessary to maintain the pH of soils at desired levels (Andy Macdonald, 2018). Liming, which is the application of calcium and magnesium compounds, is used to raise the pH of soil by decreasing proton concentrations (Filipek, 2011). Because acidic soils are deficient in terms of Ca, Mg and K amounts, liming applications increase the productivity by increasing available nutrient concentrations in soils as well as preventing Al toxicity (Han et al., 2019). Figures 4.11 and 12 demonstrate the high concentrations of Ca, K and Mg at pH 7.1 and the decreasing trend as pH lowers. Because high pH plots receive lime, the high concentration of these nutrients is reasonable and consistencies consistent with the literature (Otieno, 2018). Moreover, the available concentration of K is nearly 10-orders of magnitude lower than available Ca concentrations. The reason of this significant difference might arise from the competition between K and Ca ions where K uptake by plants is inhibited by high concentrations of Ca (main component of lime) in soils (Otieno, 2018).

Fe is one of the essential elements for plants that it is required for chlorophyll synthesis and enzyme activation. However, iron is poorly soluble because the salts (ferric and ferrous forms) can hydrolyse to hydroxides rapidly around neutral pH. Although plants can take-up the ferric form from soil, the availability of $Fe^{3+}_{(aq)}$ ions in neutral soils is too low (Panadda

Tansupoa, 2008; Colombo et al., 2014). As seen in Figure 4.11, available Fe concentration is quite low at pH 7.1 and 6.4, whilst it increases at pH 5.2 and 4.1 because acidic conditions promote the mobilisation of Fe ions. Moreover, the oxidation of Fe salts decreases the soil pH by increasing the dissolved sulfate. Therefore, the chemical speciation, relative distribution and chemical forms of Cu and Zn ions change (Reddy et al., 1995). Cu is necessary for the growth of both plants and its retention is significantly correlated with soil pH (Elbana et al., 2011). The mobility of Cu increases in low pH environments due to the change in chemical form of Cu. The results in Figure 4.11 agree with the literature findings that the available Cu concentration in acidic soils is highest while it is quite low in neutral soils. Similarly, available Zn concentration increases with pH decrease and neutral soils are generally Zn deficient (Mertens J., 2013).

The soil's ability to supply nutrients is based on its cation and anion exchange capacities, which are affected by soil pH. The nutrient availability is affected by soil pH because hydrogen (H^+) ions take place on the negatively charged soil surfaces. At high pH, the positively charged small metal ions (such as Cu, Fe, Zn) tightly stick to these negative surfaces and become less available whilst these ions cannot stick to these surfaces at low pH because they are already occupied by H^+ ions that makes these ions more plant available (McCauley et al., 2017). Figures 4.11 and 4.12 demonstrate that Cu, Fe, and Zn ions are more plant available at low pH and their plant availability decreases with increasing soil pH. Conversely, large ions (such as Ca, Mg, K) cannot stick to the walls even at high pH because of their size and they become more available at high pH. However, these ions replace with H^+ ions at low pH and generally they are lost by leaching that makes these ions less plant available. The results indicate that plant availability of Ca, Mg and K ions are highest at high pH whilst their concentration decreases with lowering pH (Figures 4.11 and 4.12). Besides the liming application of high pH plots, the availability of these ions is related with their adsorption behaviours on soil surface. Thus, **[H6]** is established by these findings.

Considering the pH of sheep-manure is in the alkaline region (generally between 8–12), it can be assumed that incubation of soils with sheep-manure raises the pH of soils and changes the soil physical properties. However, the difference between control and manure applied soils is so close that there are no significant differences in most of our samples (see Appendix for details about significance levels). For Mg concentrations, there is a significant increase in all pH plots after the 10 week incubation because of the nutrients supplied by sheep-manure (ANOVA, $P<0.02$).

4.5. Conclusion

This study investigated the effect of soil pH and leaching compounds from sheep-manure on microbial community structure and micronutrient availability. According to the findings, low pH soils tend towards peat formation resulting in high OM accretion on the top layer and, if these top layers are removed, the OM decomposition decreases through soil profile (Efretuei, 2016). Moreover, manure addition affects the microbial community structure indirectly by changing pH and any shifts in pH alter this structure. Since the pH difference between manure and the acidic soils is greater than the difference between the neutral soils, the microbial communities in the acidic soils are affected to a great extent after manure addition. The abundance of different microbial communities depends on their preference to soil pH that fungal communities prefer to live at acidic conditions that the results show a high fungi to bacteria ratio at low soil pH. Moreover, the addition of sheep-manure affects the TC and TN percentages in different ways for soils from different pH plots. The extracellular enzymes take place for the soils from low pH with high moisture content, whilst soil respiration brings forth the loss of carbon for high pH soils.

Any shift in pH results indifferent available forms of micronutrients and changes their solubility and mobilisation. Because micronutrients are available in different forms at different pH soils, their mobilities change with pH. For example, Fe hydrolyses to hydroxides at neutral pH, its uptake by plants reduces (Colombo et al., 2014). Additionally, the liming or chalk applications, which maintains the pH of soils at desired levels, increases the Ca, K and Mg levels at neutral soils. Otherwise, the cation exchange capacity of soils is directly effective on the plant nutrient availability. Due to the hydrogen ions in acidic soils, the plant uptake of small ions (such as Cu, Fe, Zn) are improved whilst the availability of larger ions like Ca, Mg, and K is inhibited due to their leaching through soil. Thus, availability of micronutrients is based on various mechanisms in soil including liming applications, soil pH, cation exchange capacity, manure addition, mobility and solubility of ions that all are interrelated with each other.

5. OVERVIEW AND FURTHER WORK

5.1. Overview

The overarching aim of the work performed presented in this thesis was to improve understanding of how environmental factors affect the mineral nutrient flow and microbial structure in pasture soils. In this context, soils obtained from Rothamsted Research were analysed in terms of their available micronutrient amounts and microbial community. The effect of soil depth, plant type (shallow- or deep-rooting), soil pH and sheep-manure treatment were investigated. The primary findings were as follows:

- The grass rooting type affects both microbial community and micronutrients where both are significantly higher in topsoils than subsoils. Topsoils provide better nutrients to microbial communities which decrease with soil depth due to the nutrient limitation in deeper regions (§ Chapter 3, Figure 3.4, 3.7, 3.8, 3.9, 3.10 and 3.11).
- Deep rooting grasses exhibit a higher concentration of biomarkers and micronutrients in the deeper regions compared to the shallow rooting grasses. Their higher concentrations might arise from in situ nutrient supply through the pores created by roots and vertical leaching of nutrients (§ Chapter 3, Figure 3.4, 3.8 and 3.9).
- Micronutrients are generally more available in topsoil except for Zn where plant roots release Zn chelators, which enhance the mobility of Zn ions (§ Chapter 3, Figure 3.10).
- Acidic soils exhibit a surficial layer of peat formation, most likely due to the accumulation of fatty acids and in the absence of iron oxide and some specific clay minerals (§ Chapter 4, Figure 4.7).
- High amounts of actinomycete biomarkers in acidic soils are likely a sign of acidophilic actinomycetes (§ Chapter 4, Figure 4.8).
- Because manure addition and nutrients leaching from sheep-manure shift the pH of soil, the microbial community and micronutrients are affected indirectly by manure addition. Because of the alkaline nature of manure, acidic soils are affected more from manure addition and a significant decrease in both microbial biomass and micronutrients is observed in acidic soils (§ Chapter 4, Figure 4.7 and 4.8).

- According to the fungal to bacteria ratio results, fungal communities prefer acidic soils rather than neutral ones (§ Chapter 4, Figure 4.9).
- Any shift in pH changes the cation exchange capacity of soil as well as the plant available form of micronutrients and, in conjunction with this, the mobility of micronutrients through soil profile changes. Thus, available micronutrients are affected by soil pH directly and manure addition indirectly (§ Chapter 4, Figure 4.11 and 4.12).

As a final conclusion, the changes in microbial communities have been interrelated with the distribution of micronutrients through soil profile. The findings show that microbial communities take role for the various soil processes including soil aggregation, porosity formation, mineralization, solubility, and mobilisation of micronutrients. Thus, the presence of microbes is directly effective on the micronutrient distribution that the availability of Fe, K, Ni and Zn ions are directly related with these processes. Manure amendment on neutral pH soils is useful in terms of being ineffective on pH whilst it affects the pH of acidic soils harshly and causes a loss in microbial communities. Thus, liming applications in neutral soils can be supported with manure amendments to improve the soil microbial biomass and bioavailable micronutrients. The increasing concentrations of particular micronutrients (Fe, Mg, Mn, Ni, and Zn) after 10 week incubation indicates the importance of long-term manure amendments and for agricultural applications, longer manure amendments can be considered to improve the bioavailability of these micronutrients.

5.2.Further work

This thesis provides a required fundamental information about what happens when soil is amended by a single type nutrient source before moving on to compare differences between nutrient sources. In future studies, assessing the effect of different manure types (i.e. from sheep fed by different nutrients or from different animals) on soil microbial communities and micronutrients would give a constructive point of view for agricultural applications in real field. Also, this study investigated the leaching of water-soluble compounds from sheep-manure to the soil and their effect on microbial community structure. In further studies, the soil can be mixed entirely with sheep-manure and the effects of leaching vs. mixing processes might be compared.

Additionally, the effect of soil pH on GDGTs was investigated just for control soils. The effect of exogenous manure can be investigated for GDGTs to get further details about the archaea biomarkers. Also, different time-points for manure amendment can be chosen in real-field experiments and incubation time can be extended to see the effect of manure amendment for long term applications.

Moreover, it would be interesting to extend investigations of soil depth effect on microbial communities and micronutrients. Through this thesis, soils were chosen from three different depths but smaller intervals between analysed depths can be chosen to follow the change through soil profile.

The PLFAs are less specific biomarkers when compared with metagenomic analysis. The DNA extractions can be used correspondingly with PLFA analysis to characterize the microbial communities in terms of their abundance and diversity in soils (Dimitrov et al., 2017). These analyses give information about the population of specific microorganisms, whilst additional functional genomic applications investigate the active genes in specific reactions. In recent literature papers, the variety and distribution of microbial communities as well as their enzymatic activities have been studied (Antunes et al., 2016; Mackelprang et al., 2018; Wright et al., 2019). For the elimination of sensitivity problems in traditional microbiological methods (Martin-Laurent et al., 2001) as well as to get deeper perspective in the effect of grass type and soil pH on presence of microbial communities, metagenomic analysis can be performed in parallel with PLFA biomarkers in future studies.

The outcomes of this thesis have been turning into papers and they are planning to be published soon.

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APPENDIX I

Table A3.1 Concentration details about ICP-OES calibration solutions.

Metal ion	ppm				
	STD1	**STD2**	**STD3**	**STD4**	**STD5**
Al	25	50	75	100	125
As	0.1	0.2	0.3	0.4	0.5
Ca	10	20	30	40	50
Cd	0.0025	0.0050	0.0075	0.0100	0.0125
Co	0.05	0.10	0.15	0.20	0.25
Cr	0.1	0.2	0.3	0.4	0.5
Cu	0.4	0.8	1.2	1.6	2.0
Fe	100	200	300	400	500
K	3	6	9	12	15
Mg	1.5	3.0	4.5	6.0	7.5
Mn	2	4	6	8	10
Ni	0.1	0.2	0.3	0.4	0.5
Pb	0.5	1.0	1.5	2.0	2.5
Zn	1	2	3	4	5

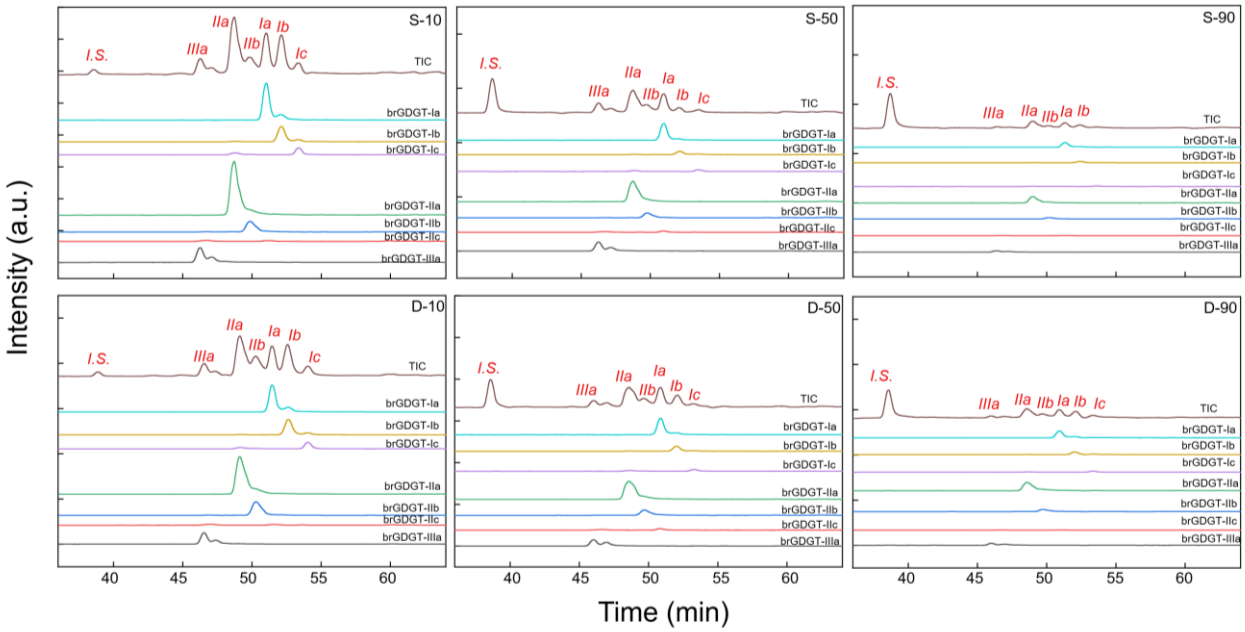

Figure A3.1 Different trials of HPLC-MS chromatograms of brGDGT fractions of soils from shallow (S) and deep root (D) grasses from different depths (S-10/D-10 refers to 10 cm, S-50/D-50 refers to 50 cm, S-90/D-90 refers to 90 cm depth).

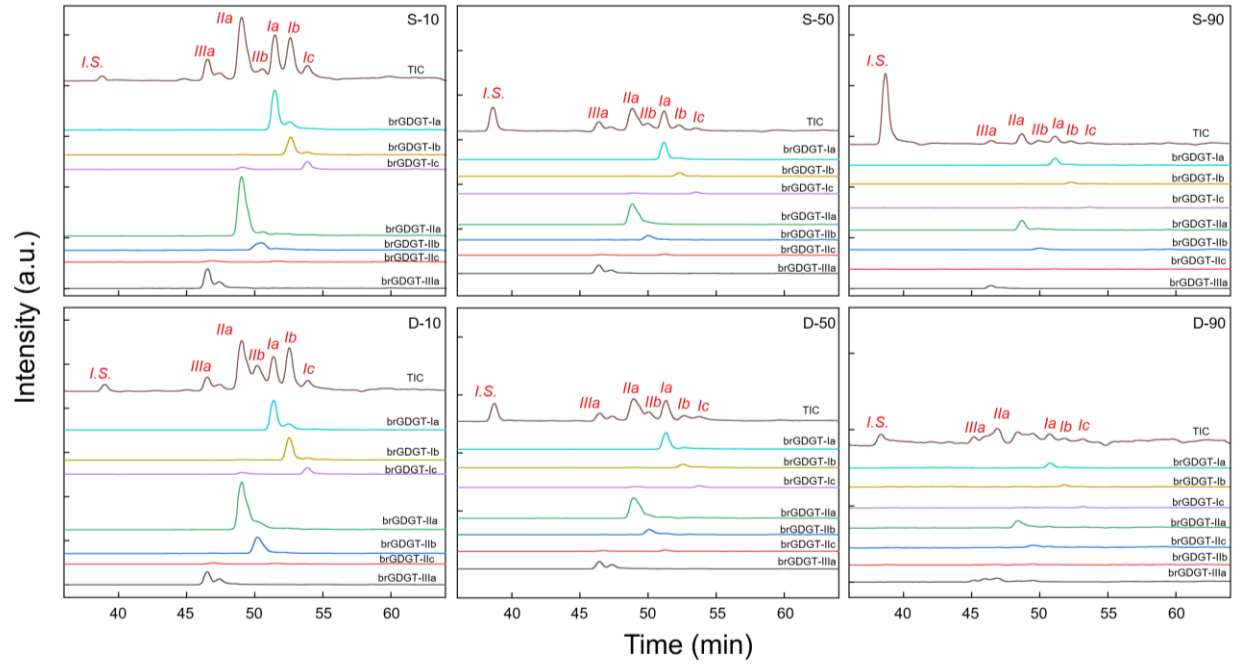

Figure A3.2 Different trials of HPLC-MS chromatograms of brGDGT fractions of soils from shallow (S) and deep root (D) grasses from different depths (S-10/D-10 refers to 10 cm, S-50/D-50 refers to 50 cm, S-90/D-90 refers to 90 cm depth).

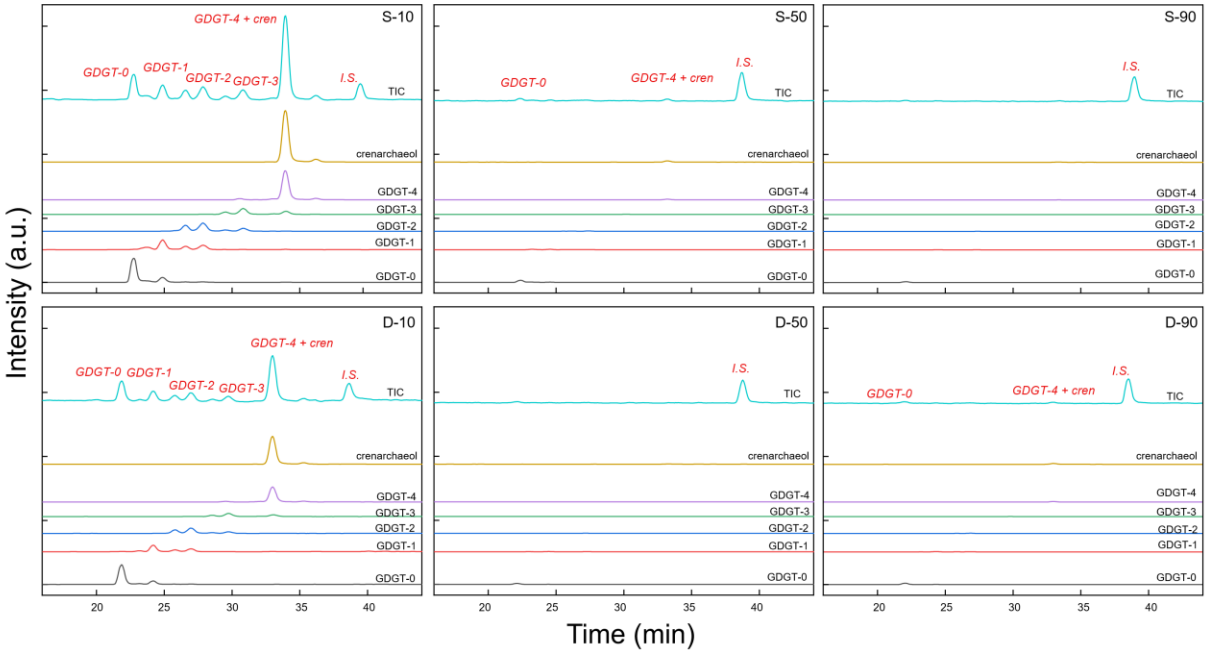

Figure A3.3 Different trials of HPLC-MS chromatograms of isoGDGT fractions of soils from shallow (S) and deep root (D) grasses from different depths (S-10/D-10 refers to 10 cm, S-50/D-50 refers to 50 cm, S-90/D-90 refers to 90 cm depth).

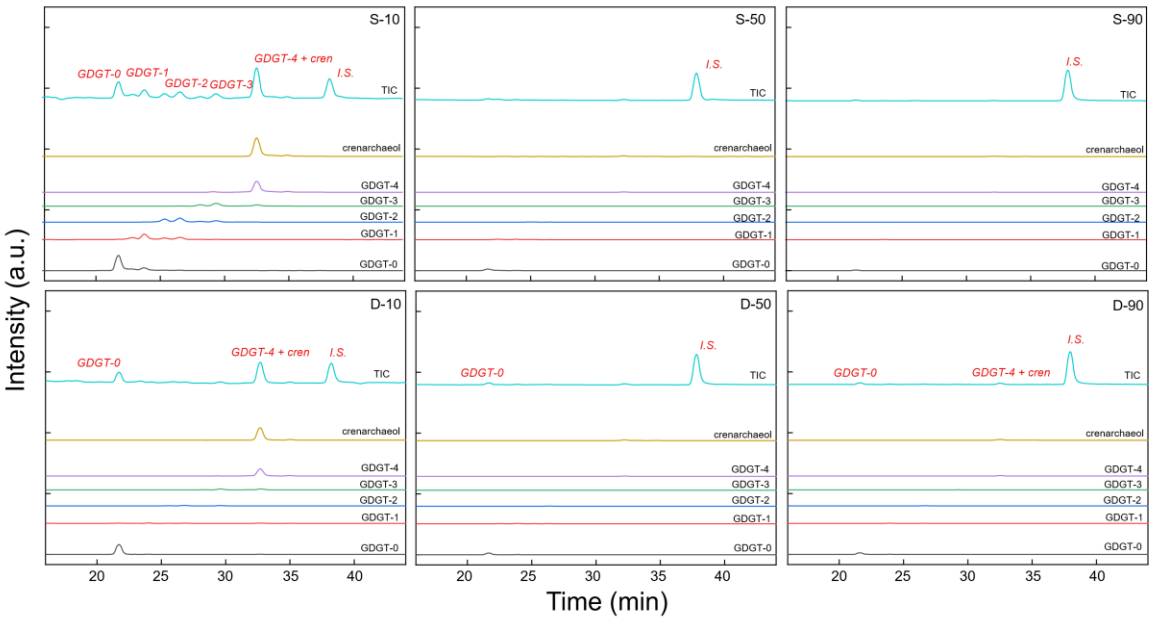

Figure A3.4 Different trials of HPLC-MS chromatograms of isoGDGT fractions of soils from shallow (S) and deep root (D) grasses from different depths (S-10/D-10 refers to 10 cm, S-50/D-50 refers to 50 cm, S-90/D-90 refers to 90 cm depth).

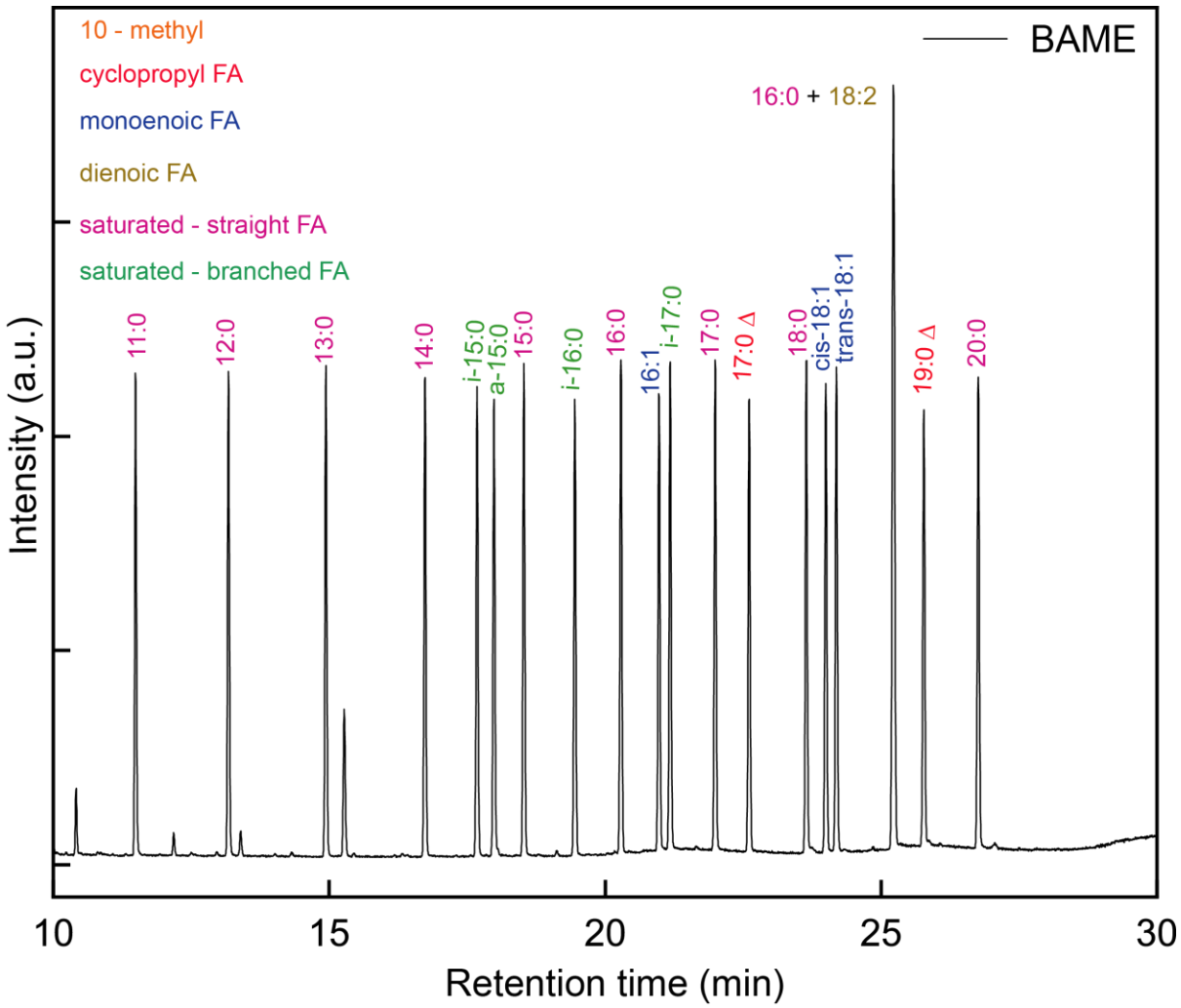

Figure A3.5 GC-MS chromatogram of BAME standard.

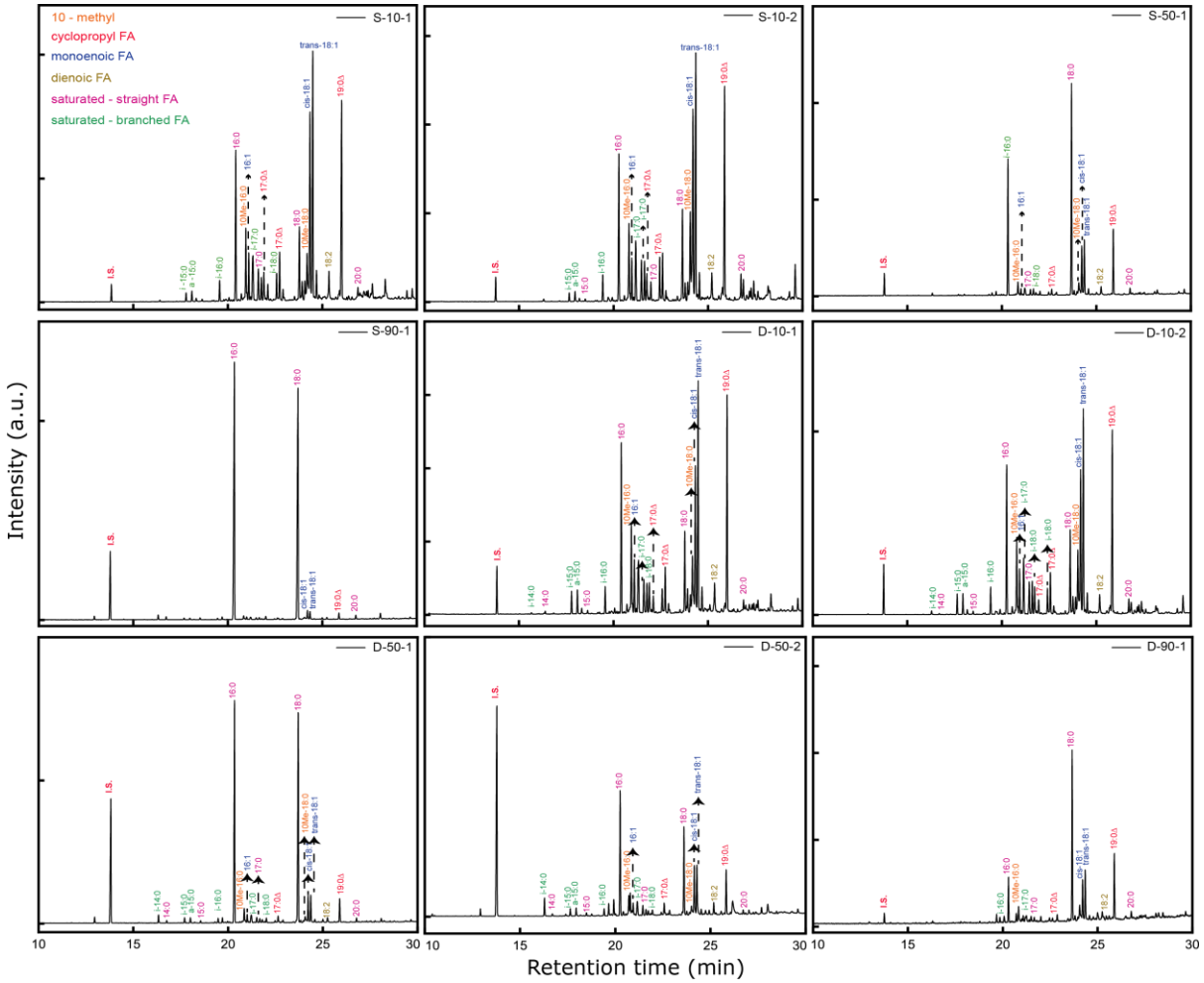

Figure A3.6 Different trials of GC-MS chromatograms of PLFA fractions of soils from shallow (S) and deep root (D) grasses from different depths (S-10/D-10 refers to 10 cm, S-50/D-50 refers to 50 cm, S-90/D-90 refers to 90 cm depth).

Table A3.2 Measurements of carbon, nitrogen and pH in soils from shallow root and deep root grasses (this data was taken from project students).

grass type	depth (cm)	C (mmol/kg)	N (mmol/kg)	pH
shallow root grass	10	2250 ± 9	247 ± 3	5.3 ± 0.1
	50	3544 ± 9	101 ± 3	6.1 ± 0.8
	90	259 ± 9	94 ± 3	6.0 ± 1.3
deep root grass	10	2355 ± 2	252 ± 9	5.8 ± 0.2
	50	347 ± 2	112 ± 47	6.8 ± 0.3
	90	335 ± 2	9 ± 9	6.2 ± 0.6

Table A3.3 Distribution of GDGTs with soil depth for both shallow root soil and deep root grass soil and their P-values calculated by one-way-ANOVA test.

Biomarker	depth (cm)	Shallow root (µg/g)	Deep root (µg/g)	P-value
brGDGTs	10	69.79 ± 6.39	53.22 ± 5.45	0.0348
	50	4.58 ± 1.77	5.89 ± 2.24	0.4254
	90	0.88 ± 0.17	1.77 ± 0.24	0.0031
isoGDGTs	10	9.96 ± 0.93	5.45 ± 0.50	0.0037
	50	0.32 ± 0.09	0.16 ± 0.06	0.0498
	90	0.08 ± 0.03	0.19± 0.06	0.0173

Table A3.4 Distribution of PLFAs with soil depth for both shallow root soil and deep root grass soil and their P-values calculated by one-way-ANOVA test.

Biomarker	depth (cm)	Shallow root (µg/g)	Deep root (µg/g)	P-value
Total FA	10	129.03 ± 27.0	62.54 ± 10.2	0.0172
	50	42.75 ± 9.7	4.51 ± 0.99	0.0017
	90	5.77 ± 0.87	4.66 ± 0.71	0.1033
Gram-positive	10	18.04 ± 1.77	10.51 ± 2.76	0.0178
	50	3.04 ± 0.76	0.45 ± 0.07	0.0011
	90	0	0.61 ± 0.09	0.0003
Gram-negative	10	34.49 ± 6.58	13.42 ± 3.21	0.0066
	50	7.17 ± 1.61	0.54 ± 0.15	0.0150
	90	0.19 ± 0.03	0.70 ± 0.11	0.0014
Actinomycetes	10	15.57 ± 3.45	6.97 ± 0.65	0.0133
	50	2.24 ± 0.66	0.31 ± 0.07	0.0044
	90	0.09 ± 0.01	0.43 ± 0.07	0.0008
Fungi	10	30.92 ± 3.05	18.41 ± 1.26	0.0040
	50	14.81 ± 1.33	0.95 ± 0.16	0.0001
	90	0.41 ± 0.06	1.37 ± 0.21	0.0026
fungi/bacteria	10	0.32 ± 0.03	0.42 ± 0.04	0.0485
	50	0.53 ± 0.05	0.27 ± 0.03	0.0043
	90	0.08 ± 0.01	0.42 ± 0.04	0.0006

Table A3.5 Distribution of nutrients with soil depth for both shallow root soil and deep root grass soil and their P-values calculated by one-way-ANOVA test.

Nutrient ion	depth (cm)	Shallow root (µg/g)	Deep root (µg/g)	P-value
Ca	10	0.98 ± 0.11	1.19 ± 0.10	0.0454
	50	0.72 ± 0.03	0.79 ± 0.08	0.1964
	90	0.39 ± 0.08	0.61 ± 0.07	0.0234
Cu	10	0.06 ± 0.003	0.049 ± 0.003	0.0101
	50	0.07 ± 0.004	0.061 ± 0.002	0.1704
	90	0.06 ± 0.001	0.045 ± 0.005	0.0113
Fe	10	23.44 ± 1.99	22.23 ± 1.84	0.3770
	50	31.77 ± 1.41	25.07 ± 1.66	0.0060
	90	33.22 ± 1.58	30.28 ± 1.91	0.1547
K	10	0.38 ± 0.02	0.41 ± 0.03	0.0558
	50	0.25 ±0.01	0.38 ± 0.01	0.0002
	90	0.36 ± 0.04	0.29 ± 0.01	0.2965
Mg	10	0.18 ± 0.002	0.16 ± 0.005	0.0148
	50	0.11 ± 0.002	0.13 ± 0.013	0.0895
	90	0.13 ± 0.006	0.11 ± 0.007	0.0047
Mn	10	0.36 ± 0.05	0.54 ± 0.05	0.0159
	50	0.22 ± 0.02	0.29 ± 0.03	0.0342
	90	0.23 ± 0.05	0.17 ± 0.02	0.1404
Ni	10	0.007 ± 0.001	0.009 ± 0.001	0.0926
	50	0.011 ± 0.001	0.009 ± 0.001	0.0382
	90	0.017 ± 0.001	0.021 ± 0.001	0.0638
Zn	10	0.049 ± 0.004	0.038 ± 0.001	0.0115
	50	0.084 ± 0.005	0.039 ± 0.004	0.0002
	90	0.071 ± 0.005	0.049 ± 0.004	0.0015

Table A3.6 Distribution of heavy metals with soil depth for both shallow root soil and deep root grass soil and their P-values calculated by one-way-ANOVA test.

Metal ion	depth (cm)	Shallow root (µg/g)	Deep root (µg/g)	P-value
Al	10	4.31 ± 0.13	4.56 ± 0.32	0.3766
	50	3.27 ± 0.11	3.89 ± 0.16	0.0031
	90	2.81 ± 0.28	2.53 ± 0.29	0.2942
As	10	0.015 ± 0.001	0.011 ± 0.001	0.0185
	50	0.014 ± 0.001	0.012 ± 0.001	0.0054
	90	0.011 ± 0.001	0.010 ± 0.001	0.8134
Cd	10	4.45e-04 ± 4.4e-05	4.07e-04 ± 3.7e-05	0.3545
	50	5.47e-04 ± 1.8e-05	4.16e-04 ± 4.7e-05	0.0087
	90	4.95e-04 ± 4.1e-05	4.97e-04 ± 4.4e-05	0.6868
Co	10	0.006 ± 0.001	0.009 ± 0.001	0.0074
	50	0.007 ± 0.001	0.012 ± 0.001	0.0001
	90	0.009 ± 0.001	0.010 ± 0.001	0.1042
Cr	10	0.013 ± 0.001	0.015 ± 0.001	0.0037
	50	0.007 ± 0.001	0.007 ± 0.001	0.1506
	90	0.008 ± 0.001	0.006 ± 0.001	0.0073
Pb	10	0.045 ± 0.004	0.041 ± 0.004	0.2357
	50	0.053 ± 0.002	0.042 ± 0.005	0.0166
	90	0.054 ± 0.005	0.049 ± 0.004	0.1232

Table A3.7 Comparison of GDGT concentration results by two-way-ANOVA test.

Biomarker	Prob > F		
	Three depths	Two grass types	Interaction btw depth and grass type
brGDGT	0.0000	0.0255	0.0025
isoGDGT	8.88e-12	5.45e-05	8.11e-06

Table A3.8 Comparison of PLFA concentration results by two-way-ANOVA test.

Biomarker	Prob > F		
	Three depths	Two grass types	Interaction btw depth and grass type
Total FA	0.0000	0.0001	0.0025
Gram-positive	0.0000	0.0004	0.0010
Gram-negative	0.0000	0.0000	0.0001
Actinomycetes	0.0000	0.0003	0.0005
Fungi	1.29e-11	8.29e-08	4.39e-06
fungi/bacteria	0.0003	0.0197	0.0000

Table A3.9 Comparison of nutrient concentration results by two-way-ANOVA test.

Nutrient ion	Prob > F		
	Three depths	Two grass types	Interaction btw depth and grass type
Ca	0.0000	0.0006	0.2253
Cu	0.0001	0.0001	0.0780
Fe	0.0000	0.0012	0.0967
K	0.0002	0.0046	0.0000
Mg	0.0000	0.0228	0.0005
Mn	0.0000	0.0069	0.0014
Ni	0.0000	0.0719	0.0065
Zn	5.04e-06	4.56e-09	3.20e-05

Table A3.10 Comparison of heavy metal ion concentration results by two-way-ANOVA test.

Nutrient ion	Prob > F		
	Three depths	Two grass types	Interaction btw depth and grass type
Al	0.0000	0.1700	0.0254
As	0.0008	0.0011	0.0058
Cd	0.0079	0.0040	0.0235
Co	0.0646	0.0000	0.0071
Cr	0.0000	0.2586	0.0000
Pb	0.0062	0.0018	0.4161

APPENDIX II

Table A4.1 Concentration details about ICP-OES calibration solutions.

Metal ion	ppm				
	STD1	STD2	STD3	STD4	STD5
Ca	20	40	60	80	100
Cu	0.02	0.04	0.06	0.08	0.10
Fe	0.25	0.50	0.75	1.00	1.25
K	4	8	12	16	20
Mg	2	4	6	8	10
Mn	0.4	0.8	1.2	1.6	2.0
Ni	0.003	0.006	0.009	0.012	0.016
Zn	0.02	0.04	0.06	0.08	0.10

Table A4.2 Percentage TC for soils from pH 7.1, 6.4, 5.2 and 4.1 through different time periods and their P-values calculated by one-way-ANOVA test.

Incubation period	pH	control soils (%)	manure applied soils (%)	P-value
5-week	7.1	5.27 ± 0.15	5.05 ± 0.15	0.1356
	6.4	4.24 ± 0.19	4.77 ± 0.16	0.0369
	5.2	5.63 ± 0.18	5.85 ± 0.14	0.1866
	4.1	4.45 ± 0.16	4.96 ± 0.12	0.0142
10-week	7.1	4.92 ± 0.15	4.70 ± 0.19	0.165
	6.4	4.50 ± 0.12	4.90 ± 0.19	0.0771
	5.2	6.05 ± 0.19	6.17 ± 0.12	0.4445
	4.1	4.55 ± 0.13	4.73 ± 0.42	0.4318

Table A4.3 Percentage TN for soils from pH 7.1, 6.4, 5.2 and 4.1 through different time periods and their P-values calculated by one-way-ANOVA test.

Incubation period	pH	control soils (%)	manure applied soils (%)	P-value
5-week	7.1	0.46 ± 0.016	0.44 ± 0.011	0.0959
	6.4	0.39 ± 0.011	0.40 ± 0.011	0.4538
	5.2	0.44 ± 0.011	0.46 ± 0.018	0.8766
	4.1	0.33 ± 0.006	0.35 ± 0.010	0.0823
10-week	7.1	0.44 ± 0.010	0.42 ± 0.011	0.3009
	6.4	0.39 ± 0.010	0.43 ± 0.010	0.5315
	5.2	0.47 ± 0.006	0.49 ± 0.013	0.0503
	4.1	0.34 ± 0.013	0.36 ± 0.010	0.2539

Table A4.4 Percentage TS for soils from pH 7.1, 6.4, 5.2 and 4.1 through different time periods and their P-values calculated by one-way-ANOVA test.

Incubation period	pH	control soils (%)	manure applied soils (%)	P-value
5-week	7.1	0.072 ± 0.002	0.062 ± 0.001	0.0006
	6.4	0.059 ± 0.002	0.061 ± 0.002	0.5243
	5.2	0.078 ± 0.003	0.073 ± 0.002	0.0448
	4.1	0.079 ± 0.002	0.083 ± 0.001	0.0389
10-week	7.1	0.062 ± 0.002	0.062 ± 0.002	0.7447
	6.4	0.065 ± 0.001	0.059 ± 0.002	0.0076
	5.2	0.071 ± 0.004	0.076 ± 0.004	0.1027
	4.1	0.072 ± 0.001	0.075 ± 0.002	0.1637

Table A4.5 C/N for soils from pH 7.1, 6.4, 5.2 and 4.1 through different time periods and their P-values calculated by one-way-ANOVA test.

Incubation period	pH	control soils (%)	manure applied soils (%)	P-value
0-week	7.1	11.53 ± 0.18	N/A	N/A
	6.4	11.58 ± 0.39	N/A	N/A
	5.2	12.97 ± 0.31	N/A	N/A
	4.1	13.70 ± 0.40	N/A	N/A
5-week	7.1	11.35 ± 0.22	11.41 ± 0.29	0.7685
	6.4	10.79 ± 0.14	11.59 ± 0.35	0.0211
	5.2	12.39 ± 0.36	12.99 ± 0.12	0.0538
	4.1	13.40 ± 0.28	14.33 ± 0.57	0.0630
10-week	7.1	11.14 ± 0.07	10.86 ± 0.44	0.3221
	6.4	11.45 ± 0.23	11.55 ± 2.02	0.9344
	5.2	12.87 ± 0.31	12.50 ± 0.22	0.1740
	4.1	13.07 ± 0.09	13.25 ± 0.91	0.7510

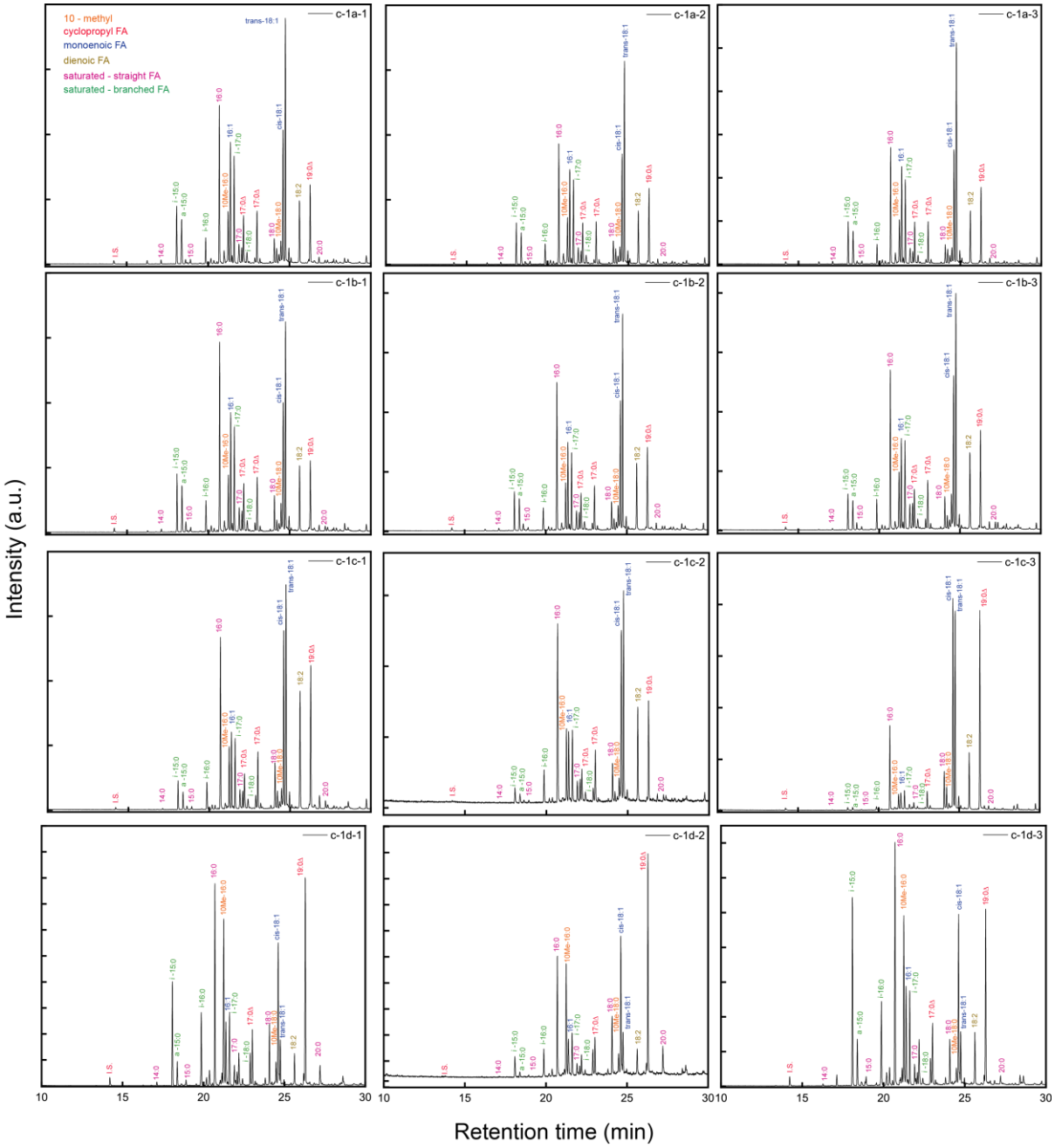

Figure A4.1 GC-MS chromatograms of PLFA fractions with triplicates of control (t=0) soils from different pH plots (plot a: 7.1, plot b: 6.4, plot c: 5.2, plot d: 4.1).

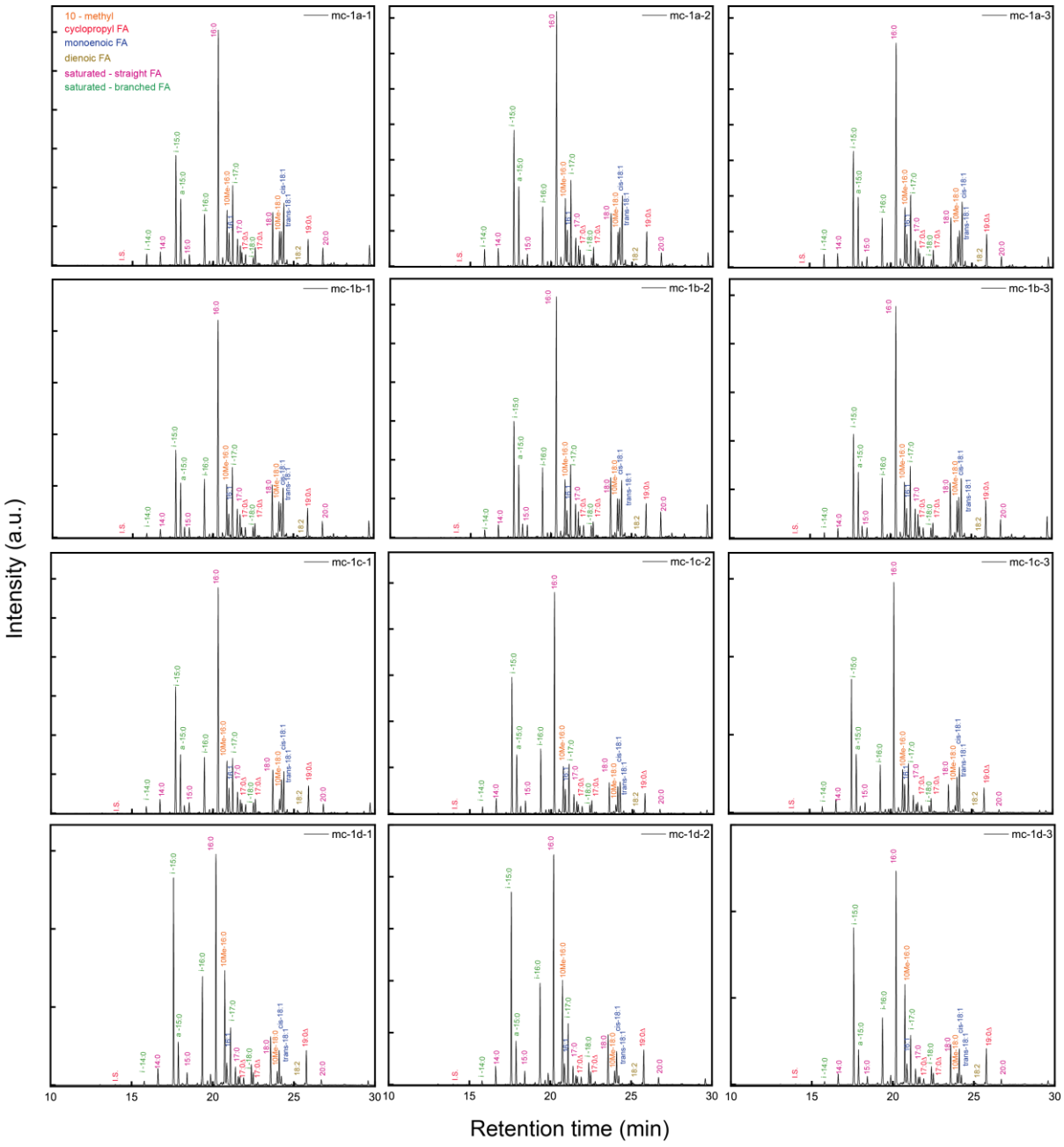

Figure A4.2 GC-MS chromatograms of PLFA fractions with triplicates of control (t=5, 5-week incubated without manure) soils from different pH plots (plot a: 7.1, plot b: 6.4, plot c: 5.2, plot d: 4.1).

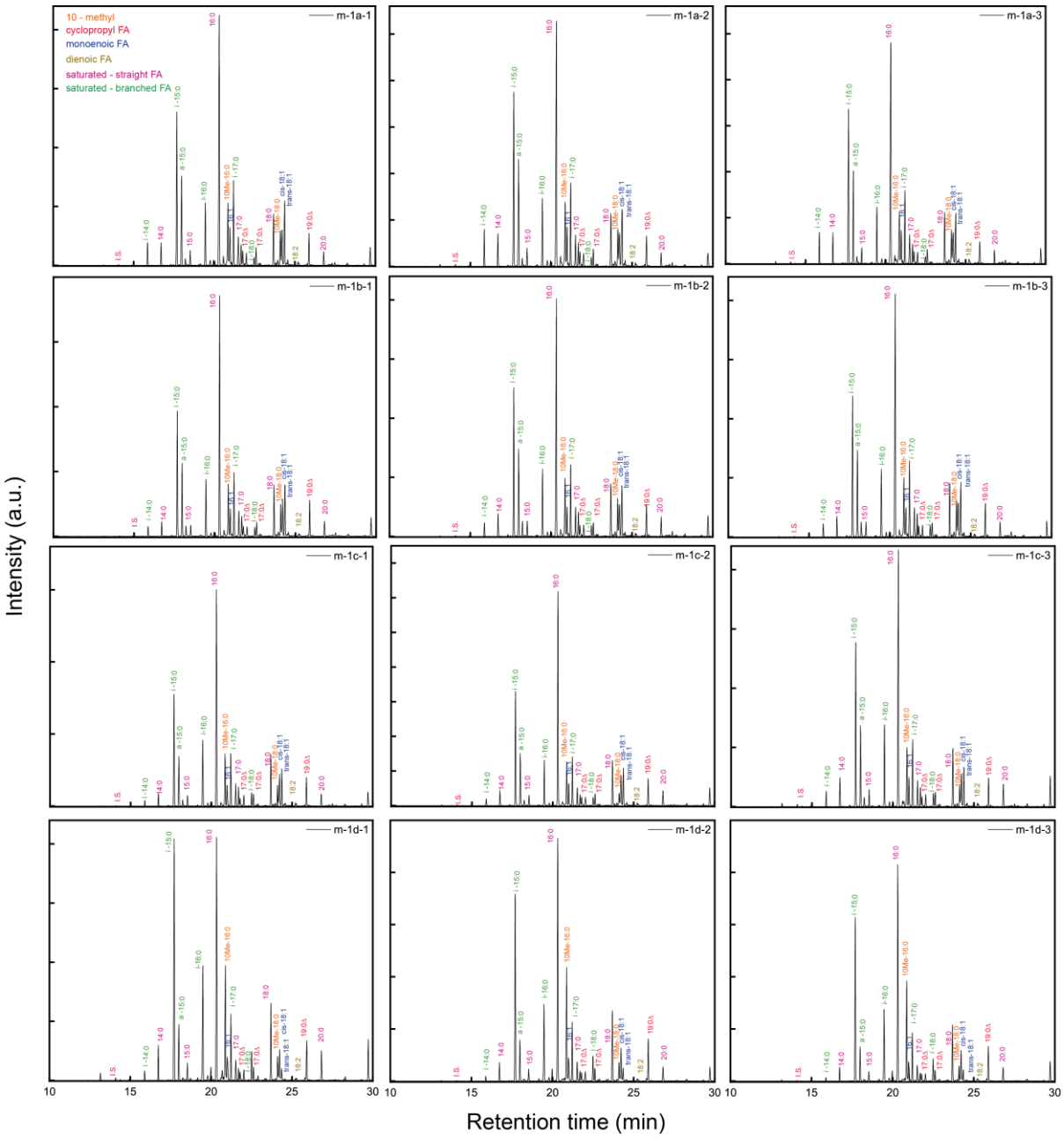

Figure A4.3 GC-MS chromatograms of PLFA fractions with triplicates of manure applied (t=5, 5-week incubated) soils from different pH plots (plot a: 7.1, plot b: 6.4, plot c: 5.2, plot d: 4.1).

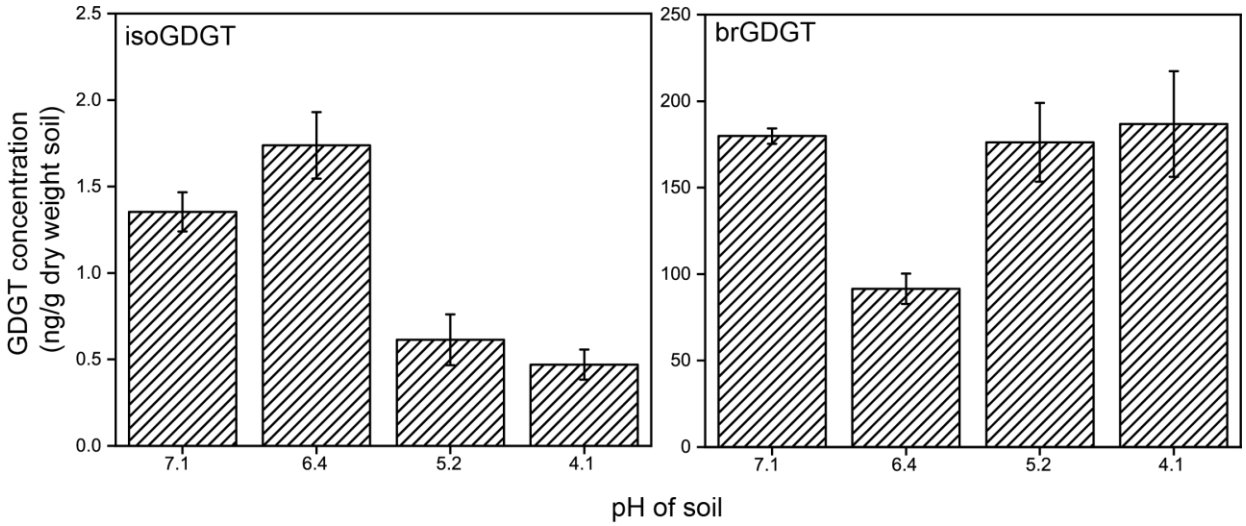

Figure A4.4 The change in amounts of isoGDGTs and brGDGTs with soil pH (see Appendix 2 for details about significance levels).

Table A4.6 Total fatty acid concentration of soils from pH 7.1, 6.4, 5.2 and 4.1 through different time periods and their P-values calculated by one-way-ANOVA test.

Incubation period	pH	control soils (μg/g)	P-value (1)	P-value (2)
0-week	7.1	299.06 ± 17.40		
	6.4	257.27 ± 17.14		
	5.2	266.44 ± 9.33		
	4.1	154.78 ± 5.42		
5-week (control)	7.1	63.32 ± 5.89		
	6.4	63.67 ± 3.36		1.75e-05
	5.2	168.27 ± 23.34	0.4042	4.24e-05
	4.1	180.49 ± 21.61	0.2120	5.83e-06
5-week (with manure)	7.1	67.63 ± 4.89	9.46e-04	8.19e-06
	6.4	58.02 ± 5.62	4.73e-04	
	5.2	46.21 ± 4.31		
	4.1	45.88 ± 3.79		

Table A4.7 Gram positive bacteria biomarker concentration of soils from pH 7.1, 6.4, 5.2 and 4.1 through different time periods and their P-values calculated by one-way-ANOVA test.

Incubation period	pH	control soils (µg/g)	P-value (1)	P-value (2)
0-week	7.1	63.41 ± 1.67		
	6.4	54.56 ± 3.65		
	5.2	36.84 ± 1.29		
	4.1	31.06 ± 1.12		
5-week (control)	7.1	17.05 ± 0.86	0.0516	1.86e-05
	6.4	15.69 ± 0.76	0.8880	5.94e-05
	5.2	47.41 ± 2.18	1.21e-04	5.37e-05
	4.1	59.91 ± 3.48	4.29e-05	1.18e-04
5-week (with manure)	7.1	19.43 ± 1.24		
	6.4	15.90 ± 1.10		
	5.2	12.41 ± 2.02		
	4.1	14.56 ± 1.33		

Table A4.8 Gram negative bacteria biomarker concentration of soils from pH 7.1, 6.4, 5.2 and 4.1 through different time periods and their P-values calculated by one-way-ANOVA test.

Incubation period	pH	control soils (µg/g)	P-value (1)	P-value (2)
0-week	7.1	76.12 ± 3.80		
	6.4	56.69 ± 3.81		
	5.2	67.88 ± 2.38		
	4.1	34.26 ± 1.20		
5-week (control)	7.1	13.11 ± 1.86	0.9172	6.36e-06
	6.4	13.43 ± 0.51	0.2730	6.25e-05
	5.2	35.42 ± 1.48	6.81e-05	4.21e-06
	4.1	39.89 ± 1.69	1.95e-05	1.56e-05
5-week (with manure)	7.1	13.29 ± 0.70		
	6.4	12.34 ± 0.60		
	5.2	9.46 ± 0.72		
	4.1	10.29 ± 0.64		

Table A4.9 Actinomycetes biomarker concentration of soils from pH 7.1, 6.4, 5.2 and 4.1 through different time periods and their P-values calculated by one-way-ANOVA test.

Incubation period	pH	control soils (µg/g)	P-value (1)	P-value (2)
0-week	7.1	19.31 ± 1.02		
	6.4	20.69 ± 0.72		
	5.2	20.19 ± 0.71		
	4.1	20.24 ± 0.71		
5-week (control)	7.1	5.23 ± 0.34	0.2635	2.92e-05
	6.4	4.94 ± 0.30	0.0960	9.49e-06
	5.2	10.75 ± 0.80	2.00e-04	1.11e-05
	4.1	19.29 ± 0.62	4.40e-06	4.88e-06
5-week (with manure)	7.1	4.80 ± 0.36		
	6.4	4.38 ± 0.56		
	5.2	3.39 ± 0.19		
	4.1	5.23 ± 0.39		

Table A4.10 Fungal biomarker concentration of soils from pH 7.1, 6.4, 5.2 and 4.1 through different time periods and their P-values calculated by one-way-ANOVA test.

Incubation period	pH	control soils (µg/g)	P-value (1)	P-value (2)
0-week	7.1	88.44 ± 3.53		
	6.4	69.74 ± 5.59		
	5.2	100.76 ± 3.53		
	4.1	20.68 ± 0.72		
5-week (control)	7.1	18.12 ± 0.64	0.7460	3.69e-06
	6.4	17.52 ± 0.67	0.0260	1.44e-04
	5.2	43.84 ± 4.45	2.31e-05	2.71e-06
	4.1	26.13 ± 1.42	1.42e-05	3.36e-05
5-week (with manure)	7.1	18.73 ± 1.60		
	6.4	14.52 ± 1.16		
	5.2	11.78 ± 0.74		
	4.1	6.59 ± 0.93		

Table A4.11 F/B of soils from pH 7.1, 6.4, 5.2 and 4.1 through different time periods and their P-values calculated by one-way-ANOVA test.

Incubation period	pH	control soils (µg/g)	P-value (1)	P-value (2)
0-week	7.1	0.42 ± 0.03		
	6.4	0.37 ± 0.02		
	5.2	0.61 ± 0.04		
	4.1	0.15 ± 0.01		
5-week (control)	7.1	0.40 ± 0.03	0.3830	0.1388
	6.4	0.38 ± 0.03	0.0320	0.0725
	5.2	0.35 ± 0.02	0.4305	5.02e-04
	4.1	0.17 ± 0.01	0.5295	0.8607
5-week (with manure)	7.1	0.38 ± 0.03		
	6.4	0.33 ± 0.02		
	5.2	0.34 ± 0.02		
	4.1	0.17 ± 0.01		

Table A4.12 GDGT concentration of soils from pH 7.1, 6.4, 5.2 and 4.1 for control soils and their P-values calculated by one-way-ANOVA test.

Biomarker type	pH	concentration (µg/g)	p-values for pH between (7.1-6.4)	(7.1-5.2)	(7.1-4.1)	(6.4-5.2)	(6.4-4.1)	(5.2-4.1)
isoGDGT	7.1	1.35 ± 0.11	0.0406	0.0023	4.29e-04	0.0013	4.80e-04	0.2173
	6.4	1.74 ± 0.19						
	5.2	0.61 ± 0.15						
	4.1	0.47 ± 0.09						
brGDGT	7.1	179.82 ± 4.51	9.96e-05	0.8025	0.7144	0.0038	0.0065	0.6555
	6.4	91.49 ± 8.74						
	5.2	176.23 ± 22.75						
	4.1	186.80 ± 30.49						

Table A4.13 Zn concentration for soils from pH 7.1, 6.4, 5.2 and 4.1 through different time periods and their P-values calculated by one-way-ANOVA test.

Incubation period	pH	control soils (%)	manure applied soils (%)	P-value
0-week	7.1	1.11E-03 ± 5.88E-05	N/A	N/A
	6.4	1.95E-03 ± 2.19E-05	N/A	N/A
	5.2	2.07E-03 ± 3.43E-04	N/A	N/A
	4.1	1.18E-03 ± 2.33E-04	N/A	N/A
5-week	7.1	1.56E-03 ± 1.75E-04	1.58E-03 ± 1.45E-04	0.8682
	6.4	2.28E-03 ± 3.59E-04	1.80E-03 ± 1.05E-04	0.0692
	5.2	1.71E-03 ± 3.09E-04	1.56E-03 ± 1.96E-04	0.8674
	4.1	1.14E-03 ± 1.08E-04	1.32E-03 ± 1.58E-04	0.1810
10-week	7.1	2.34E-03 ± 3.05E-04	2.68E-03 ± 1.18E-05	0.3221
	6.4	3.36E-03 ± 2.11E-04	3.65E-03 ± 1.29E-04	0.7945
	5.2	3.23E-03 ± 1.81E-04	4.41E-03 ± 2.43E-04	0.9923
	4.1	2.19E-03 ± 6.05E-05	2.38E-03 ± 1.53E-04	0.0068

Table A4.14 Ni concentration for soils from pH 7.1, 6.4, 5.2 and 4.1 through different time periods and their P-values calculated by one-way-ANOVA test.

Incubation period	pH	control soils (%)	manure applied soils (%)	P-value
0-week	7.1	2.15E-04 ± 3.37E-05	N/A	N/A
	6.4	3.20E-04 ± 3.79E-05	N/A	N/A
	5.2	2.87E-04 ± 6.35E-05	N/A	N/A
	4.1	2.60E-04 ± 4.00E-05	N/A	N/A
5-week	7.1	2.77E-04 ± 1.13E-05	2.86E-04 ± 1.35E-05	0.4405
	6.4	3.31E-04 ± 4.42E-05	2.79E-04 ± 4.36E-05	0.2177
	5.2	2.05E-04 ± 4.30E-05	2.62E-04 ± 2.97E-05	0.1746
	4.1	2.07E-04 ± 1.82E-05	2.29E-04 ± 2.68E-05	0.2718
10-week	7.1	3.43E-04 ± 3.01E-05	4.06E-04 ± 4.06E-05	0.0962
	6.4	3.65E-04 ± 2.47E-05	3.81E-04 ± 4.06E-05	0.6437
	5.2	2.65E-04 ± 3.23E-05	3.29E-04 ± 3.28E-05	0.0755
	4.1	3.03E-04 ± 3.60E-05	3.02E-04 ± 1.44E-05	0.9732

Table A4.15 Mn concentration for soils from pH 7.1, 6.4, 5.2 and 4.1 through different time periods and their P-values calculated by one-way-ANOVA test.

Incubation period	pH	control soils (%)	manure applied soils (%)	P-value
0-week	7.1	0.039 ± 0.003	N/A	N/A
	6.4	0.040 ± 0.003	N/A	N/A
	5.2	0.024 ± 0.001	N/A	N/A
	4.1	0.006 ± 0.001	N/A	N/A
5-week	7.1	0.042 ± 0.004	0.043 ± 0.001	0.6182
	6.4	0.034 ± 0.002	0.036 ± 0.007	0.6449
	5.2	0.019 ± 0.002	0.027 ± 0.002	0.2046
	4.1	0.005 ± 0.001	0.006 ± 0.001	0.1705
10-week	7.1	0.049 ± 0.001	0.053 ± 0.003	0.1528
	6.4	0.042 ± 0.003	0.044 ± 0.001	0.2746
	5.2	0.025 ± 0.002	0.030 ± 0.009	0.3994
	4.1	0.006 ± 0.001	0.008 ± 0.002	0.0693

Table A4.16 Mg concentration for soils from pH 7.1, 6.4, 5.2 and 4.1 through different time periods and their P-values calculated by one-way-ANOVA test.

Incubation period	pH	control soils (%)	manure applied soils (%)	P-value
0-week	7.1	0.081 ± 0.005	N/A	N/A
	6.4	0.086 ± 0.005	N/A	N/A
	5.2	0.076 ± 0.009	N/A	N/A
	4.1	0.039 ± 0.006	N/A	N/A
5-week	7.1	0.090 ± 0.007	0.100 ± 0.004	0.1128
	6.4	0.081 ± 0.006	0.083 ± 0.010	0.8433
	5.2	0.066 ± 0.008	0.075 ± 0.008	0.0991
	4.1	0.038 ± 0.001	0.055 ± 0.009	0.0389
10-week	7.1	0.094 ± 0.002	0.123 ± 0.006	0.0013
	6.4	0.086 ± 0.005	0.109 ± 0.008	0.0116
	5.2	0.074 ± 0.005	0.097 ± 0.008	0.0142
	4.1	0.042 ± 0.001	0.068 ±0.004	3.89e-04

Table A4.17 K concentration for soils from pH 7.1, 6.4, 5.2 and 4.1 through different time periods and their P-values calculated by one-way-ANOVA test.

Incubation period	pH	control soils (%)	manure applied soils (%)	P-value
0-week	7.1	0.406 ± 0.026	N/A	N/A
	6.4	0.469 ± 0.029	N/A	N/A
	5.2	0.398 ± 0.023	N/A	N/A
	4.1	0.308 ± 0.046	N/A	N/A
5-week	7.1	0.454 ± 0.043	0.510 ± 0.017	0.1077
	6.4	0.422 ± 0.029	0.429 ± 0.056	0.8572
	5.2	0.373 ± 0.044	0.411 ± 0.027	0.3816
	4.1	0.306 ± 0.011	0.329 ± 0.039	0.3826
10-week	7.1	0.478 ± 0.010	0.544 ± 0.026	0.0154
	6.4	0.468 ± 0.020	0.519 ± 0.022	0.0411
	5.2	0.432 ± 0.032	0.447 ± 0.034	0.6178
	4.1	0.350 ± 0.014	0.368 ± 0.005	0.1184

Table A4.18 Fe concentration for soils from pH 7.1, 6.4, 5.2 and 4.1 through different time periods and their P-values calculated by one-way-ANOVA test.

Incubation period	pH	control soils (%)	manure applied soils (%)	P-value
0-week	7.1	0.007 ± 0.001	N/A	N/A
	6.4	0.007 ± 0.001	N/A	N/A
	5.2	0.019 ± 0.005	N/A	N/A
	4.1	0.033 ± 0.001	N/A	N/A
5-week	7.1	0.007 ± 0.001	0.011 ± 0.004	0.1931
	6.4	0.012 ± 0.008	0.008 ±0.001	0.3862
	5.2	0.024 ± 0.005	0.018 ±0.005	0.2722
	4.1	0.031 ± 0.002	0.035 ± 0.005	0.2495
10-week	7.1	0.008 ± 0.001	0.008 ± 0.001	0.9263
	6.4	0.008 ± 0.001	0.009 ± 0.001	0.5344
	5.2	0.024 ± 0.001	0.020 ± 0.004	0.1041
	4.1	0.030 ± 0.001	0.068 ± 0.008	7.31e-04

Table A4.19 Cu concentration for soils from pH 7.1, 6.4, 5.2 and 4.1 through different time periods and their P-values calculated by one-way-ANOVA test.

Incubation period	pH	control soils (%)	manure applied soils (%)	P-value
0-week	7.1	2.68E-04 ± 2.38E-05	N/A	N/A
	6.4	3.19E-04 ± 5.38E-05	N/A	N/A
	5.2	2.68E-04 ± 7.74E-06	N/A	N/A
	4.1	8.14E-04 ± 3.73E-05	N/A	N/A
5-week	7.1	4.05E-04 ± 1.98E-04	3.28E-04 ± 3.32E-05	0.5417
	6.4	3.38E-04 ± 3.03E-05	3.16E-04 ± 7.60E-05	0.5304
	5.2	3.19E-04 ± 1.34E-04	4.24E-04 ± 5.22E-05	0.1640
	4.1	7.47E-04 ± 5.58E-05	7.42E-04 ± 3.57E-05	0.9136
10-week	7.1	3.12E-04 ± 6.82E-05	2.49E-04 ± 5.89E-05	0.2930
	6.4	2.22E-04 ± 1.93E-05	2.18E-04 ± 3.48E-05	0.8476
	5.2	2.39E-04 ± 2.17E-05	1.81E-04 ± 3.27E-05	0.0630
	4.1	6.58E-04 ± 2.59E-05	6.58E-04 ± 5.95E-05	0.9860

Table A4.20 Ca concentration for soils from pH 7.1, 6.4, 5.2 and 4.1 through different time periods and their P-values calculated by one-way-ANOVA test.

Incubation period	pH	control soils (%)	manure applied soils (%)	P-value
0-week	7.1	3.347 ± 0.086	N/A	N/A
	6.4	1.917 ± 0.062	N/A	N/A
	5.2	1.336 ± 0.177	N/A	N/A
	4.1	0.156 ± 0.022	N/A	N/A
5-week	7.1	3.893 ± 0.186	3.775 ± 0.078	0.3912
	6.4	1.733 ± 0.067	1.644 ± 0.200	0.5060
	5.2	1.152 ± 0.144	1.178 ± 0.079	0.8027
	4.1	0.153 ± 0.006	0.203 ± 0.023	0.0219
10-week	7.1	3.799 ± 0.036	4.340 ± 0.044	8.22e-05
	6.4	1.834 ± 0.077	1.958 ± 0.062	0.0958
	5.2	1.276 ± 0.084	1.343 ± 0.087	0.3931
	4.1	0.156 ± 0.005	0.229 ± 0.010	3.07e-04

Table A4.21 Comparison of PLFA concentration results by two-way-ANOVA test.

Biomarker	Prob > F		
	Incubation period	Soil pH	Interaction btw incubation period and soil pH
Total FA	9.94e-06	4.04e-22	5.01e-14
Gram-positive	3.54e-05	1.59e-31	6.59e-20
Gram-negative	1.52e-09	1.06e-25	1.97e-17
Actinomycetes	5.76e-14	2.97e-26	4.56e-15
Fungi	3.82e-18	1.75e-25	5.74e-18
fungi/bacteria	0.0000	0.0003	0.0000