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Metabolomic and volatilomic profiling for the assessment of soil carbon cycling and biological quality

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Declaration

I hereby declare that this thesis is the results of my own investigations, except where otherwise stated. All other sources are acknowledged by bibliographic references. This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree unless, as agreed by the University, for approved dual awards.

Yr wyf drwy hyn yn datgan mai canlyniad fy ymchwil fy hun yw'r thesis hwn, ac eithrio lle nodir yn wahanol. Caiff ffynonellau eraill eu cydnabod gan droednodiadau yn rhoi cyfeiriadau eglur. Nid yw sylwedd y gwaith hwn wedi cael ei dderbyn o'r blaen ar gyfer unrhyw radd, ac nid yw'n cael ei gyflwyno ar yr un pryd mewn ymgeisiaeth am unrhyw radd oni bai ei fod, fel y cytunwyd gan y Brifysgol, am gymwysterau deuol cymeradwy.

Signed: R. Brown   Date: 29th October 2021

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Thesis Summary

Soil is the universal substrate which underpins agricultural productivity, providing plants and soil organisms with water and nutrient resources, as well as a plethora of additional anthropogenic ecosystem services. However, the sustained intensity at which we are using soil resources and the increasing frequency and intensity of extreme weather events is leading to a serious decline in soil quality, often defined as 'the capacity of the soil to function', and associated ecosystem function and service delivery. Better understanding and monitoring soil quality is key to slowing and reversing this decline. Soil biology (and related biochemistry) has often been underutilised as an indicator of soil quality; however, it is one of the most reactive and sensitive indicators. This thesis explores novel methods of profiling the small organic molecules (i.e., metabolites) in the soil; produced by the biological community during the catabolism of substrates and anabolism of cellular metabolites. It examines methods of profiling both primary (i.e., compounds involved directly in the growth, development and reproduction of organisms) and secondary (i.e., compounds performing additional functions) metabolites in relation to soil quality and carbon (C) cycling. Specifically, I applied untargeted primary and secondary metabolomic methods to 'real world' field conditions and laboratory mesocosm experiments, assessing their applicability and aiming to further understand the complex biochemical interactions within the soil under a range of conditions, combining this data with a suite of physicochemical measurements to make conclusions about changes in soil quality and function. Here, I showed that, under field drought conditions, the primary metabolome shows similar trends to previous laboratory-based research, with significant increases in drought 'biomarker' compounds and storage lipids during drought, followed by a significant, rapid decrease in those compounds under post-drought conditions. Overall, soil functionality showed a high resilience to drought. Additionally, I showed that pure microplastic (MP) addition has little impact on the biological functioning of soil over a field season, even at unrealistically high loading rates. From the biological, physical and chemical indicators measured, few significant effects relative to no MP application were observed. However, it was concluded that while in the short-to-medium term MPs are recalcitrant and inert, pure plastic loading is unrealistic, and further research should be undertaken on the effect of plastic additives on soil health. Further, I mechanistically disentangled the effect of nutrient addition (C:N:P) on the soil microbial metabolite profile and C use efficiency. Demonstrating that; nitrogen (N) addition had the greatest impact on the ability of the soil microbial community to utilise excess C substrates, while phosphorus (P) addition led to significant increases in the synthesis of fatty acids. I concluded that inorganic nutrient enrichment of soils is likely to have substantial implications for labile and recalcitrant C cycling and microbial resource partitioning within the soil system. Additionally, I explored soil-derived secondary metabolites as an indicator of soil quality, by applying a headspace-solid phase microextraction (HS-SPME) method to profile the volatile organic compounds (VOCs) under a variety of induced 'soil qualities'. I identified compounds associated with the differences between treatments, showing that substrate availability and quality are key in the production and emission of VOCs. Also, I evaluated a novel HS-SPME-trap-enrichment method to improve compound recovery and sensitivity, comparing it with other HS-VOC extraction methods. I concluded that metabolomic and volatilomic methods provide another sensitive tool in the kit for the characterisation and elucidation of soil biochemistry and chemical ecology, to aid the understanding of the complex small molecule interactions taking place within soils. The ultimate aim being the integration of metabolomics with other 'omics platforms, with an emphasis on providing a greater functional understanding of key soil processes and the development of new soil health metrics.

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Project Caveat

This KESS II funded studentship started as a collaborative initiative between two industrial partners and Bangor University with the aim of investigating phospholipid fatty acid (PLFA) biomarkers for soil biological quality, tailoring a system that had been validated for Australian soils to UK soils. Unfortunately, within the first year of the studentship commencing, intellectual property (IP) issues became impassable, leading to both industrial partners withdrawing from the project. Subsequently, the studentship was entirely supported by KESS II funding.

In order to avoid infringing IP, the project subsequently took a significant diversion from its original aim, with a greater focus on the metabolomic and volatilomic techniques. This change brought increased research freedom, allowing for a more dynamic project, responding to events and collaborating with several different institutions. The subsequent collection of chapters brings together the themes of soil biogenic primary and secondary metabolism as a result of this freedom and collaboration.

Chapter 1: Introduction

1.1. General introduction

Soil is a complex, heterogenous substrate, on which, much of the earth's life, directly or indirectly, depends. It is the intersection of the biosphere, hydrosphere, lithosphere and atmosphere, and is one of the most biodiverse habitats on earth, with estimates that a quarter of the earth's biodiversity resides in soil (Guerra et al., 2021). In an anthropogenic context, soils are extremely useful, producing a wide range of ecosystem services; goods obtained from ecosystems that benefit people's well-being (Pereira et al., 2018), including climate regulation, nutrient cycling, water purification, contaminant removal and the provision of food, fuel and fibre (Bardgett and van der Putten, 2014; Baveye et al., 2016). Soil is a finite and non-renewable resource on a human timescale, taking up to 1000 years to produce 2 – 3 cm of soil. However, it is estimated that up to one-third of the world's soil are degraded, with up to 90% of soils potentially becoming degraded by 2050, due to intensification of use, mainly as a result of agriculture (UNFAO, 2015). Understanding soil quality, often defined broadly as; 'the capacity of the soil to function' (Karlen et al., 1997), its trends and inherent dynamism, is key to ensuring sustainability into the future.

1.2. Background and rationale

The constitution of soil, with its solid, liquid, and gaseous phases, underpinned by biological processing and turnover, makes it one of the most complicated and dynamic substrates on earth, varying considerably over both space and time (Bünemann et al., 2018). Within the context of the agroecosystem, on which this thesis is based, inherent quality is governed by extrinsic factors, for example, parent material, climate, topography, as well as land use and management.

While records are not contemporaneous, the first formal documentation of soil assessment originates from the Zhou dynasty (1048 – 256 BCE) in China (Harrison et al., 2010), while other cultures throughout ancient history, including the Romans, also valued soil assessment highly (Warkentin, 1995). Moving towards the present, agricultural soil quality assessment has developed at a substantial rate since the 1970's moving from a small number of indicators, focusing on the productivity at a field or farm scale to a plethora of indicators, with the aim of assessing the multi-functionality of soil, provision of ecosystem services as well as resistance and resilience to perturbation (Bünemann et al., 2018). Soil quality

assessment remains highly relevant to understanding and monitoring soil resources particularly as agricultural intensification continues, due to the requirement of feeding a growing global population. Until relatively recently (circa 2010), soil quality was assessed using physical and chemical measurements. However, biological, and particularly biochemical, metrics were often neglected due to a lack of analytical approaches, their complexity in interpretation and sensitivity to abiotic, e.g., changes in the chemistry (pH, salinity, organic matter, aeration/ saturation, available nutrients, application of agrichemicals) or physical conditions (soil structure and texture) and biotic factors (for example, aboveground biology) (Bünemann et al., 2018). Arguably this dynamism is one of biology's strengths, allowing effective indication over a much shorter timeframe than chemical and physical indicators, with soil biology driving soil function (Wagg et al., 2014).

Soil organic matter consists of the living organisms within soil (e.g., the soil micro- and macro-ecology), fresh residues (e.g., plant, animal, and microbial necro mass or excreta) i.e., the substrate for prokaryotic and eukaryotic growth, as well as the transformation products of these residues in a range of forms, broadly defined as humic and non-humic matter (Nieder and Benbi, 2008). The carbon (C) stored within organic matter represents a larger store than global and atmospheric C combined (Lehmann and Kleber, 2015). As such, understanding the biological and biochemical processes that form the basis of C cycling within the soil is of high impetus, having implications for not only soil quality, but also climate stability and food production, and more general ecosystem service provision.

The concept of metabolism, the catabolic (breaking down) and anabolic (building up) reactions that allows an organism to function (i.e. grow, develop, reproduce and interact) by synthesising new organic material, has widely been applied in individual organisms (Blanco and Blanco, 2017). However, the concept may also be applied to the collection of organisms within systems in an ecological context i.e. soil (Sardans et al., 2011). The application of metabolomics, the large-scale study of small molecules (substrates, intermediates, and products) within a sample, to soil has been used to aid the understanding of small molecule cycling as well as the interactions between organisms in fine detail (Canarini et al., 2019; Overy et al., 2021; Swenson et al., 2015; Tyc et al., 2015; Withers et al., 2020).

Primary metabolomics, referring to the analysis of the compounds directly involved in growth, development, reproduction (e.g. sugars, organic acids, amino acids, phenolics, fatty acids etc), and secondary metabolomics, compounds not directly involved in growth,

development, reproduction (a chemically diverse group of compounds with functions ranging from anti-biotic/fungal and quorum ensuing molecules to degradative enzymes), together form the fundamental biochemical building blocks on which C and nutrient cycling, pools and fluxes within and from the soil are based. The metabolic profile for the soil is as a result of several levels of 'omic' interaction. The genome is determined by the soil biome composition. This, in turn, determines the transcriptome (ribosome) and proteome (enzyme profile), which in turn determines the output of the metabolome. The metabolome therefore reflects the abiotic and environmental selection conditions as well as biotic selection and adaption.

As alluded to in section 1.1, soil is the foundation of many ecosystem services, and a well-functioning and healthy soil system is key to their successful provision. Understanding the ability of the soil and its microbial community to respond to both climate change (for example, increased risk of drought and flooding (Hamidov et al., 2018)) and anthropogenic management (for example the addition of microplastics into the soil system (de Souza Machado et al., 2020)) perturbation, in a chemical ecology and biochemical context, furthers our knowledge of ecosystem function, as well as potentially identifying new 'biomarker' compounds for evaluating stress conditions or change.

1.3. Thesis outline and aims

Broadly, the aim of this thesis is to explore the soil primary and secondary metabolism and its intrinsic link to function within the context of the agroecosystem. Comprising of eight chapters, the overarching objective is to advance the current state of knowledge and perception of metabolomics within the field of soil science (Fig. 1.1).
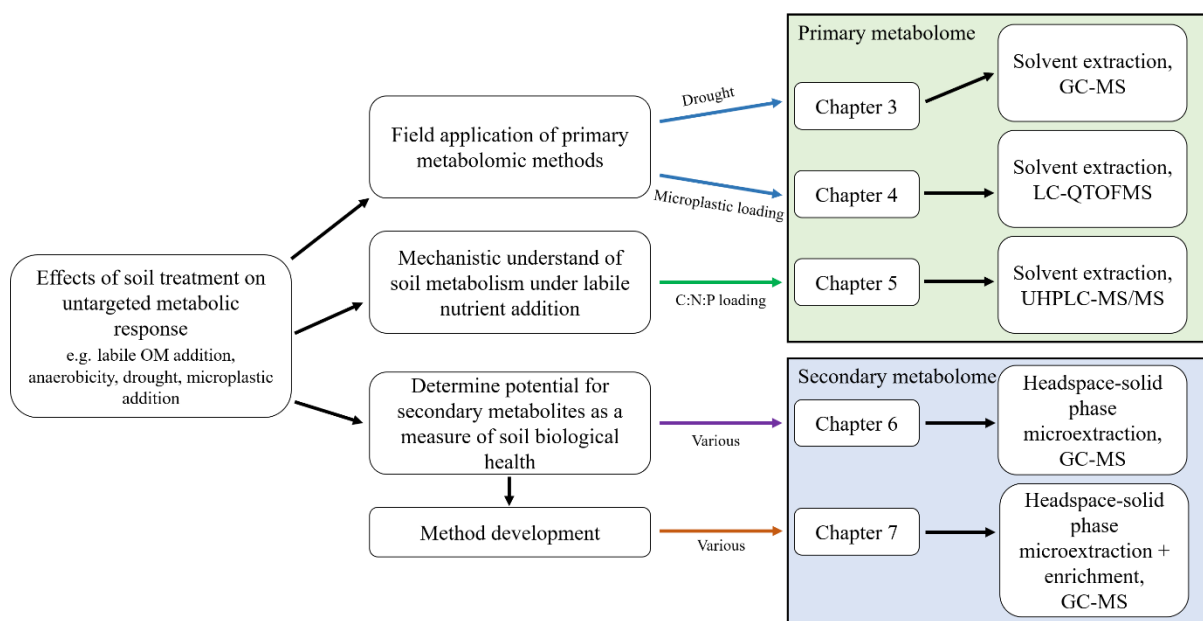
Figure 1.1. *Schematic diagram of the experimental chapters contained within this thesis.*

Chapter 2 comprises a review synthesising the current state of research within the field of soil metabolomics and its relationship to soil quality. Examining primary, secondary and lipid metabolism and the factors affecting them. Advances in the measurement of metabolites, and their potential for use as a biochemical quality indicator are also addressed.

Chapters 3, 4 and 5 apply metabolomic profiling methods to field and laboratory-based experiments. Specifically, chapter 3 focuses on primary metabolomic and lipid profiling, microbial community structure and greenhouse gas fluxes from soil under natural drought conditions, the likelihood of which is increasing with climate change. It aims to examine the untargeted microbial metabolomic and lipidomic responses to drought conditions, under natural, field conditions, particularly focusing on resilience of the system. Chapter 4, examines the effect of microplastic (MP) loading on soil health over a cropping season, combining novel metabolomics methods (biogenic amine profiling), with soil biological community, physiochemistry, greenhouse gas emissions, and crop health analysis. It aims to provide a comprehensive overview of the impact of MP introduction on agroecosystem health. And, chapter 5 examines the effects of stoichiometrically balanced nutrient (C:N:P) input to soil, on the primary metabolites synthesised. With the aim of providing a mechanistic understanding of biogenic nutrient processing and C cycling within an agricultural soil.

Chapters 6 and 7 broadly focus on soil secondary metabolism, in particular the analysis of volatile organic compounds (VOCs). Chapter 6 describes the utilisation of a headspace solid phase micro-extraction (HS-SPME) method combined with gas chromatography mass spectrometry (GC-MS) to analyse the soil VOC profile. It aimed to understand the methods' ability to separate soils based on their quality, compared to an established measure of soil biological quality, phospholipid fatty acid (PLFA) profiling, and identify potential condition specific 'biomarker' compounds. Chapter 7 builds on the SPME methods described in chapter 6, comparing it with other headspace (HS) extraction methods and exploring the possibility of enrichment methods to increase sensitivity. It aimed to compare the sensitivity and recovery of each method, and to explore the VOC emissions profile associated with each soil treatment.

Chapter 8 provides a discussion of the use of metabolomics as a tool for the analysis of organic matter and C cycling in soils including its strengths and weaknesses, highlighting areas of future research and summarising the main conclusions from this body of work. Finally, appendices one to five include supplementary material from the data chapters (three to seven), and appendix six contains a further study performed and written during this research project but which does not fit within the overarching themes of this thesis.

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Chapter 2: Literature review - Soil metabolism and its intrinsic link to soil quality

2.1. Introduction

In the UK, 71% of land is used for agriculture, relying on soil for production (DEFRA, 2016). Additionally, environmental quality, which ultimately determines human health, relies on good soil, air, water and ecosystem quality (Karaca et al., 2010). Soil quality, defined by Karlen et al. (1997) as "the capacity of a specific kind of soil to function, within natural or managed ecosystem boundaries, to sustain plant and animal productivity, maintain or enhance water and air quality, and support human health and habitation", is often considered a more dynamic subset of overall land quality (Bünemann et al., 2018). Soil provides numerous environmental benefits through the provisioning of ecosystem services, summarised in Fig. 2.1. There are many factors that affect the quality of soil in an agricultural context; these can be intrinsic (the factors determining pedogenesis), although in most modern settings it is extrinsic factors, namely the management regime and environmental extremes, that have a significant impact on soil quality (Dai et al., 2019; Li et al., 2017; Suddarth et al., 2019).
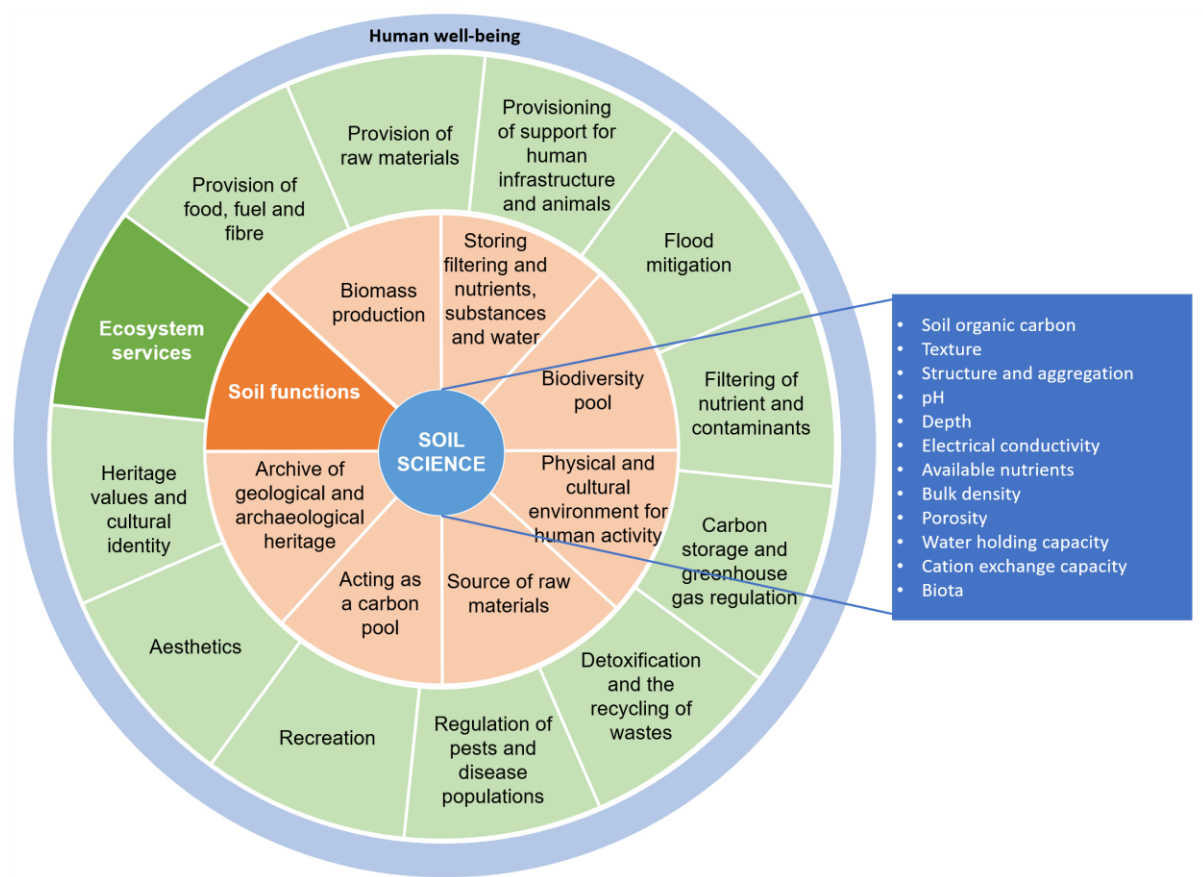
Figure 2.1. *Examples of soil functions that provide ecosystem services, adapted from Keesstra et al. (2016) and Adhikari and Hartemink (2016).*

2.1.1 Criteria for soil quality assessment

Currently, there is no agreed standard criteria for the assessment of soil quality, instead the criteria vary in relation to the requirements of the assessment e.g. management goals. However, Bünemann et al. (2018) summarized criteria of soil quality indicators from various publications into four broad categories: conceptual, practical, sensitive and interpretable. In summary, good indicators must; have relevance and relate to soil function or ecosystem processes, integrate biological, chemical and physical properties and be important at an applicable scale (Doran and Parkin, 1996; Larson and Pierce, 1994; Macdonald et al., 1998; Ritz et al., 2009). Additionally, indicators must be easy to sample or measure, with high reliability and relatively low cost (Bone et al., 2012; Doran and Parkin, 1996; Merrington, 2006). Measurements must be sensitive to spatial and temporal variation as well as changes in land use or management (Nortcliff, 2002; Oberholzer et al., 2012), while also being easy to interpret, comparable with routine sampling and be able to show trends (Doran and Parkin,

1996; Idowu et al., 2008; Ritz et al., 2009). If these criteria can be met, then the resulting soil quality index and interpretation will be informative in land management decision making.

2.1.2 Current quality indicators

Soil quality can be assessed using physical, chemical and biological indicators (Bünemann et al., 2018). The focus has been on physical and chemical indicators, due to their ease of sampling and analysis. But biological indicators are now receiving greater interest.

Physical indicators relate well to the water characteristics, in addition to the stability and support of the soil. It is likely they are some of the oldest soil assessment tools as they can be assessed qualitatively with little equipment required. Common indicators include water storage and infiltration rates (Lowery et al., 1996), bulk density (Arshad et al., 1996) and texture, structure and structural stability (Bronick and Lal, 2004).

Chemical measurements are often indicative of soil buffering and nutrient cycling functions and relate to soil water interactions. Traditionally chemical indicators have been most widely used to characterise soil quality (Bünemann et al., 2018). These include soil organic matter (SOM) (Rasmussen and Collins, 1991), pH (Arshad and Martin, 2002), available nitrogen (N) and phosphorus (K) (Dinkins and Jones, 2013) and total N (Kennedy, 1999).

Presently, one of the best indicators of soil biological health is simply presence/abundance and ecotypes of earthworm (Stroud, 2019). As microbiology within the soil system is complex and variable, highly dependent on the physical (soil structure and texture) and chemical (pH, salinity, organic matter, aeration/ saturation, available nutrients, application of agrichemicals) soil properties as well as a range of other factors (climate, vegetation type, management and disturbance). Historically, there has been a lack of understanding about the link between microbial diversity and soil function (Nannipieri et al., 2003). For these reasons, biological indicators have often been omitted from soil quality indexes (Bünemann et al., 2018). However, their critical role in ecosystem function, as well as improvements in the measurement and understanding of biophysicochemical interactions of microbiology with the environment, makes them increasingly hard to ignore as part of a broader soil quality indexing tool (Enriqueta Arias et al., 2005; Schloter et al., 2003; Visser and Parkinson, 1992).

Additionally, biological indicators can also be effective indicators over much shorter temporal periods than traditional physical and chemical indicators (Bastida et al., 2006).

Commonly used metrics include soil respiration (Ölinger et al., 1996), microbial biomass (Cardoso et al., 2013), phospholipid fatty acids (PLFAs) (Frostegård et al., 1993), N mineralisation (Saez et al., 2012) and soil invertebrate surveys (Kanianska et al., 2016). However, advancements in technology and reducing cost of techniques have allowed for more advanced exploration of soil biology. For example, the assessment of community composition through metabarcoding and metagenomic approaches (Feng et al., 2018), gene expression rates and impact through transcriptomics (Perazzolli et al., 2016), as well as the processing rate and functional impact on the community through metabolomic profiling (Judd et al., 2006; Schimel and Schaeffer, 2012).

2.1.3 Importance of soil assessment

Soil is a non-renewable resource on a human timescale. Yet it is key, alongside air and water, to global ecosystem and agroecosystem functioning as well as the provisioning of a wide range of ecosystem functions and services, making it an incredibly valuable resource (Dominati et al., 2010). Expansion of agriculture in order to meet future demand for food, fuel and fibre is likely to require a combination of sustainable intensification of current agricultural systems as well as the need for additional land. This will inevitably lead to greater pressure on soil resources (Gomiero and Tiziano, 2016).

Measurement of soil quality is often used to inform stakeholders (land managers, farmers and policy makers) about the current state of their soil and the agroecosystem as a whole. Soil degradation from agricultural intensification is an increasing issue, costing the UK an estimated £1.2 billion per year, with loss of organic matter, compaction and erosion the greatest contributors (Graves et al., 2015). Additionally, soils vary markedly both temporally and spatially, so a greater level of agri-statistical information is needed to inform targeted approaches to applying agrochemicals as well as interventions such as subsoiling and drainage, in order to optimise crop growth at a sub-field scale. More information on soil quality will inevitably lead to improved resource use efficiencies, resulting in reduced waste and increased benefit to the farmer, while in turn also lowering the risk of environmental pollution. A major challenge in developing a soil quality index capable of mainstream adoption is the establishment of guidelines for selecting assays that reflect changes in specific agroecosystems and the productivity, environmental, and health components of soil quality (Granatstein and Bezdicek, 1992).

2.2. Metabolism in a soil context

The concept of soil metabolism was first introduced by Quastel (1955) in order to understand the chemical interrelationships affecting biological cell behaviour and processes. The metabolome refers to the entirety of small molecules (< 1500 Da) found within a biological sample (Klassen et al., 2017). Primary metabolism is key in the sustenance of life. This complex set of biochemical catabolic and anabolic processes determines the ability of organisms to access the energy within their foodstuff, convert this energy into useful 'building blocks' i.e. proteins, lipids, nucleic acids and carbohydrates, and dispose of compounds that are no longer useful. Therefore, primary metabolites are any small molecule involved directly in the growth, development and reproduction of an organism (Rojas et al., 2014). In all organisms, primary metabolism is governed by strict metabolic pathways, with the transformation of chemicals being facilitated and regulated by specific enzymes (Fig. 2.2), which are in turn encoded by an organism's genes (van der Knaap and Verrijzer, 2016). However, the metabolic pathways currently represented on metabolic charts only represent a small proportion of the total number present in an organism or environment. This unknown in metabolomics research is often referred to as 'metabolic dark matter' (Markley et al., 2017).
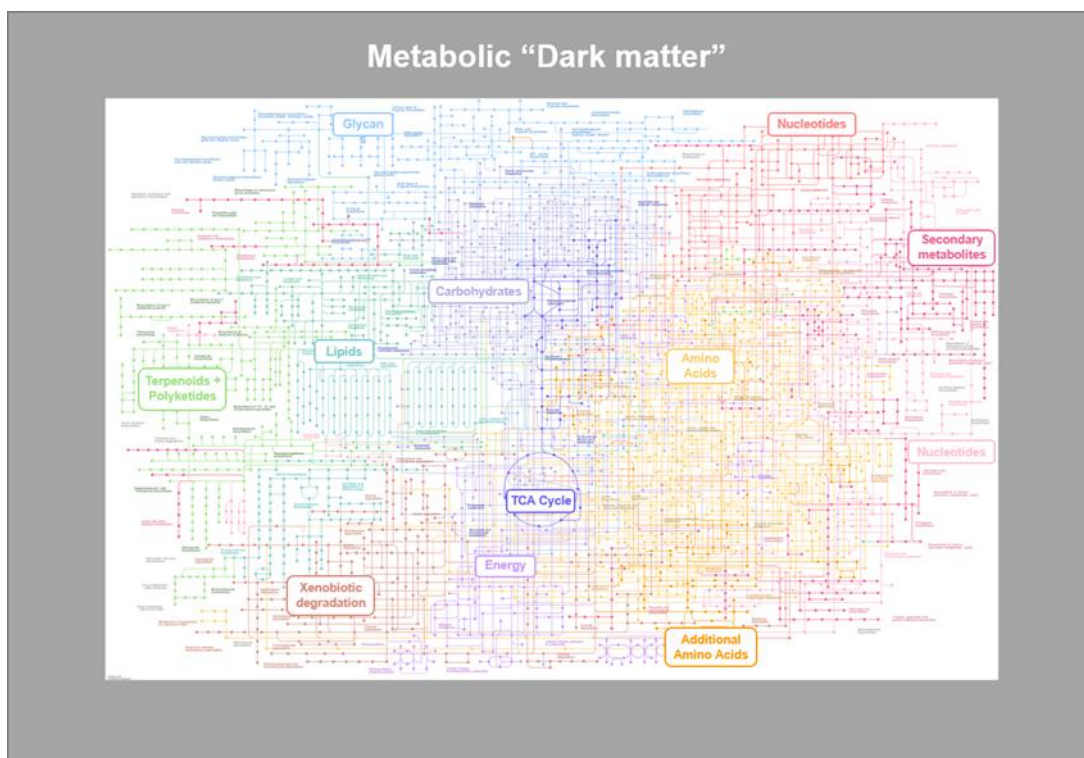
Figure 2.2. *The Kyoto Encyclopedia of Genes and Genomes (KEGG) schematic representation of known metabolomic pathways (Kanehisa et al., 2016), however the database is not extensive, with the potential for many other, as yet unidentified, metabolic pathways, leading to metabolic 'dark matter'.*

Generally, primary metabolism is conserved across most life forms (Peregrín-Alvarez et al., 2009). The most basic conserved pathway being glycolysis in sugar metabolism, driving the citric acid cycle eventually producing adenosine triphosphate (ATP) for energy and nicotinamide adenine dinucleotide (NADH) as a reducing agent, ultimately releasing carbon dioxide (CO_2) (Kalucka et al., 2015). However, soil respiration as well as metabolic quotient (qCO_2), while barometers of soil metabolic activity, do not reflect the primary metabolism as they do not describe carbon (C) accumulation rates or variations in C accumulation in biomass (Doi et al., 2010; Manzoni et al., 2010).

In contrast, secondary metabolism (sometimes referred to as specialised metabolism) concerns all other small molecules that are not directly involved in the growth, development or reproduction of an organism. In general, their synthesis comes from primary metabolites, either directly or as a by-product, and in this sense the two systems are inextricably linked (Ramakrishna and Ravishankar, 2011). Secondary metabolites may be involved in a range of non-essential roles, as well as serving as mediators for inter- and intra-species signalling and

interactions, for example, pathogen growth inhibition, beneficial or symbiotic attraction, growth regulation or abiotic stress mitigation (Brilli et al., 2019). As such, they are much more varied in their role and chemical composition, with much less conservation across taxa, with different groups of organisms often producing unique compounds to their benefit (Holopainen et al., 2018). Broadly, secondary metabolites can be grouped by their volatility, either low volatility (for example, tannins, organic acids and some alkaloids) or high volatility (for example, phenolics, terpenes, alkanes and some sulphides); this characteristic has a major effect on their ability to diffuse through the soil and thus the sphere of influence of a compound or organism (Rowan, 2011; Tyc et al., 2017b).

Traditionally, metabolomics has been thought about on the level of a single cell or organism (Witting et al., 2018; Zenobi, 2013). However, more recently the idea of metabolism has been used in systems biology; monitoring the changes within the metabolome, and from this, inferring the physiological state of a cell (Damiani et al., 2020). This process may also be applied to larger and more complex systems and communities of organisms; allowing the characterisation of the function of an ecosystem at a macro-scale based on the interactions and reactions (to abiotic or biotic environmental change) of the members of the microbial community at a micro-scale (Abram, 2015). In this sense, the measurement of a systems' metabolism transcends traditional approaches to looking at soil biology, going beyond species, phylum or domain level.

Soil is an extremely complex and dynamic matrix due to its intrinsically linked mixture of biology, chemistry and physics. A simplified representation of the soil system's primary and secondary metabolism is displayed in Fig. 2.3. However, the soil system is further complicated by its spatial heterogeneity, caused by a number of reasons, for example factors of soil formation (climate, geology, flora and fauna, topography and time) and anthropogenic management (Burke et al., 1999). However, this may also be an advantage, as each soil type and level of health, in theory, will have its own unique metabolic state.
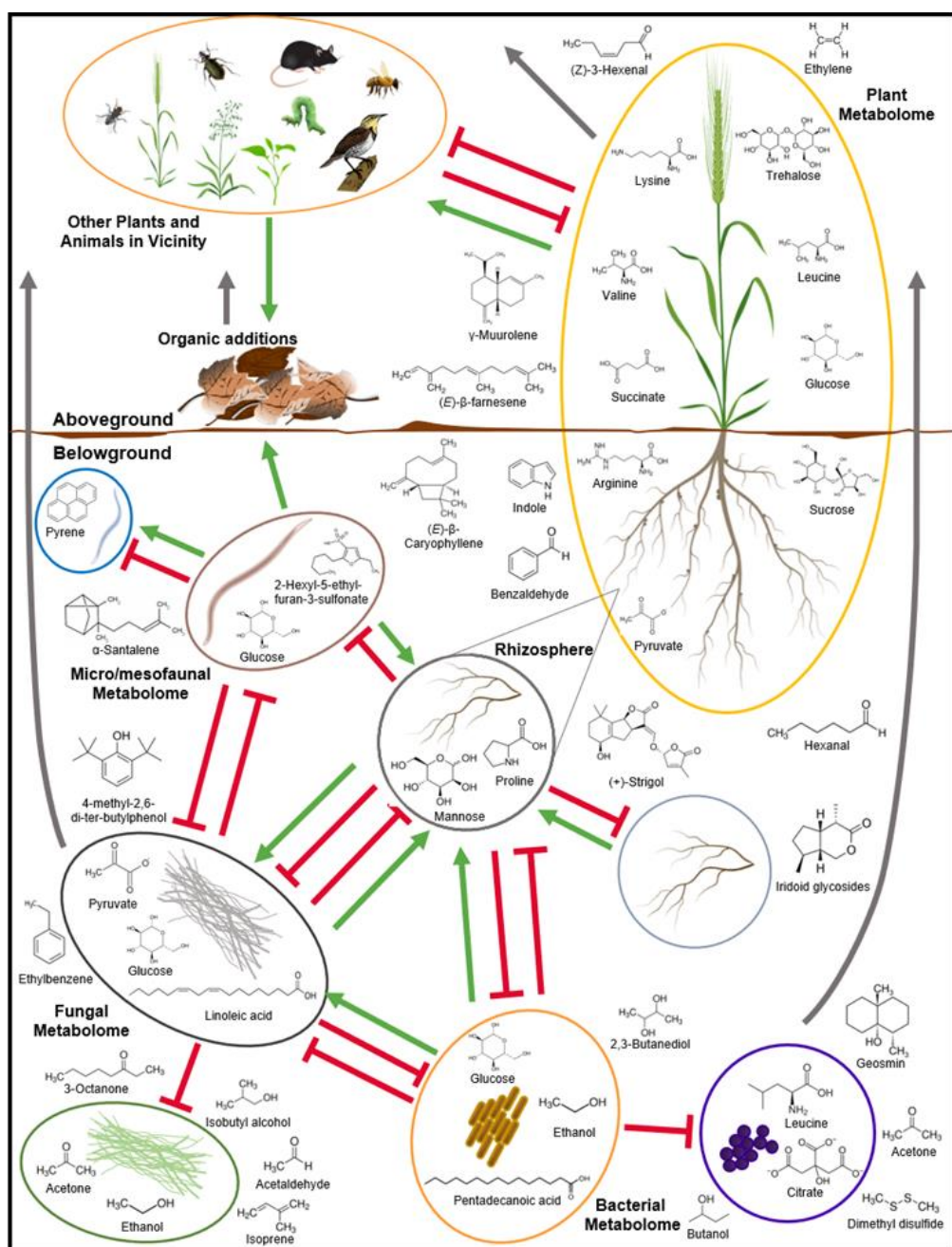
Figure 2.3. *A generalised schematic of example primary (within spheres) and secondary (outside spheres) metabolic interactions in the soil system. Red interactions represent potential negative effects (i.e. growth inhibition or toxicity), green interactions represent potential positive effects (i.e. growth promotion or attraction of predators in tri-tropic interactions) and grey arrows represent loss of volatile organic compounds (VOCs) from the system.*

2.2.1. Genetic and enzymatic control of metabolic pathways

Enzymes underpin an organism's ability to perform metabolic reactions. Enzymes are proteins that allow more rapid chemical reactions, while not undergoing any permanent alteration themselves, combining with their specific substrates in a specific fashion as to change the electronic configuration around certain susceptible bonds (Tabatabai, 1983). The unique intracellular enzymes contained within a bacterial cell number in their thousands (Renneberg et al., 2017), while enzymes characterised in plants range from tens to thousands depending on the species (Engqvist, 2016). In turn, the ability of an organism to encode for and produce enzymes within the proteome, is dependent on the genetic information carried in the genome (DNA (deoxyribonucleic acid)) and transcriptome (RNA (ribonucleic acid)) (Busk et al., 2014; Takahashi et al., 2012; van der Knaap and Verrijzer, 2016; Young et al., 2012).

Briefly, the genome, carries the genetic information for the development, function, growth and reproduction of an organism, i.e. gene function (Cooper, 2000). Of the entire genome, ~1 - 2% consist of coding regions (i.e. codes for proteins) in eukaryotes (International Human Genome Consortium, 2004; Manzoni et al., 2016). The transcriptome is responsible for coding, decoding, regulation and expression of genes. In eukaryotes, 1 - 4% of RNA consists of messenger RNA (mRNA) that allows for protein synthesis (Manzoni et al., 2016). The transcriptome is then translated into a set of proteins (the proteome). Together, the transcriptome and proteome constitute the potential function of an organism, i.e. the ability to perform metabolic reactions catalysed by enzyme activities. Subsequently, the set of metabolites that make up the metabolome is produced. Ultimately, there is a biological hierarchy that culminates with the metabolome, this offers the possibility of capturing several layers of underlying biological activity within one analysis (Fig. 2.4).

In addition to the transcriptional mechanisms, it has been speculated that non-transcriptional mechanisms (e.g. metabolite–protein interactions and protein phosphorylation) are highly relevant in controlling metabolic output (Heinemann and Sauer, 2010; Humphrey et al., 2015; Yang et al., 2012). The consequences of non-transcriptional processes; post-transcriptional, post-translational, and pleiotropic, are likely to have unexpected consequences on regulatory networks and possibly affect functionality (Ray et al., 2011). For example, affecting the virulence of *Salmonella* (Shin et al., 2006).
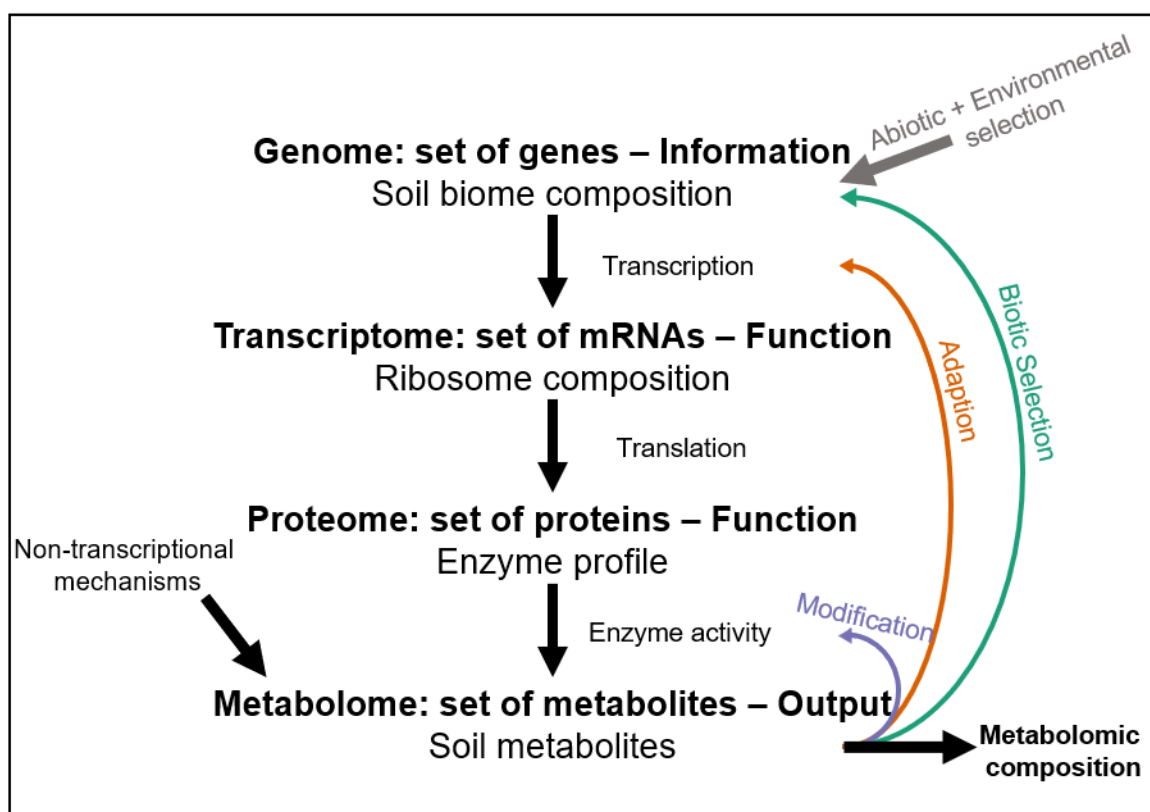
Figure 2.4. *A conceptual representation of the biological hierarchy from starting with the genome and culminating with the metabolome, which is sensitive to environmental and organismal change. Adapted from (Takahashi et al., 2012).*

2.2.2 Origins of metabolites in the soil system

The makeup and speed of metabolic processing of the soil is dependent on the soil's inherent biological, chemical and physical properties. Many soil organisms also process and produce metabolites during the decomposition of litter and nutrient cycling. Harder to break down structural components (e.g. lignin and cellulose) and presence of secondary metabolites (e.g. tannins and phenolics) increase the recalcitrance of litter and make it less palatable to decomposers (Pavao-Zuckerman, 2008). Ultimately, if conditions are unfavourable for the biological community, the rate of nutrient processing and metabolite production will slow. Small molecule metabolites have two main sources in the soil; plants and microorganisms, on which this review will focus.

2.2.2.1 Plant metabolites in the soil system

Plants represent the largest source of metabolites in the soil. The production and fluxes of both primary and secondary metabolites reflects a plant's current physiological status.

Although, abiotic processes can produce metabolites, for example through ultraviolet (UV) light breakdown of organic material (Sulzberger et al., 2019). It has been estimated that plants exude 21 - 25% of C through their roots (Dessaux et al., 2016; Jones et al., 2009). This major rhizospheric C source is then metabolised by the microbial community (Sasse et al., 2018). This exudation may not only be of benefit to the plant, but may also promote relationships between the plant, microbial, fungal and mesofaunal communities.

2.2.2.1.1 Plant root primary metabolism

Plant root primary metabolism is generally endogenous, concerning the small molecule interactions within the root, and is very similar in nature to the metabolism of the above ground-biomass. However, roots also represent a sink organ, as they rely exclusively on the import of sugars from the above ground biomass, which is used by different root tissues for metabolism or storage (Hennion et al., 2019). Storage of sucrose, as well as other proteins in roots, blur the boundaries between primary and secondary metabolism. In this regard, Bais et al. (2001) speculated that some major proteins in underground storage organs have evolved more than one function, including defence (e.g., insecticidal properties of palatin in potatoes (Strickland et al., 1995)). Roots have also been shown to change their primary metabolic composition, particularly carbohydrates and amino acids, in response to insect attack (Zhou et al., 2015). The physiological consequences of this change are often exhibited as reduced water and nutrient uptake, reduced growth and biomass, as well as the depletion of below-ground storage compounds (Johnson et al., 2016). While under environmental stress, e.g. reduced water or nutrient conditions, plants may increase the concentration of primary metabolites in their tissues, particularly in the roots, in order to enhance the uptake of water and nutrients (Gargallo-Garriga et al., 2014; Mundim and Pringle, 2018).

2.2.2.1.2 Plant root secondary metabolism

Some endogenous primary metabolites (for example, sugars and sugar alcohols, amino acids and organic acids) may also be employed as exogenous secondary metabolites as they are actively excreted into the rhizosphere by roots to stimulate microbial response, providing microbial nutrients (Sasse et al., 2018). This process of engineering the rhizosphere or 'feeding your friends' may be beneficial to the plant; by bioengineering a non-pathogenic, or even symbiotic, biofilm serving as protection from the wider soil biosphere (Dessaux et al., 2016; Sasse et al., 2018).

Roots are extremely leaky, with root exudation estimated to represent between 10 – 40 % of assimilated C and 15% of N (Hennion et al., 2019; Venturi and Keel, 2016). The rhizodeposition of sugars and amino acids and other large, non-volatile molecules is complex, requiring transport through the plasma membrane. While the plasma membrane of cells is permeable to some gases (e.g. oxygen (O_2) and CO_2) and other small hydrophobic molecules, larger molecules permeate much more slowly (Yang and Hinner, 2015). Therefore, larger molecules require molecule specific transmembrane proteins to facilitate their passage through the lipid bilayer, for example sugars use the SWEET transport family (Hennion et al., 2019; Manck-Götzenberger and Requena, 2016; Williams et al., 2000) and organic acids use; ALMT/malate and MATE/citrate transporters (Meyer et al., 2010). Once out of the phloem cell, they are likely excreted via the apoplastic transport pathway, in some plants. Microorganisms in the rhizosphere subsequently utilise and metabolise root exudates, lowering the concentration in the soil which promotes further exudation, by increasing the diffusion gradient (Jones and Darrah, 1996; Vranova et al., 2013). However, there is mixed evidence for this theory with some studies showing enhancement of exudation in the absence of microorganisms. This may be due to metabolic differences in plant species but being a bidirectional exchange, it is very difficult to directly measure (Groleau-Renaud et al., 2000; Valentinuzzi et al., 2015).

It is evident that plants are adaptable as rhizosphere engineers, 'sensing' changing soil conditions and changing their nutrient foraging strategies accordingly (Gent and Forde, 2017). For example, in relation to N availability, plants have been shown to be responsive to the concentration of amino acids-to-ammonium (NO_3^-) in the soil and adapt their N foraging strategy accordingly (amino acids tend to dominate the available N pool in low fertility conditions and NO_3^- dominates in high fertility) (Henry and Jefferies, 2003; Padgett and Leonard, 1993; Schimel and Bennett, 2004).

Plant root volatile organic compounds (VOCs) are estimated to make up about 1% of plant secondary metabolites, however, they represent a very diverse group of compounds, which due to their inherent chemical properties, can easily diffuse through gas- and water-filled pores and therefore have a wide effective range in soil (Venturi and Keel, 2016). Less is known about the transport and release of VOCs from plant roots (Weston et al., 2012). Smaller and more volatile compounds are likely to be able to diffuse through cell walls. However, others may require transport pathways to move them across membranes. These are likely to be similar

to those elsewhere in the plant (i.e. the leaves), for example membrane-bound transport proteins including ABC and MATE proteins (Weston et al., 2012).

2.2.2.1.3 Non-volatile exo-metabolites and rhizosphere interactions

Roots, and in particular, root tips are hot spots for root exudation, particularly amino acids (e.g. glycine, glutamate), organic acids (e.g. citrate, malate) and sugars (e.g. glucose and sucrose), offering a rich source of C and N to surrounding microorganisms. This aids the establishment of beneficial relationships for example with symbiotic bacteria (e.g. rhizobium) and fungi (e.g. arbuscular mycorrhiza) (Li et al., 2016). For instance, legumes exude flavonoids to attract N fixing bacteria (Bolton et al., 1986) and the exudation of benzoxazinoids by maize has been shown to attract growth-promoting bacteria (e.g. *Pseudomonas putida*) (Neal et al., 2012). However, in other cereals, the function of benzoxaziniods has been shown to vary (e.g. insecticidal, antimicrobial or allelopathic) (Wouters et al., 2016).

Mycorrhizal fungi form an extremely important relationship with roots in 90% of all terrestrial plants (Canarini et al., 2019). Initial mycorrhizal fungi colonisation of roots is also likely to be regulated by the exudation of specific secondary metabolites, however little is known about this initial signalling interaction (Parniske, 2008). Once a relationship is established, the mycorrhizal fungi receive a large flux of C from their plant host in return for other nutrients (van der Heijden et al., 2015). Despite the importance of mycorrhizal fungi there is very little understanding of its role and response to root exudation, signalling and metabolism.

Root exudates may not all be released for the benefit of microorganisms. Non-volatile exudates can have a considerable effect on rhizosphere architecture, particularly in the transition zone (the root zone following the root tip (meristematic zone)), which is key in signalling directional root growth, depending on the physiological and nutrient status of a plant (Baluška and Mancuso, 2013; Canarini et al., 2019). Exudation of non-volatile metabolites may also have a number of other functions, for example the release of organic acid anions can be triggered in response to P or iron (Fe) deficiency, making P or Fe more available (Jones and Darrah, 1994), carboxylic acids can complex potentially toxic metals (Mench et al., 1987; Morel et al., 1986), and terpenoids such as momilactones can act as antimicrobial and allelopathic agents (Kato-Noguchi et al., 2008).

2.2.2.1.4 VOC metabolite emission and soil interactions

VOCs represent a much smaller pool of secondary metabolites as opposed to non-volatile secondary metabolites (Venturi and Keel, 2016). However, their much larger diffusive potential and mobility means their influence is likely to extend far beyond the rhizosphere, with Schulz-Bohm et al. (2018) showing VOC diffusion up to 12 cm from roots. This 'volatisphere', may contain a wide variety of molecules with an extensive range of functions, the emission of which will almost always have a net positive benefit for the emitting plant. Over 40 years ago Vančura and Stotzky (1976) characterised a number of VOCs produced from germinating seedlings and suggested their potential ecological impacts. While research techniques have improved significantly, the functional attributes of compounds often remain elusive. Below we examine two key roles of VOCs in plant-soil interaction, namely, attraction and defence.

2.2.2.1.4.1 Attraction of beneficial organisms

Interactions between beneficial or plant growth inducing bacteria aid plants to overcome environmental stresses, e.g. water or nutrient limitation, pathogenic infections and herbivory. It has been shown that plants can recruit or attract beneficial bacteria to their roots over distances greater than the rhizosphere. For example, Schulz-Bohm et al. (2018) showed that migration of 'distant' soil bacteria outside the rhizosphere can be stimulated by plant root VOCs. While no specific VOCs were attributed to the attraction, the majority of VOCs identified were aromatic and ester compounds. Other studies have been able to identify specific compounds that are involved in the attraction, for example Rudrappa et al. (2008) showed that malic acid secreted by roots of *Arabidopsis thaliana* was a selective signal to recruit *Bacillus subtilis* a beneficial rhizobacteria. Some microorganisms also have the capability to use specific compounds as sole C sources, an example of this being the ability both *Pseudomonas fluorescens* and *Alcaligenes xylosoxidans* to using α-pinene (Kleinheinz et al., 1999). Root emission of these compounds are likely to encourage growth of these bacteria. CO_2 is also crucial as a subsoil plant volatile compound for attraction, location and growth stimulation. This is demonstrated by the ability of soybeans to attract beneficial *Pseudomonas fluorescens* using CO_2 (Scher et al., 1985). However, the release of CO_2 may also have negative consequences, allowing herbivores or pathogenic organisms to sense root location, though these signals may be masked by the emission of other secondary metabolites (Dreher et al., 2019; Johnson et al., 2006; Johnson and Gregory, 2006; Reinecke et al., 2008).

2.2.2.1.4.2 Defence from hostile organisms

There are two methods in which plants can counteract root herbivory and attack, either release defensive secondary metabolites themselves or release compounds that attract other organismal predators of the antagonist or use a combination of both (Erb and Kliebenstein, 2020). The size of the antagonistic organism is likely to affect the mode of defence employed, with smaller infections or attacks being dealt with by the plant alone. While for larger infections and attacks the plant may employ entomopathogenic organism in addition to their own response. Plant root anti-fungal secondary metabolite defences are well documented in the literature, with a large percentage being terpenoids; for example cuminaldehyde and p-cymene from *Bunium persicum* effective against *Fusarium oxysporum* (Sekine et al., 2007), β-phellandrene from *Smyrnium olusatrum* and *Rhodiola rosea* effective against *Fomes annosus* (Bertoli et al., 2004; Rohloff, 2002) and methyl propanoate and methyl prop-2-enoate are effective against common barley pathogens (*Fusarium culmorum* and *Cochliobolus sativus*) (Kaddes et al., 2019). Equally, anti-microbial potential has also been shown by 1,8-cineol against a number of microbes (Kalemba et al., 2002; Vilela et al., 2009). Methyl ketones may also provide defence mechanisms against larger attacking organisms (Ntalli et al., 2011; Williams et al., 1980).

The release of secondary metabolites for example, alkaloids (Dawson, 1941), glucosinolates (Kaplan et al., 2008), phenolics and benzoxazinoids (Niemeyer, 2009), may also reduce the plant quality for the feeding herbivores. The release and production of these compounds is likely to decrease the digestive enzyme efficiency of an organism or create a toxic response within the organism (Dobler et al., 2011; Houseman et al., 1992). Ultimately, making the root tissue a less desirable food source. Terpenoids may also be employed as signalling molecules to attract entomopathogenic organisms to respond and limit herbivory. A well cited example of tri-trophic signalling is the sesquiterterpene (E)-β-caryophyllene, released by maize roots being attacked by *Diabrotica virgifera*, this volatile signal attracts entomopathogenic nematodes, parasitizing and killing the *Diabrotica* and protecting the plant from further herbivory (Ali et al., 2010; Rasmann et al., 2005).

2.2.2.2 Summary

It is evident that, even examining a limited range of examples, that the plant metabolome is extremely varied and complex. Primary metabolites govern the ability of a plant to function and survive on a basic level, while secondary metabolites mediate the relationships

that the plant has with the surrounding soil biological and physico-chemical environment, in many cases protecting and enhancing its health. In general, study of the plant root metabolome has focused on changes and defence mechanisms regarding insect or pathogen attack (Kaddes et al., 2019; Rasmann et al., 2005; Schwachtje and Baldwin, 2008). However, much of this work is carried out under laboratory conditions. The complexity of the system in a field environment may limit the applicability of these results. Further work exploring the role of secondary metabolite exudates and VOCs on the complex interaction between plants, fungi and bacteria, particularly under field conditions is needed. Equally, little work has been performed on the change in metabolic function under abiotic stresses (nutrient limitation and water limitation among others), as these are likely to be key regulators of the types of compounds emitted.

2.2.3 Microbial metabolites in the soil system

Microorganisms provide the second greatest contribution to metabolites in the soil. Similarly, to a plant, the production and flux of both primary and secondary metabolites can reflect a microorganism's current physiological status, as this will affect the efficiency of transfer and processing of C between environmental and cellular compartments, as summarised in Fig. 2.5 (Gougoulias et al., 2014). Being a much more metabolically constrained system, the amount of energy and C expended on producing secondary metabolites is likely to be relatively larger than plants and larger organisms. As such, microorganisms specialise in producing a smaller number of bioactive secondary metabolites (Malik, 1980; Singh et al., 2019). Here we will focus on the primary and secondary metabolism of bacteria and fungi in relation to the soil system.
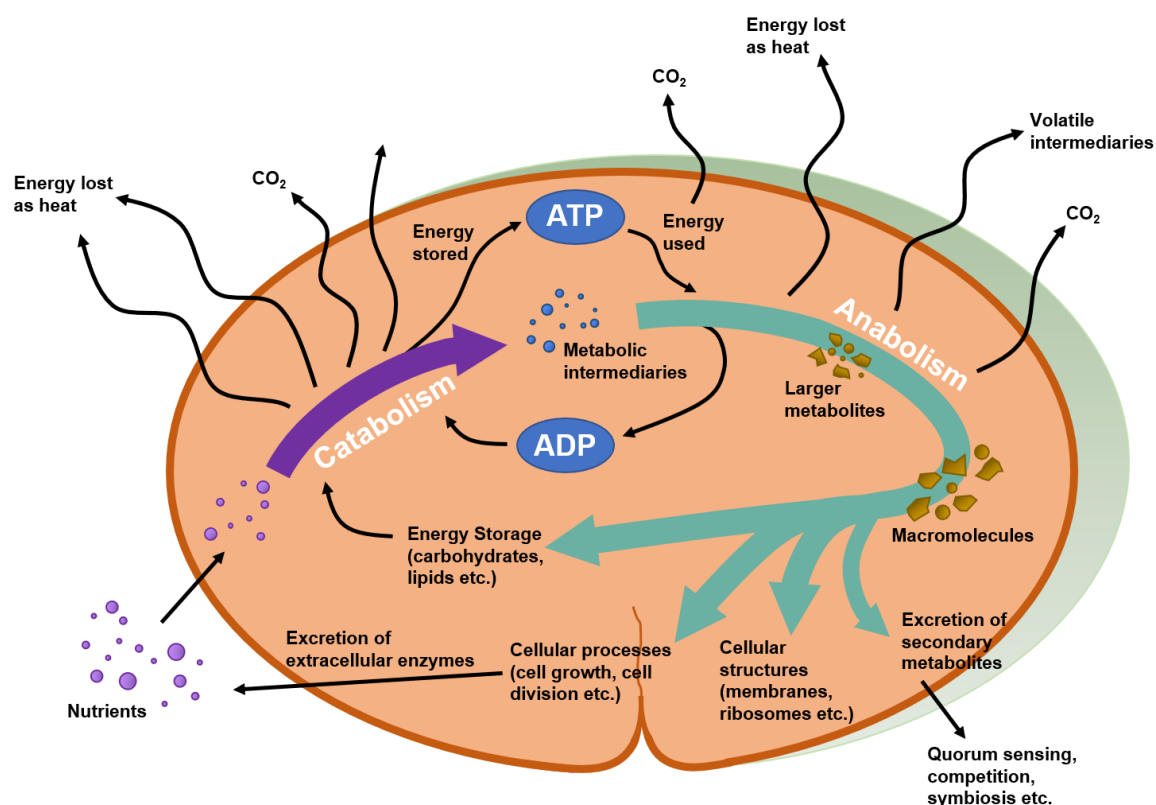
Figure 2.5. *Schematic overview of generalised microbial metabolism, summarising the major processes of anabolism and catabolism which underpin cellular function. Adapted from Varman et al. (2014) and Kadier et al. (2016).*

2.2.3.1 Microbial primary metabolism

As discussed above, primary metabolism is conserved across most organisms (Peregrín-Alvarez et al., 2009). Bacteria and fungi are no exception, relying on the breakdown of sugars and other carbon-based molecules to produce energy; key to an organism's survival and fitness. However, microbes use a diverse range of metabolic strategies and in many cases can be differentiated based on metabolic characteristics, helping different microbes to establish an ecological niche. In the soil system, this translates into a complex patchwork of territorial niches on a micro-scale, with one, or a small number of co-existing microorganisms, dominating under the specific physicochemical properties and environmental conditions present (Bauer et al., 2018). Fungi and most bacteria in the soil are heterotrophic (notable exceptions include autotrophic bacteria involved in nitrification e.g. *Nitrosomonas europaea*

and *Nitrobacter winogradskyi*), or autotrophic obtaining their carbon from organic compounds (Marten, 2005; Ritz, 2005). Different specialisations of heterotrophic metabolism are explored briefly below.

Heterotrophic microbes are true chemoorganoheterotrophs; utilising organic compounds as both C and energy sources. These organic compounds are often scavenged from live hosts (i.e. parasites or symbionts) or dead organic matter (e.g. saprophages). Microbial organisms can account for up to 90% of SOM processing, and microbial biomass contributes to about 80 % of SOM (Miltner et al., 2012; Schmidt et al., 2011). The most accessible, labile organic components are utilised first, and more inaccessible, recalcitrant forms (e.g. cellulose and lignin) having longer residence times, often requiring a succession of several different organisms to break them down (Lavelle et al., 1993; Thevenot et al., 2010; Yan et al., 2007).

As discussed above, glycolysis and the citric acid cycle play a central role in metabolic models, however prokaryotic heterotopic metabolism allows for more versatility in terms of sugar metabolism. For example, the use of the Entner-Doudoroff pathway in *Pseudomonas* (Chavarría et al., 2013; Wilkes et al., 2019), or the pentose phosphate pathway as a parallel to glycolysis (Stincone et al., 2015), both allowing for greater oxidative stress tolerance. Fermentation metabolism either obligately or facultatively, uses other molecules as a terminal electron acceptor instead of oxygen (thus requiring an alternative method to supply NAD^+ to maintain primary metabolism), and represents another survival method allowing organisms to continue producing energy under anaerobic conditions (Jurtshuk, 1996). In general, ATP is produced using substrate-level phosphorylation conversion from adenosine diphosphate (ADP), rather than using ATP synthase during respiration (Dimroth and Schink, 1998). Other, more uncommon, metabolic pathways may include methylotrophy, the ability to use mono-carbon molecules as energy sources for example *Methylomonas* and *Methylobacter* (Chistoserdova et al., 2009), or syntrophy, the interaction of two or more species to achieve what would normally be an energetically unfavourable reaction, for example *Syntrophomonas* and methanogens (Morris et al., 2013; Zhang et al., 2004).

It is also important to note that many microorganisms in the soil have the ability to switch their metabolic state very rapidly. This allows for long periods of dormancy, allowing genetic code to be maintained at low or zero metabolic activity to prevent loss of metabolic function and biomass (Lennon and Jones, 2011). As conditions become more favourable, growth can re-occur rapidly (Joergensen and Wichern, 2018). Due to the transient nature of the

microbial biomass, it is extremely important to understand the current physiochemical properties associated to that metabolic state, as even small changes may change enzyme expression rates and thus the metabolic activity of the soil. This may in turn have a large effect on the cycling of nutrients and soil organic carbon (Blagodatsky et al., 2000).

2.2.3.2 Lipid metabolism

Lipids are key metabolites; they are diverse in their structure and role and arguably bridge the gap between primary and secondary metabolism. It may be argued that membrane lipids, predominantly phospholipids, glycolipids, and cholesterol, are primary metabolites due to their importance in the protection and shielding of both prokaryotic and eukaryotic cells from the external environment (Harayama and Riezman, 2018). However, the storage of energy and carbon in lipids, largely as poly(hydroxyalkanoates) (PHAs), triacylglycerols (TAGs) and wax esters (WEs), may be considered secondary metabolism. As, although they are not essential for growth and development of the organism, these are required for its survival in unfavourable environments i.e. the maintenance of normal metabolism under starvation or stressed conditions (Wältermann and Steinbüchel, 2005).

2.2.3.2.1 Membrane lipids

Lipids are the main component in cellular structures providing a function barrier between the subcellular compartments as well as the cell and the environment. Typically, lipids can be characterised by the polarity of their head groups, neutral lipids (e.g. acylglycerols, free fatty acids, sterols, sterols esters, waxes and hydrophobic pigments) which are used in cells to store energy and polar lipids (e.g. phospholipids, glycolipids) which are essential components of cell membranes (Alberts et al., 2002; Meullemiestre et al., 2015).

Membrane lipids are very diverse, both compositionally and chemically. The lipid composition has a large effect on the physical properties of the membrane as well as the effect of membrane protein functions (i.e. ion channels) (Harayama and Riezman, 2018). Since the early days of microbiology, bacteria have been classified by their cell wall properties via the Gram stain test (Bartholomew and Mitterwer, 1952; Friedländer, 1883). Gram-positive bacteria are characterised by a cytoplasmatic membrane and a thick murein cell wall comprising many layers, whereas Gram-negative bacteria are characterized by the presence of two distinct membranes, and a thin murein cell wall between them.

In most cases membranes are formed by glycerophospholipids, however, bacteria may also form phosphorus-free membrane lipids e.g. ornithine lipids (OLs), sulfolipids and diacylglycerol (DAG) among others (Sohlenkamp and Geiger, 2016). Fungal membrane lipids are also very varied, often comprising of sterols and sphingolipids, which the majority of bacteria are unable to synthesise (Olsen and Jantzen, 2001; Volkman, 2003).

In bacteria, membrane lipid synthesis pathways are best characterised in *E. coli*, which for a long time was seen as the model organism. However, development of more detailed analysis methods (genomics, transcriptomics and enzymology) have revealed that lipid metabolism is not totally uniform across the bacterial kingdom, with *E. coli* presenting a relatively simple membrane composition in comparison to other bacteria (Sohlenkamp and Geiger, 2016). Generally, the type II fatty acid synthetic pathway (the conversion of carbohydrate derived acetyl-CoA, and NADPH to fatty acids) is key in the production of the initial building blocks in lipid synthesis within the cytosol. This pathway is particularly important in the production of phospholipid acyl chains in order to maintain membrane physicality and function (De Kroon et al., 2013; Zhang and Rock, 2008). However, while the metabolism of specific phospholipids is extremely complex, the synthesis of the three most common phospholipid groups, phosphatidylethanolamines, phosphatidylglycerols and cardiolipins occur from the central metabolite cytidine diphosphate-diacylglycerol (Sohlenkamp and Geiger, 2016). The synthesis or modification of other phospholipids, for example aminoacylated phosphatidylglycerols or aminoacylated cardiolipins, which can be used to lower the net negative charge of their membrane to evade antibacterial agents (e.g. cationic antimicrobial peptides) or other environmental stresses (Arendt et al., 2012; Dare et al., 2014; Fischer and Leopold, 1999; Roy, 2009).

Non-phospholipid membrane lipids are also minor components of bacterial membranes; examples include, diacylglycerol-based glycolipids which are structurally diverse, but important in regulating the physical properties of the membrane as well as the activities of some membrane-related enzymes (Goñi and Alonso, 1999). Equally, ornithine lipids which are seemly unique to prokaryotes, form under stress to change membrane properties by modification of existing lipids, rather than synthesising new lipids (Vences-Guzmán et al., 2012). It is well documented that microorganisms under stress conditions are able to adapt the composition and fluidity of their cell membranes in order to better cope with their environment (Bossio and Scow, 1998; Córdova-Kreylos et al., 2006). For example, the phospholipid fatty acid (PLFA) content in a culture-grown bacterium (*Phyllobacterium myrsinacearum*)

constituted <0.1% of cell dry weight, which was 50-90 times smaller than in bacterial cells extracted from soil, likely due higher stress in the natural environment (Blagodatskaya and Kuzyakov, 2013).

In fungi, phospholipids are the most abundant lipids in cells, constituting up to 60% of cellular lipid content, with glycerophospholipids accounting for the majority of structural lipids in eukaryotic cells (Casares et al., 2019; van Meer et al., 2008). Additionally, sterols are key in functional organisation and sphingolipids are important in membrane structure and as signalling molecules (Pan et al., 2018). As in bacteria, fatty acid synthesis, performed in the cytosol by the hexameric fatty acid synthase (FAS) complex, forms the basis of lipid metabolism (Pan et al., 2018). Fungal lipid membrane composition and glycolipid concentration has been shown to affect the virulence of fungal pathogens and membrane fluidity (Florek et al., 2018; Rella et al., 2016). Generally, fungal membrane lipids are more restricted in diversity compared to bacterial lipids.

In summary, membrane lipids are key in both bacterial and fungal survival, in terms of homeostatic regulation (Agmon and Stockwell, 2017). As well as modulating communication between organisms (for example plant-fungi or bacteria-plant or bacteria-bacteria) (Siebers et al., 2016) and providing a physical barrier protecting an organism from the environment (Casares et al., 2019).

2.2.3.2.2 Storage lipids

Lipids are also key molecules in endogenous energy storage and accumulation in the cells, of almost all organisms (except yeasts) (Sandager et al., 2002; Zhang and Liu, 2017). Fatty acids are reduced and anhydrous, thus have a high energy yield compared to other molecules (e.g. carbohydrates) (Berg et al., 2002). Their storage in simple, neutral lipids (triacylglycerols, sterol esters or wax esters), allows for very efficient storage, permitting non-polar, anhydrous fat to store six times more energy per weight as the equivalent in hydrated glycogen (Berg et al., 2002). Storage lipids are often grouped into lipid droplet (LD) organelles within the cell, consisting of a core of neutral lipids surrounded by a phospholipid monolayer interspersed with proteins (Olzmann and Carvalho, 2019).

It has been speculated that LDs probably evolved in microorganisms to temporarily store additional alimentary lipids, surplus to the immediate requirements in membrane formation or turnover (Murphy, 2012). Over time it is likely that they evolved a role in long-

term carbon storage that enabled organisms to survive episodic lack of nutrients, being utilised for either energy production or membrane phospholipid synthesis (Olzmann and Carvalho, 2019). As such, they are very dynamic bodies, accumulating in favourable conditions and depleting under unfavourable conditions. Additionally, LDs demonstrate a wide range of functions for example increasing resilience to stress (Zhang and Liu, 2017), regulating and buffering lipotoxicity within cells (Rambold et al., 2015; Schaffer, 2003) as well as regulating host-organism infection (Libbing et al., 2019).

LD synthesis is still poorly understood but thought to occur using similar pathways in prokaryotes and eukaryotes (Wältermann et al., 2004). In eukaryotes, it is thought that they form from coalescence of neutral lipids in the endoplasmic reticulum which are subsequently secreted, forming buds with high membrane surface tension to minimise contact with the aqueous cytosol, amalgamation and growth then occurs (Olzmann and Carvalho, 2019). It is evident that formation is highly reliant on protein and enzyme activity. For example, biosynthesis of triacylglycerol through diacylglycerol acyltransferases and the facilitation of budding by fat storage-inducing transmembrane protein 2 (FITM2) and Coat protein complex II (COPII) (Choudhary et al., 2015; Jensen and Schekman, 2011; Wältermann et al., 2007; Wilfling et al., 2013). However, little work has focused on identifying the genes involved in lipid storage, particularly in bacteria and fungi (Walther and Farese, 2012).

Utilisation of stored fatty acids is performed in a three-step process. Mobilisation, through enzymatic lipolysis of triacylglycerols to fatty acids and glycerol (Berg et al., 2002). Fatty acid degradation then occurs either in the cytosol in prokaryotes or the mitochondria in eukaryotes through the β-oxidation cycle (Jimenez-Diaz et al., 2017). This produces acetyl-coenzyme A (CoA), which can be further metabolized to acquire energy and precursors for cellular biosynthesis (Shi and Tu, 2015; Wältermann et al., 2007).

2.2.3.3 Secondary metabolism

Bacterial and fungal secondary metabolites dominate soil ecological chemistry and their production is highly diverse in both structure and quantity, with the function of many still yet to be discovered (Insam and Seewald, 2010). The fitness and survival of an organism is, in many cases, dependent on how its unique blend of secondary metabolites are adapted to its immediate environment, including the effectiveness of the countermeasures it employs (i.e. detoxification, resistance (antibiotic/fungal)) to prevent other organisms occupying its niche (Giubergia et al., 2016; Karlovsky, 2008).

In general, compounds are either soluble, having influence on a nano scale, or volatile and have influence on a larger spatial scale. This is largely affected by temperature, polarity of molecule, pressure and molecular size. Also, concentration gradients of VOCs in soil are expected to be much more stable than in the plant canopy due to the limited air convention. Growth inhibitors and allelopathic compounds (e.g. bacteriocins (Cotter et al., 2005; Riley and Wertz, 2002), lipopeptides (Raaijmakers et al., 2006; Raaijmakers and Mazzola, 2012), alkaloids (Evans, 2009)) make up many soluble compounds excreted by microorganisms. Due to their relatively large molecular size, it is likely that these compounds are actively exuded through the membrane (Araújo et al., 2005).

Emission of VOCs may occur actively as metabolic end products or passively through diffusion of low molecular weight compounds (Insam and Seewald, 2010). While their role is diverse, generally they can be functionally categorised into the following: quorum sensing, biological control, or inter-/ intra-organismal interactions (Keller, 2019; Evans, 2009; Yergeau et al., 2017). Examples of the diversity and effects of VOCs include, 2,3-butanediol and acetoin, emitted by bacteria, promoting plant growth (Ryu et al., 2003), with similar effects from the fungal emission of isobutyl alcohol, isopentyl alcohol and 3-methylbutanal (Hung et al., 2013). Inhibitory, promotion and interaction effects of VOCs have been shown to be produced by bacteria species against one another, fungi and other species (e.g. nematodes and plants) (Audrain et al., 2015; Bitas et al., 2013; Garbeva et al., 2014; Kanchiswamy et al., 2015; Marmulla and Harder, 2014; Syed-Ab-Rahman et al., 2019; Xu et al., 2015). Equally, VOCs have been shown to have the ability to promote distinct post-transcriptionally regulated responses in plants (García-Gómez et al., 2019). Hence there is a growing interest in the use of VOCs as agrochemicals (Lamy et al., 2017; Song et al., 2013).

2.3. Factors affecting metabolism

Metabolic responses may vary over varying scales, with short term perturbation and rapid stress of the microbial community potentially presenting 'biomarker' responses from a limited number of compounds. In the longer term a relatively stable system (i.e. little perturbation) the metabolome is likely to reflect the health and function of the population of the environment. However, the biological complexity of the system increases significantly when examining whole community responses (Fig. 2.6).

Metabolism and, in a wider ecosystem service context, nutrient cycling relies on enzymes to catalyse the underlying biochemical reactions. In the past, measures of metabolic

activity have been inferred through measuring enzyme activity (Caldwell, 2005; Casida, 1977; Makoi and Ndakidemi, 2008; Perucci, 1992). The efficacy of enzymes driving metabolism as well as the loss of secondary metabolites is affected by a number of factors including biological, chemical, physical and management as is explored below.

Short term   **Long term**

Biological complexity, response time, ecological significance

| Pollution chemicals and radionucleotides | Molecular and cellular | Tissue | Organs | Whole organism | Population |

Signal:noise ratio, responsiveness, detectability

Rapid distress (biomarkers)

Deficiency, disturbance, stress

Population, biodiversity, ecosystem and environmental change

Figure 2.6. The association between detectably of metabolites as ecological stress signals and their ecological relevance, adapted from Moore et al. (2004) and Lankadurai et al. (2013).

2.3.1 Biological

Enzymes, in both exo- and endo- metabolomics, fundamentally underpin the ability of an organism to perform primary and secondary metabolism, allowing the transformation of molecules and release of energy, i.e. function (Shuler et al., 1979; Von Bertalanffy, 1957). In culture, bacteria have distinct, defined growth phases; lag, exponential, stationary, decline and death, with cell growth and DNA replication taking place throughout most of the cycle (Bertranda, 2019). Eukaryotes growth varies from species to species, with the cell cycle being more complex than prokaryotes. The secondary metabolites produced by an organism are often highly related to its stage of development (ontogenetic stage). In bacteria they are primarily produced in the stationary phase, in eukaryotes the production and type of secondary metabolites tend to vary with development stage (Calvo et al., 2002; Isah, 2019; Ramakrishna and Ravishankar, 2011; Ruiz et al., 2010). However, this is likely to differ in environmental

settings due to external abiotic and biotic factors providing physiological triggers for production (Austen et al., 2019; Bibb, 2005).

Soil metabolism is likely to be very dependent on the activity of organisms. Plant roots are highly metabolically active (Bais et al., 2001). Whereas, Blagodatskaya and Kuzyakov (2013) suggest that only 0.1 - 0.2% of total microbial biomass is actually active (i.e. utilising substrates and performing biochemical transformations), with potentially active microorganisms (i.e. can switch on utilisation of substrates within minutes to hours) contributing up to 60% (De Nobili et al., 2001). This is a potential strength of soil metabolism as a measure of soil biology and function as it is only measuring organisms that are active, and thus is representative.

2.3.2 Chemical

pH represents the chemical activity of protons, which participate in metabolism related reactions as well as interacting with cellular components and structures (Jin and Kirk, 2018a). Each soil organism, like each enzyme, will have a definite pH range in which it will achieve optimum activity (Dotaniya et al., 2019; Frankenberger and Johanson, 1982). For plants, this is usually between pH 5 – 7, as this is the range at which the most macronutrients are most accessible from the soil (Goulding, 2016). The composition of bacterial communities has been strongly correlated to soil pH (Cho et al., 2016; Fierer and Jackson, 2006; Wang et al., 2019), whereas fungal communities are less affected by pH (Rousk et al., 2010; Ullah et al., 2019). Nevertheless, pH can strongly affect the metabolic function of the soil, plant and microbial community (Ye et al., 2012). This is likely to particularly affect chemotrophs, as they liberate chemical energy from their environment by catalysing redox reactions. Thus, factors that impact on the energy availability in the environment are likely to affect the metabolic activity of microbial communities (Jin and Kirk, 2018b).

The metabolic activity for all organisms is highly dependent on the availability of substrates. Under normal, unstressed conditions external substrates are utilised, whereas under starvation conditions, cell biomass decreases, and endogenous substrates are broken down (Hockin et al., 2012; Lima et al., 2014; Plaxton and Tran, 2011). Thus, the nutritional status of the soil is important, particularly the C:N ratio due to the importance of carbon and nitrogen in fundamental cell activities (Zheng, 2009). In general, inorganic nutrient inputs will induce a change in the microbial community, increasing the abundance of faster-growing, copiotrophic bacteria and reducing fungal prevalence (Carrero-Colón et al., 2006; Fang et al., 2018; Hartman

and Richardson, 2013; Leff et al., 2015). A change in either the environment or biotic selection will result in a change in metabolic output from the system, as illustrated in Fig. 2.4. Soil microorganisms are also key to the transformation, breakdown and stabilisation of soil organic matter (SOM), an important source and sink of biological nutrients and have a large influence on the physical, chemical and biological functions of soil (Nieder and Benbi, 2008; Powlson et al., 2001). Increased SOM levels generally show an enhancement of microbial biomass and functional diversity, yet little work has been performed relating SOM to soil metabolism (Blankinship et al., 2014). However, exoenzyme activity, microbial substrate utilisation and metabolic diversity have been shown to be important in SOM breakdown and biological soil quality (Bending et al., 2000; Blankinship et al., 2014).

An organism's stress levels can have a large impact on its metabolic efficacy. Stress effects are various and may be induced by, for example, injuries, parasites, extreme temperatures, osmotic stress (i.e. drought), or soil and atmospheric pollutants, such as trace metals or ozone, respectively (Cho et al., 2016; Darko et al., 2019; Gaudinier et al., 2015; Jenerette and Chatterjee, 2012; Kesselmeier and Staudt, 1999; Price and Sowers, 2004). Temperature is also an important factor in determining VOC production, and gaseous diffusion rates, as well as having a direct effect of the efficiency of enzyme activity (Asensio et al., 2007).

2.3.3 Physical

Physical factors constrain the ability of soil biology to use organic matter and nutrients in soil, often limiting the ability of organisms to access substrates, promoting their recalcitrance (Ladd et al., 1993). The structure and the structural stability of soil is very important for plant growth, allowing the transmission and storage of water, nutrients and gases, as well as supporting healthy, unconstrained root growth (Bengough, 2003; Oades, 1984). In this sense, structure is also key to a healthy microbial community, however on a much smaller scale, with the composition of micro- (<250 μm) and macroaggregates (0.25 to 2 mm) shaping and regulating the ecological niches that exist on them (Wilpiszeski et al., 2019). This being said, less than 1% of soil surface area is typically occupied by microbes; leading to biological islands as hotspots of biochemical activity (Young and Crawford, 2004).

Poor soil structure often leads to anaerobic conditions, favouring organisms with facultative or obligate anaerobic metabolism. Whereas, under a good, aerobic soil structure it is likely that aerobic organisms will dominate, although, aerobic soil has been shown to contain

anaerobic microsites (Keiluweit et al., 2017). The oxygen status, as well as the redox potential of the soil, have been shown to be a major influence on the VOCs formed (Brzezińska et al., 1998; Silver et al., 1999). For example, under reducing conditions, production of volatile sulphur compounds becomes more prevalent (Devai and DeLaune, 1995). Under aerobic conditions, organic C sources are predominantly used for energy production and primary metabolism, leaving only small amounts of substrate to be utilised for secondary metabolite production. However, under anaerobic conditions more C finishes as end products of fermentative processes, leading to an increase in the diversity and amount of VOC emitted (Insam and Seewald, 2010; Stotzky et al., 1976).

Equally, in terms of inter- and intra-species interaction, diffusion of solutes and gases are highly dependent on pore size, connectivity and water saturation. Under drought conditions the exudation of soluble secondary metabolites and signalling molecules are likely significantly reduced as energy will be better used in the production of osmolytes for preservation. Conversely, under saturated conditions volatile secondary metabolites cannot diffuse and instead maybe dissolved. For quorum-sensing bacteria, accumulation of signalling molecules aggregates can change microbial pathogenesis, biofilm formation capabilities, motility, and production of secondary metabolites (Wu and Gschwend, 1986).

2.3.4 Management

Land use type and land use change have a large impact on the ability of soil to function, particularly in terms of biology. Under agriculture, perturbation through soil turnover and agrochemical inputs are likely to change the selection pressure on the microbial community present leading to a change or reduction in the microbial community present, often implying a loss of metabolic and functional resilience (Fig. 2.4) (MacRae, 1989; Mazzetto et al., 2016; Van Heerden et al., 2002). Management of a soil is likely to affect the substrate (OM) quantity and quality as well as the structure and pH, among other factors. As previously mentioned, soil biology is sensitive to changes in soil management and may be an early indicator of changing function (Bending et al., 2000). As such, this will have a direct and rapid effect on the primary and secondary metabolism of the soil biological community. For example, organic amendments have been shown to drive soil VOC emissions (Potard et al., 2017). However, there is little evidence of the study of soil metabolism across land use types. Equally, xenobiotics, i.e. organic or inorganic pollutants, are likely to have a large effect on the ability of soil organisms to effectively metabolise. Pollutants will cause a significant shift in microbial

and biological community (Gao et al., 2010; Singleton, 1994; Sutton et al., 2013), impacting soil enzyme function (García-Gil et al., 2013) and transcription (Van Straalen and Roelofs, 2008).

2.4. Recent advances in analytical techniques

One of the main challenges in the analysis of the metabolome is its complexity and chemical diversity ranging from lipids, organic acids, carbohydrates, amino acids, nucleotides and steroids. In comparison, genes and proteins are chemically homogenous as genes are based on four basic nucleotides, while each protein contains a mixture of 20 amino acids. The variability in chemical structure results in a large variety of physicochemical properties, i.e. polarity, solubility, size and volatility. As such, there is no single metabolomic analysis methodology that can measure the entire metabolome accurately. This is particularly true when performing untargeted analysis; comprehensive analysis of all measurable analytes within a sample, correlated to reference libraries, providing qualitative results. The workflow for untargeted analysis is summarised in Fig. 2.7. The alternative; targeted analysis; the measurement of defined and biochemically annotated metabolites quantified with reference standards, can be more easily optimised to ensure high sensitivity toward the target analyte. This section will briefly discuss current and emerging metabolomic analysis techniques.
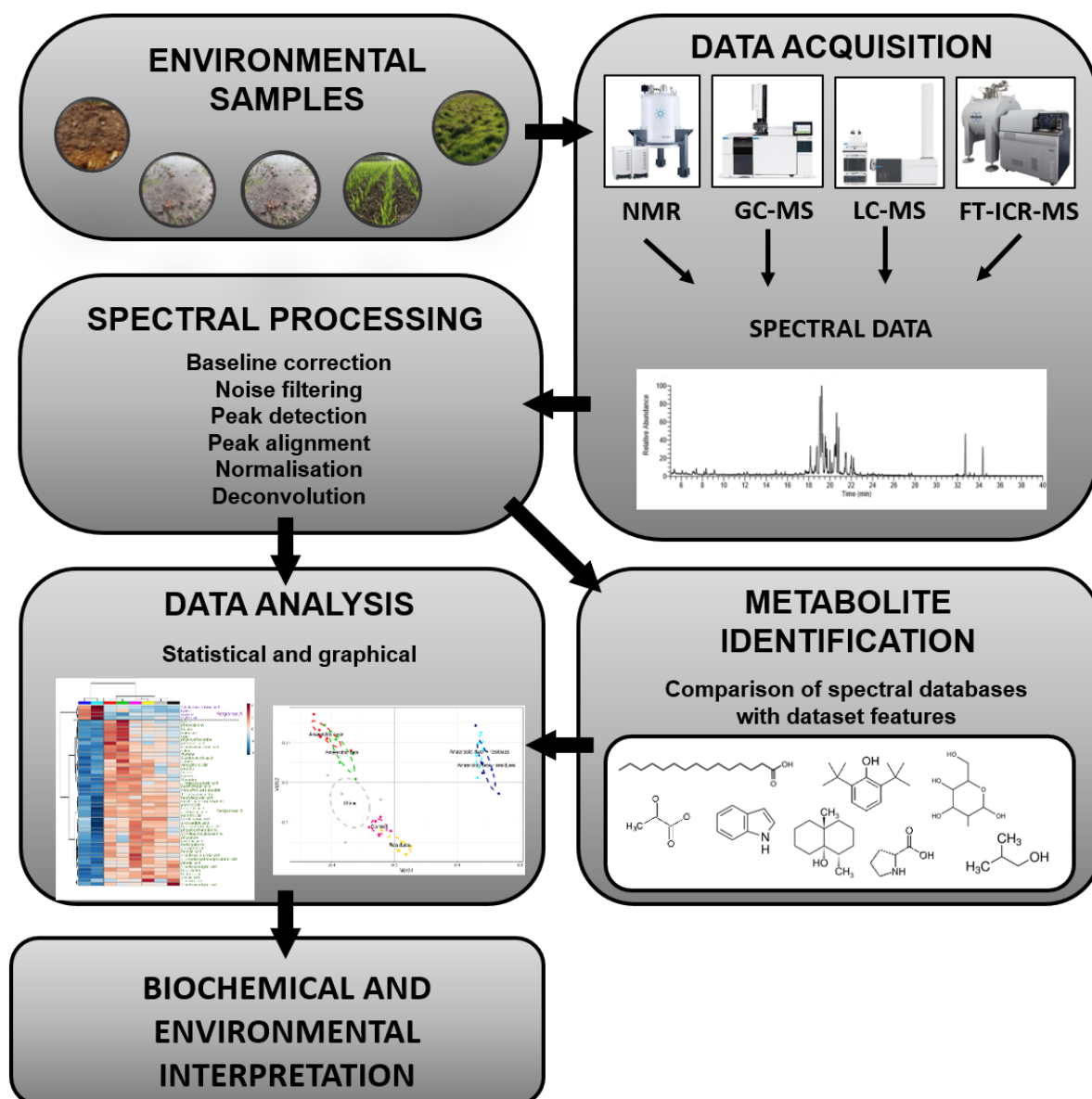
Figure 2.7. *A workflow for analysis in the study of untargeted metabolomics. Adapted from Alonso et al. (2015).*

2.4.1 Primary metabolomics

Primary metabolite extraction methods from soils differ depending on the type of analysis required. Untargeted analysis is likely to employ a solvent or solvent mixture that will extract a large range of metabolites; polar, non-polar and protein precipitates. Generally, extractions for untargeted metabolite analysis are performed using a mixture of organic solvent (i.e. methanol, ethanol, acetone, chloroform or acetonitrile) and water to ensure broad coverage of metabolite groups (Lankadurai et al., 2013). Examples include methanol/chloroform/water

(2:2:1.8) (Bligh and Dyer, 1959; Lin et al., 2007), isopropanol/acetonitrile/water (3:3:2) (Fiehn et al., 2008; Swenson et al., 2015). For targeted metabolite analysis, the most efficient solvent for liberating the metabolite class of interest must be chosen, for example a water-only extraction for water soluble metabolites (Swenson et al., 2015).

Mechanical and chemical techniques may also be used to improve extraction efficiency, for example chloroform fumigation of samples in order to lyse microbial cells and limit microbial processing of metabolites (Jenkins et al., 2017), or grinding to homogenise and increase the surface area to volume ratio of the soil and pulverise tissues (Lu et al., 2017; Naz et al., 2014). In order to limit metabolite change pre-extraction, metabolic (and enzyme) activity must be quenched as soon as possible after sampling, this is most often performed by flash-freezing (to < -80 °C). Storage to limit changes in metabolite composition is also important. Short term storage at -20°C does not significantly affect the metabolite profile, however, long-term storage should be conducted at -80°C (Smith et al., 2020; Wandro et al., 2017).

Primary metabolomic analysis is commonly performed on gas chromatography mass spectrometry (GC-MS), liquid chromatography mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR) systems (Emwas et al., 2019). These techniques and application are briefly summarised below.

MS systems are considered to be the most sensitive, with broad metabolome coverage (Theodoridis et al., 2011). Of these, LC-MS systems are considered the most versatile; briefly analytes are separated on a column, ionised by an ion source before being separated by a mass analyser and subsequently detected. However, a major consideration is that LC-MS requires metabolites to ionise in order to detect them, therefore it is not applicable for non-ionisable molecules (Lu et al., 2017).

The principals of GC-MS separation are similar to that of LC-MS. GC-MS strengths lie in the analysis of low-molecular weight and volatile analytes, which often are not well retained or poorly ionised on LC systems, namely alcohols, hydroxy acids and sugars (Papadimitropoulos et al., 2018; Roessner et al., 2000). Extensive mass spectral libraries also exist for accurate compound identification. In many cases, derivatisation is needed in order to increase compound volatility and separation. However, the high temperatures associated with the injection and evaporation of compounds may lead to breakdown or loss of thermolabile compounds (Kaspar et al., 2008).

NMR exploits the spin of a nucleus. On the application of an external magnetic field to a sample, the energy transfer (between base energy and the higher energy level) is measured, this corresponds to a specific radio frequency. From this, details of the structure and functional groups of a molecule can be determined. NMR, has a number of unique advantages over other metabolomic analysis techniques, particularly sampling is non-destructive and requires little sample treatment as well as being able to identify the structures of unknown compounds (Markley et al., 2017). However, its lack of sensitivity is the greatest drawback, with metabolite yields typically only 20% of those measured using MS techniques (Emwas et al., 2019).

Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS), while not commonly used in metabolomics research, possibly due to its comparative relative expense, complexity and slow acquisition times, holds great future research potential (Ghaste et al., 2016). Briefly, mass-to-charge ratios of ions are determined based on the frequency of rotation (cyclotron frequency) under a fixed magnet field (Comisarow and Marshall, 1996; Marshall and Hendrickson, 2002). Ions, under high vacuum and extremely low temperature are passed into a strong magnetic field, where they are forced into a circular motion around the magnetic field (perpendicular to the Lorentz Force) and prevented from leaving the cell by trapping plates at each end. Excitation of individual mass-to-charge ratios is achieved by a radio frequency pulse across the cell, this induces an alternating current which can be detected. The frequency of the current produced is equal to the cyclotron frequency of the ions and the intensity is proportional to the number of ions. FT-ICR-MS enables high mass (< 1 ppm) and resolving accuracy (Ghaste et al., 2016; Han et al., 2008). Allowing the potential for more accurate metabolic separation between samples; as shown in whisky characteristics (Roullier-Gall et al., 2018), grapevine leaves (Maia et al., 2016), cloudwater (Bianco et al., 2019) and freshwater (Valle et al., 2018). This offers a great amount of potential in the soil sciences, where metabolic separation may potentially be quite subtle.

2.4.2 Lipids

As discussed above, lipids may be considered either primary or secondary metabolites depending on their classification. Membrane lipids (phospholipids and glycolipids) require a different approach to extraction compared to storage lipids (triacylglycerols, sterol esters or wax esters). Analysis of lipids in soils has traditionally had a strong focus on PLFAs as a measure of the soil biological community structure (Frostegård et al., 2011). Applications have included impacts on the soil microbial community through changes in land use (Wu et al.,

2016), pollution (Chodak et al., 2013), nutrient addition (Marschner et al., 2003), environmental conditions (Pétriacq et al., 2017), management practices (García-Orenes et al., 2013) and seasonal change (Moore-Kucera and Dick, 2008).

A multi-phase extraction is usually required for membrane lipid analysis from the soil. Initial extraction of lipids from the sample is usually performed using a modified Bligh and Dyer (1959) method using chloroform, methanol and a (phosphate or citrate) buffer (Chowdhury and Dick, 2012). This is followed by fractionation of whole extracted lipids into individual lipid classes (for example neutral lipids, glycol-lipids and phospholipids), generally performed by liquid chromatography using packed solid phase extraction (SPE) cartridges. From these, methylation or derivatization converts lipids into more volatile methyl derivatives. The method by which this is performed can have a large effect on the FA profile (Chowdhury and Dick, 2012). However, higher throughput methods, with reduced reagent usage have been developed (Buyer and Sasser, 2012). Analysis of fatty acid methyl esters (FAMEs) is then performed by GC equipped with either a MS or flame ionisation detector (FID) (Dodds et al., 2005).

Non-polar lipids, generally involved in storage and organism signalling (e.g. triacylglycerols), are treated differently. While still based on the principles of the classical Bligh and Dyer (1959) and Folch (1957) methods, the widely applied Matyash et al. (2008) method utilises a methyl tert-butyl ether (MTBE), methanol, water solvent mix for rapid and clean lipid extraction. However, thus far this method has not been optimised for soil.

2.4.3 Secondary metabolites

Secondary metabolites represent many different classes of compounds and the current extraction and analysis methods reflect this variability. Generally, compounds of interest are either volatile (i.e. VOCs), soluble (i.e. water extractable organic matter (WEOM)) or non-volatile and insoluble (i.e. complex carbohydrates). Like primary metabolites, there is no one method that will effectively extract all classes of secondary metabolite and therefore targeted extraction methods must be employed. In soil, research has mostly focused on VOCs and soluble metabolites as these are often the most involved in inter- and intra-species interactions (Chomel et al., 2016; Insam and Seewald, 2010; Tyc et al., 2017a).

Extraction of volatile compounds can be performed using a variety of different methods. Generally, methods either require the adsorption of volatile molecules onto a material

before being desorbed into a GC-MS, or the direct analysis of headspace gases. Thermal desorption (TD) techniques are commonly used for a wide range of applications from the food and drink industry (Gong et al., 2017) to environmental pollution assessment (Llompart et al., 2019). Headspace sampling is most commonly used on complex matrices such as soil, to reduce fouling of the sorbent and matrix effects (Orazbayeva et al., 2017). The most common solvent-free techniques for the extraction of volatiles from soil are passive head space (HS) solid phase micro-extraction (SPME) for *ex-situ* sampling (Wypych and Mañko, 2002) and sorbent tube extraction for *in-situ* sampling, i.e. insertion into ground in a permeable container (Woolfenden, 2010). Many factors affect the efficacy of compound extraction, for example, extraction temperature and sorbent exposure time, sorbent composition (generally a mixed polarity coating is most appropriate for environmental applications (e.g. Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS)), as well as soil moisture and structure (Peñuelas et al., 2014; Turner et al., 2019). Improving the partition coefficient (ratio of analyte in headspace-to-analyte in sample) and the phase ratio (volume of headspace-to-volume of sample) will generally improve the recovery rate of analytes, however, analysis is not extensive and recovery rates are usually deviate from 100% (Tena and Carrillo, 2007).

A major drawback of SPME extraction is the large amount of competition for sorbent phase, leading to only the most competitive VOCs being adsorbed to the fibre and consequently being analysed. This makes the choice of sorbent compound and exposure period critical for sensitive and targeted extraction (Pawliszyn, 2000). Equally, passive *in-situ* sorbent tube sampling can lead to fouling and contamination of the sorbent due to the relatively long exposure times required to target compounds at low concentrations (Namieśnik et al., 2005). Both methods allow for thermal desorption directly into the GC-MS for analysis.

For dynamic headspace sampling (i.e. purging, or purge and trapping) direct injection to the GC can increase the sensitivity of analysis and ensure maximum extraction of analytes. However, it has been shown that purge and trap methods lead to poor recovery due to the large amount of VOC loss on transfer to sample vessels (Voice and Kolb, 1993). For direct analysis of headspace gases in real time, proton-transfer-reaction mass spectrometry (PTR-MS) offers a sensitive approach to quantification and identification (Mancuso et al., 2015; Park et al., 2002). For higher mass resolutions a time-of-flight (TOF) MS may be used, however, ions require pulsing into the TOFMS, so there is some loss of ions from the constant stream generated by the PTR (Tadjimukhamedov and Forbes, 2015).

Research on water-soluble secondary metabolites in the natural environment has largely focused on plants (Kosmas et al., 2014). However, recently more interest has been expressed in the role of dissolved organic matter (plant and microbial exometabolites) in organic matter cycling and biological utilisation, as generally it is the most accessible component of SOM (Swenson et al., 2015). The limitation of exometabolite studies is the complexity of soil as a substrate, leading to studies being performed on non-environmentally relevant media (e.g. an optimised culture) for ease of extraction (Baran et al., 2015; Liebeke and Lalk, 2014). To address this problem intra- and extra-cellular approaches are used to reflect the potentially accessible microbial metabolites that may be present in soil, i.e. cells were lysed by chloroform fumigation before extraction with water (Jenkins et al., 2017; Swenson et al., 2015). Analysis of soluble secondary metabolites can be performed by either GC-MS or LC-MS, depending on the compounds of interest. Generally, LC-MS is considered to provide the broadest range of compounds, however, sugars are better resolved using GC-MS (Jenkins et al., 2017; Pétriacq et al., 2017).

2.4.4 Data and statistical processing and interpretation

The metabolomics standards initiative (MSI) have released minimum requirements for the reporting of data transformation and statistics (Fiehn et al., 2008; Sumner et al., 2007). Data pre-processing (i.e. peak picking and alignment) and annotation is extremely important. Extraction of metabolite information from raw chromatograms has improved considerably from initial manual integration, with many analysis programmes (for example MassHunter Workstation Profinder (Agilent Technologies, Palo Alto, USA) or ChromSpace (StepSolve Analytical, Peterborough, UK)) allowing chromatogram alignment, deconvolution and feature extraction to be conducted within one programme using supervised feature extraction algorithms. Databases of metabolites and mass spectra are fundamental to compound identification. Commonly used databases include the Kyoto Encyclopaedia of Genes and Genomes (KEGG) (Kanehisa and Goto, 2000), Human Metabolome Database (HMDB) (Wishart et al., 2018) and National Institute of Standards and Technology (NIST) high resolution spectral database. However, care must be taken when reporting identified compounds; if solely identified through database searches and not confirmed with analytical standards compounds may only be referred to as 'tentatively identified as X'.

Metabolomics data, like any large biological dataset are often difficult to interpret. However, a large number of assessable statistical tools exist to aid extraction of meaningful

conclusions (Chagoyen and Pazos, 2013), many of which have been recently and thoroughly reviewed by Gardinassi et al. (2017). These tools are complimentary to more traditional statistical analysis in programmes such as R (Costa et al., 2016; Grace and Hudson, 2016). As concluded by Gardinassi et al. (2017), there is still work to be done to bridge the gap between purely analytical and biological and environmental applications. However, metabolomics is becoming an ever-more accessible field through the development of these tools.

2.5. Use of biological soil quality measurements for land use and management assessment

As discussed previously, soil quality is a complex concept, consisting of the interaction between physical, chemical and biological factors. As Bünemann et al. (2018) suggests, biological and biochemical indicators have the potential to be highly sensitive to environmental and management change, but are under-represented. This is likely due to the complexity of datasets and subsequent interpretation compared to more traditional chemical and physical measurements. However, as the accessibility of metabolomics increases, with decreasing cost and increasing availability of analytical and bioinformatic tools, it has the potential to become a powerful addition to the soil analysis toolkit available to farmers, policy makers and land managers.

Lipid metabolism, through the use of PLFAs, represents the most extensively utilised metabolomic analysis method in the soil sciences (Quideau et al., 2016). However, there are several limitations of this method namely; PLFA biomarkers are often not specific to either species or group (for example, Gram-positive, Gram-negative and fungi) making sampling context (soil conditions, management level, likelihood of bacterial or fungal dominance) extremely important in interpretation (Bárcenas-Moreno and Bååth, 2009; Frostegård et al., 2011). There is also an inability to differentiate between metabolically active and non-active microorganisms (Blagodatskaya and Kuzyakov, 2013) and a lack of standardised extraction and analysis method across the literature (Philippot et al., 2012). Ultimately these factors make comparison of results between studies difficult.

Metabolically active (i.e. sensitive, dynamic and relevant) measures will be much more useful in the understanding of soil processes by land managers. Metabolomics may be able to overcome some of the drawbacks of taxonomic diversity indices and microbial networks as indicators of soil quality, namely the lack of sensitivity and specificity, and the prediction of process rates from the presence and quantity of genes and transcripts is yet to be clearly

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established (Karimi et al., 2017; Rocca et al., 2015). Equally, the production and analysis of so called "big data" from sequencing still poses a challenge in terms of time, computational capability and meaningful interpretation, as the functional and taxonomic characterisation of a large proportion of soil organism still remains elusive (Mardis, 2016; Schloter et al., 2018). In this sense, metabolomics may yield potentially suitable soil quality indicators with direct links to ecosystem processes (Bending et al., 2004, 2000; Insam and Seewald, 2010). However, the integration of either primary or secondary metabolomics into soil quality indices would require understanding the links and relationships to traditional soil chemical and physical measurements, an area of research which remains largely unexplored. It also requires an understanding of how metabolic function changes over time.

2.6. Outlook and conclusions

Understanding how anthropogenic disturbance and management affects soil quality is not only key to improving global food production but will also help build resilience to climate change and improve the quality of the wider environment. Some of the most sensitive indicators of soil quality; biological and biochemical metrics, are underrepresented in existing soil health indices. While the environmental application of metabolomics is still in its infancy, its importance in understanding the biochemical system in relation to regulation, management and the underpinning of the delivery of ecosystem services is beginning to be understood; in particular, the complex links between organisms, as well as the fundamental ability of the biological community to process and cycle key nutrients. The metabolome offers the possibility of capturing several broad layers of underlying biological activity within one analysis method, i.e. the genome, transcriptome and proteome. Primary and secondary metabolomics and lipidomics potentially offer a powerful addition to the soil scientist's and land manager's toolkit. Volatilomics is of particular interest to ecologists, in terms of interactions between organism, as they have the potential to be captured non-invasively.

However, there remain many challenges in the environmental application of metabolomics, particularly in complex, heterogeneous matrices such as soil. Summarised are some of the key research bottlenecks in the field:

i) Much of the metabolic network is not yet fully elucidated. Greater understanding of the enzymatic links between metabolite anabolism and catabolism is therefore required to completely elucidate the function of the system. Consequently, more data is needed to understand the entire system.

ii) There is a lack of understanding of the contribution of biotic versus abiotic processes to soil VOC production and consumption in the field. There is little data to confirm the sources of VOCs. While evidence points to most VOCs being of biological origin, abiotic processes, for example photodegradation or chemical hydrolysis, have the potential to contribute significantly to overall production.

iii) Little research has focused on the differences in the whole metabolome (i.e. primary, secondary and lipid metabolites) under different soil qualities and their implications for system functioning, particularly in relation to physicochemical properties. Would a biomarker or profiling approach be more appropriate?

vi) For meaningful integration of indicators based on metabolomic methods into soil quality assessments, standardized and optimised extraction and analysis techniques and a reference system are required to ensure the ability to compare between studies. While extraction methods do exist, there is little evidence that they have been optimised for a soil matrix. Equally, an acknowledgement of the bias within a method is important. Although reference systems and databases exist, they are largely based on the human metabolome which has been subject to a great amount of research, but which may not always be applicable in environmental research.

vii) An enhanced understanding of the functional role of metabolites in soil is required in order to integrate metabolomic research, as measures of chemical ecology, into future methods of quality assessment.

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Chapter 3: Use of metabolomics to quantify changes in soil microbial function in response to fertiliser nitrogen supply and extreme drought

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RB, DC and DJ conceptually conceived and designed the experiment. RB collected the experimental data, with the exception of the greenhouse gas data which was provided by HZ. RB performed data analysis and interpretation and wrote the first draft of the manuscript. All authors contributed to subsequent revisions.

3.1 Abstract

Climate change is expected to increase the frequency and severity of droughts in many regions of the world. Soil health is likely to be negatively impacted by these extreme events. It is therefore important to understand the impact of drought on soil functioning and the delivery of soil-related ecosystem services. This study aimed to assess the resilience and change in physiological status of the microbial community under extreme moisture stress conditions using novel metabolic profiling approaches, namely complex lipids and untargeted primary metabolites. In addition, phospholipid fatty acid (PLFA) profiling was used to identify changes in microbial community structure. Soil samples were collected during a natural, extreme drought event and post-drought from replicated grassland split plots, planted with either deep-rooting *Festulolium* (cv. AberNiche) or *Lolium perenne* L. (cv. AberEcho), receiving nitrogen (N) fertiliser loading rates at either 0 or 300 kg N ha^{-1} yr^{-1}. These plots were split at the start of the drought period, and half of each subplot was irrigated with water throughout the drought period at a rate of 50 mm week^{-1} to alleviate moisture stress. PLFA analysis revealed a distinct shift in microbial community between drought and post-drought conditions, primarily driven by N loading and water deficit. Complex lipid analysis identified 239 compounds and untargeted analysis of primary metabolites identified 155 compounds. Both soil complex lipids and primary metabolites showed significant changes under drought conditions. Additionally, the irrigated 'reference' plots had a significantly higher cumulative greenhouse gas (CO_2 and N_2O) flux over the period of sampling. Recovery of the microbial lipidome and metabolome to reference plot levels post-drought was rapid (within days). Considerable changes in soil primary metabolomic and lipidomic concentrations shown in this study demonstrate that while soil metabolism was strongly affected by moisture stress, the system (plant and soil) was highly resilient to an intense drought.

Keywords: Ecosystem resilience, Extreme weather, Metabolic profiling, Osmotic stress, Soil health.

3.2. Introduction

Climate change is predicted to alter precipitation regimes leading to an increased frequency, duration and severity of drought in many regions of the world (Garner et al., 2017; Dodd et al., 2021). Generally, natural ecosystems are considered to be at greatest long-term risk to these extreme droughts, for example through wildfires (Nolan et al., 2020) or combinations of heat and water deficit that can induce complete biome collapse (Matusick et al., 2013). However, extreme weather events (e.g. droughts, floods, ground level O_3) can also have severe short-term impacts on agroecosystems leading to significant yield and economic losses (Environment Agency, 2006; Mills et al., 2011). Understanding the impact and regional risk and consequences of extreme weather events is therefore key to the design of more resilient land management systems (Dodd et al., 2021). While the impact of drought on crop plants and the development of strategies to overcome water stress are well advanced in above-ground plant components (Tardieu et al., 2018), our understanding of below-ground responses to drought still remains fragmented. This is partly due to the complex interrelationships between plant roots, their symbionts, the wider microbial community and the physical soil matrix.

A well-functioning and healthy soil system is crucial for the provision of ecosystem services, particularly for food production. This requires a good holistic understanding of soil biological and physicochemical properties and their interrelationships. Biological soil quality is an understudied, yet dynamic and responsive aspect of soil quality, underpinning many soil functions, such as nutrient cycling and carbon (C) storage (Wagg et al., 2014; Hatfield et al., 2017; Bünemann et al., 2018). The advance of molecular methods has allowed us to better understand taxonomically how microbial communities respond to extreme events. However, this provides little information on soil functioning under drought, especially given the high level of functional redundancy which exists within the soil microbial community (Briones, 2014; Cortois and De Deyn, 2012). Although soil respiration provides an integrated measure of total below-ground metabolic activity, it cannot easily differentiate between CO_2 derived from plant roots, mesofauna and the soil microbial community (Phillips and Nickerson, 2015; Geyer et al., 2016). It is therefore susceptible to misinterpretation in terms of ascribing cause and effect to observed CO_2 responses (Luo et al., 2006).

One potential alternative to better understand below-ground functioning is to analyse the biochemical pathways that underpin microbial functioning, especially the flow of C into structural biomass (e.g. cell walls), storage pools (e.g. fatty acid, acylglycerol and sterol lipids), and respiratory pathways (Dijkstra et al., 2011; Wu et al., 2020). This involves the extraction

and quantification of primary metabolites; directly involved in growth, development and physiological function, present in the microbial community (e.g. sugars, organic acids, amino acids, phenolics, fatty acids etc). Metabolites are products or intermediates of enzymatic reactions, providing informative proxies of biochemical activity within an organism, or in this case the whole soil microbiome. This metabolomic approach has been used extensively in biomedical science (Armitage and Barbas, 2014; Gupta et al., 2018; Meyer et al., 2013), plant biology (Gomez-Casati et al., 2013; Putri et al., 2013), and for investigating the biochemical responses of single microbial species (Brauer et al., 2006; Jozefczuk et al., 2010). However, its application to soils, particularly under field conditions, remains rare, where the focus has tended to be on specific metabolites (Warren, 2020a). Recent studies, however, have suggested that the metabolome is very sensitive to differences in ecosystem productivity (Withers et al., 2020) and can provide functional information on soil microbial responses to abiotic stress (Li et al., 2019; Miura et al., 2020).

In this study we also used simple (fatty acids, glycerols and alcohols) and complex lipids (lipids usually containing three or more chemical identities); compounds insoluble in water, to explore how soils respond to and recover from drought stress. Focusing mainly on phospholipid fatty acids (PLFAs; found in high concentrations in the cell membrane) and triacylglycerols (TAGs; intracellular storage lipids). Lipids are a diverse and ubiquitous group of compounds which play many key biological functions, including their core role in microbial cell membranes, serving as energy storage sources and participating in signalling pathways. Further, the relative diversity and abundance of lipids has been used extensively to investigate organismal responses to a range of external stressors (Furse and Shearman, 2018). While the majority of previous research in soil has focused on membrane composition through the study of PLFAs (Frostegård and Bååth, 1996; Mathew et al., 2012; Lupwayi et al., 2017), and recently, intact polar lipids (Ding et al., 2020), understanding the response and use of intracellular lipids, e.g. TAGs under drought, may also provide useful insights to changes in microbial function. One of the key challenges in this field of research, however, is the translation of observed changes in complex lipid or metabolite profiles into soil health indicators (i.e. are the observed changes indicative of adverse stress or tolerance?). Under stress (e.g. osmotic, oxidative and temperature stresses), the metabolic and lipid profiles of organisms will shift in order to provide osmotic adjustment and allow continual organismal function. Soil dwelling microorganisms have a number of regulated adaptive responses, many of which have been well studied, for example substitution of membrane lipids (Dawaliby et al., 2016; Wang

and Levin, 2009) or upregulation of osmoprotectant compounds e.g. ectoine, betaine, proline, trehalose, and arabitol (Warren, 2014).

The cycling and fluxes of carbon (e.g. carbon dioxide (CO_2)) and nitrogen compounds in soil, as a consequence of biological cycling, has long been the benchmark in understanding soil function (Geyer et al., 2016), representing the capacity of soil to support soil life. The measurement of the CO_2 flux produced from soil and plant respiration is well correlated with factors that influence plant growth and microbial activity. For example, soil moisture, temperature, and the availability of nutrients and carbon substrates (Phillips and Nickerson, 2015). The effect of drought on soil and plant respiration and nitrification is well documented; supressed CO_2 and nitrous oxide (N_2O) emissions during drought followed by a sudden but short-lived loss of CO_2 and N_2O upon rewetting as re-metabolization occurs, often referred to as the "Birch effect", it is a good proxy for whole system functionality (Barrat et al., 2020; Sun et al., 2019; Unger et al., 2010).

To advance our understanding of soil metabolic profiling, the aim of this field-based study was to examine the microbial metabolomic and lipidomic responses to drought conditions, taking advantage of an exceptional period of low rainfall in the summer of 2018. We hypothesised that, 1) primary metabolites related to coping with osmotic stress will increase under drought conditions, 2) microbial storage lipid composition will increase during drought conditions, as soil C and other nutrients become inaccessible, 3) the soil microbial community will alter PLFA membrane composition under osmotic stress conditions, 4) the microbial community will show a lasting metabolic legacy after alleviation of the drought, and 5) the post-drought rewetting would result in a flush of CO_2 ("Birch effect") and N_2O emissions, compared to the reference treatment.

3.3. Materials and methods

3.3.1. Experimental setup

The study took place at the Henfaes Agricultural Research Station, Abergwyngregyn, North Wales (53°14′N, 4°01′W) (Apprendix 1 Fig. 1). The site has a sandy clay loam textured Eutric Cambisol soil type overlying a glacial till parent material, a temperate-oceanic climate regime with mean annual rainfall of 1060 mm and temperature of 10°C. The experimental plots were initially established in September 2016 and arranged in a randomised, complete block, split plot design to create independent replicates of each treatment ($n = 4$). Within each block

(12×52 m), half the plots were seeded with *Festulolium* (cv. AberNiche), a cross between *Festuca pratensis* L. and *Lolium multiflorum* L. which has an improved drought tolerance (relative to *L. multiflorum*), while the other half of the plots were seeded with *Lolium perenne* L. (cv. AberEcho) which possesses high levels of water-soluble carbohydrate (Harper et al., 2018). In comparison to *L. perenne*, *Festulolium* has a deeper root system (Humphreys et al., 2018). Each plot (3.5×2 m) was then split into two, with one half receiving NH_4NO_3 fertiliser at a rate of 300 kg N ha^{-1} yr^{-1} spread over three applications (20th April, 15th June, 17th August) and the other half receiving no N fertiliser. At the start of the growing season, P and K was applied to all plots at a rate of 20 and 90 kg ha^{-1} yr^{-1}, respectively, to satisfy crop needs based on soil test results (AHDB, 2020). Each plot was divided into two equal sections (1.5×2 m) with a 0.5 m buffer zone placed between (Appendix 1 Fig. 2). This study did not use rainfall exclusion roofs, which may themselves create a range of artifacts and not be truly representative of 'real world' conditions (Vogel et al., 2013; Kreyling et al., 2017). Instead, we took advantage of a period of unprecedented natural drought in the summer of 2018 (Ramonet et al., 2018), during which one half of the sub-plots were manually irrigated with low ionic strength dechlorinated mains water at a rate of 50 l m^{-2} $week^{-1}$ (50 mm $week^{-1}$ equivalent) to alleviate drought stress and form control plots (henceforth referred to as 'reference' plots), while the other half was left un-watered to represent drought conditions (Fig. 3.1, Appendix 1 Fig. 3). Irrigation water properties are summarised in Appendix 1 Table 1. As drought conditions were naturally occurring, we were not in a position to take soil samples pre-drought. Local climatic data were recorded using an automated weather station located adjacent to the field plots (Fig. 3.1).

3.3.2. Soil sampling and analysis

The first soil sampling took place on the 11th July 2018, 45 days after the last significant rainfall event (> 2 mm) and after 24 consecutive days of no rainfall (i.e. drought sampling). A second sampling (i.e. recovery sampling) was undertaken on the 16th August 2018. In the 10 days prior to this second sampling 18.3 mm of rain had fallen (Fig. 3.1). On each occasion, multiple soil cores ($n = 12$; $\phi = 1$ cm; depth 0-10 cm) were randomly sampled from each plot and homogenised to obtain a representative soil sample per sub-plot. Soil was then sieved through a 2 mm mesh to remove stones and plant and root material. Soil moisture content was determined gravimetrically by oven drying (105°C, 24 h). Soil pH and electrical conductivity

(EC) were measured using standard electrodes submerged in 1:5 (w/v) soil-to-distilled water suspensions.
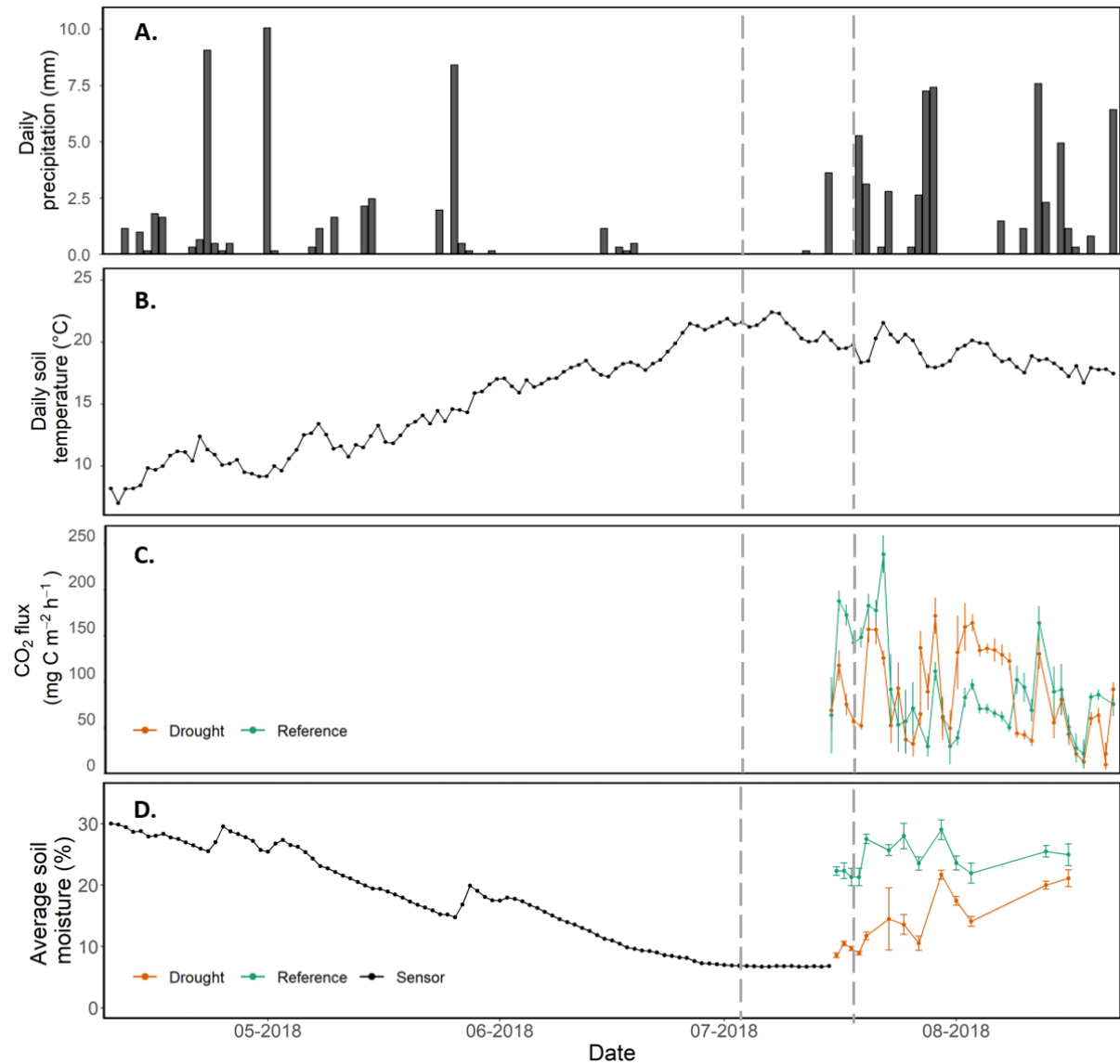
Figure 3.1. *Metadata for the experimental field site from mid-Spring to mid-Summer of 2018. A. Daily precipitation (sum of hourly precipitation over 24 h). B. Daily surface soil temperature (mean of hourly soil temperature over 24 h). C. Daily CO2 flux, (mean of hourly soil temperature over 24 h), error bars represent ± SEM (n = 6). D. Soil moisture, black line represents daily (Acclima) sensor data (10 cm depth) from an unirrigated adjacent field trial until soil sampling started, error bars represent ± SEM (n = 6). Grey dashed lines represent the start and end of control plot irrigation.*

Total soil C and N were determined on oven-dried, ground soil using a TruSpec$^{®}$ Analyzer (Leco Corp., St. Joseph, MI, USA). Within 24 h of soil collection, 1:5 (w/v) soil-to-0.5 M K_2SO_4 and 1:5 (w/v) soil-to-0.5 M AcOH extractions were performed. Total organic C and total extractable N (mineral and organic) was determined on K_2SO_4 extracts using a Multi N/C 2100S Analyzer (AnalytikJena, Jena, Germany). Nitrate (NO_3-N), ammonium (NH_4-N) within the 0.5 M K_2SO_4 extracts were measured by the colorimetric methods of Miranda et al. (2001) and Mulvaney (1996), respectively. Phosphate (P) was determined using the AcOH extracts using the method of Murphy and Riley (1962). Exchangeable potassium (K) was measured on the 1:5 (w/v) soil-to-0.5 M AcOH extracts using a Sherwood Model 410 Flame Photometer (Sherwood Scientific Ltd, Cambridge, UK). Soil characteristics are summarised in Table 3.1.

Table 3.1. *Influence of drought and N fertiliser rate on topsoil (0 - 10 cm depth) quality. Soil was sampled either during the drought or in the subsequent post drought (recovery) period. Results are expressed on mean dry soil weight basis ± SEM (n = 4). Letters denote significant differences between treatments using an ANOVA with a Tukey Post-hoc test (p < 0.05).*

	Drought period				Recovery period			
	0 kg N ha^{-1}		300 kg N ha^{-1}		0 kg N ha^{-1}		300 kg N ha^{-1}	
	Drought	**Reference**	**Drought**	**Reference**	**Drought**	**Reference**	**Drought**	**Reference**
Soil moisture (%)	$7.38 \pm 0.35^{A,C,E,G,L}$	$24.7 \pm 0.62^{A,B,J}$	$7.04 \pm 0.39^{D,F,H,M}$	$22.9 \pm 0.91^{C,D}$	$21.9 \pm 0.4^{E,F,I}$	$25.8 \pm 0.6^{G,H,I,K}$	$20.4 \pm 0.4^{J,K,N}$	$24.1 \pm 0.6^{L,M,N}$
pH	6.06 ± 0.04^{A}	$6.19 \pm 0.04^{B,C}$	5.97 ± 0.19^{D}	6.01 ± 0.06^{E}	5.92 ± 0.06	6.00 ± 0.09^{F}	$5.57 \pm 0.03^{A,B,D,E,F}$	5.78 ± 0.06^{C}
EC (µS cm^{-1})	90 ± 12	71 ± 11	108 ± 27	57 ± 10	66 ± 6	45 ± 2	88 ± 9	45 ± 3
Total C (%)	2.58 ± 0.11	2.54 ± 0.11	3.09 ± 0.44	2.49 ± 0.17	2.85 ± 0.12	2.89 ± 0.11	2.58 ± 0.08	2.83 ± 0.15
Total N (%)	$0.28 \pm 0.01^{B,F,J}$	$0.29 \pm 0.01^{C,G,K}$	$0.29 \pm 0.01^{D,H}$	$0.27 \pm 0.01^{A,E,I,L}$	0.15 ± 0.01^{A}	$0.16 \pm 0.01^{B,C,D,E}$	$0.17 \pm 0.01^{F,G,H,I}$	$0.16 \pm 0.00^{J,K,L}$
C:N ratio	9.2 ± 0.3	8.8 ± 0.3	10.4 ± 1.1	9.2 ± 0.5	19.3 ± 1.7	18.1 ± 1.3	15.4 ± 0.5	17.7 ± 1.2
Dissolved organic C (mg C kg^{-1})	152 ± 12	134 ± 13	$203 \pm 31^{A,B}$	134 ± 22	122 ± 3^{A}	114 ± 3^{B}	134 ± 8	134 ± 6
Total dissolved N (mg N kg^{-1})	16.6 ± 3.3	11.1 ± 0.8^{A}	$33.8 \pm 6.4^{A,B,C}$	20.9 ± 6.4	13.6 ± 1.9^{B}	12.4 ± 0.8^{C}	24.7 ± 4.0	21.2 ± 3.8
Extractable NO$_3^-$ (mg N kg^{-1})	$1.59 \pm 0.38^{A,B,C,D,E}$	$1.90 \pm 0.28^{F,G,H,I,J}$	$21.4 \pm 4.3^{A,F,K}$	$4.98 \pm 1.93^{K,L}$	$8.82 \pm 0.77^{B,G,M}$	$7.75 \pm 0.46^{C,H,N}$	$28.9 \pm 5.9^{D,I,L,M,N,O}$	$9.79 \pm 2.61^{E,J,O}$
Extractable NH$_4^+$ (mg N kg^{-1})	$5.70 \pm 0.59^{A,B}$	6.41 ± 0.62^{C}	$28.7 \pm 5.80^{A,D,E,F}$	$16.7 \pm 4.31^{G,H}$	$6.42 \pm 2.72^{D,H,I}$	$3.32 \pm 0.80^{C,E,G}$	$24.7 \pm 4.15^{B,I}$	6.77 ± 3.27^{F}
Extractable PO$_4$ (mg P kg^{-1})	$5.60 \pm 0.52^{A,E,I,M}$	$7.06 \pm 1.23^{B,F,J,N}$	$7.06 \pm 1.16^{C,G,K,O}$	$6.72 \pm 1.71^{D,H,L,P}$	$31.4 \pm 3.6^{A,B,C,D}$	$25.6 \pm 2.1^{E,F,G,H}$	$26.7 \pm 2.9^{I,J,K,L}$	$23.1 \pm 1.7^{M,N,O,P}$
Exchangeable K (mg kg^{-1})	210 ± 20^{A}	$694 \pm 201^{B,C,D,E}$	250 ± 88^{F}	$319 \pm 51^{G,H}$	$145 \pm 25^{B,E}$	44 ± 12^{C}	$88 \pm 19^{D,G}$	$78 \pm 7^{A,E,F,H,I}$
Bacterial/Fungal PLFA ratio	0.10 ± 0.00	$0.12 \pm 0.00^{A,D,F,H,J}$	$0.09 \pm 0.00^{A,C}$	$0.13 \pm 0.00^{B,C,E,G,I,K}$	$0.09 \pm 0.00^{D,E}$	$0.09 \pm 0.00^{F,G}$	$0.09 \pm 0.00^{H,I}$	$0.09 \pm 0.00^{J,K}$
Microbial PLFA biomass (µmol PLFA kg^{-1})	$149 \pm 4^{A,E,I,M}$	$173 \pm 5^{B,F,J,N}$	$140 \pm 9^{C,G,K,O}$	$163 \pm 6^{D,H,L,P}$	$113 \pm 3^{A,B,C,D}$	$112 \pm 3^{E,F,G,H}$	$108 \pm 3^{I,J,K,L}$	$114 \pm 4^{M,N,O,P}$

EC - electrical conductivity

3.3.3. Phospholipid fatty acid (PLFA) profiling of the microbial community

Soil was subsampled from homogenised samples described in section 3.2.2 immediately after sieving and stored at -80°C. Samples were then lyophilised using a Modulyo Freeze Dryer with RV pump (Edwards Ltd., Crawley, UK) and the samples stored again at -80°C. Samples were then shipped on dry ice (-78.5°C) to Microbial ID Inc. (Newark, DE, USA) and extracted, fractionated, and transesterified according to the method of Buyer and Sasser (2012). Subsequently, samples were analysed using a 6890 gas chromatograph (GC) (Agilent Technologies, Wilmington, DE, USA) equipped with autosampler, split–splitless inlet, and flame ionization detector. The system was controlled with MIS Sherlock® (MIDI, Inc., Newark, DE, USA) and Agilent ChemStation software. GC-FID specification, analysis parameters and standards are as described in Buyer and Sasser (2012). Fatty acids contributing to each taxonomic group are summarised in Appendix 1 Table 3.

3.3.4. Untargeted primary metabolites and complex lipids

Additional multiple soil cores ($n = 5$; $\emptyset = 1$ cm; depth 0 – 10 cm) were randomly taken across each sub-plot and homogenised to obtain a representative soil sample. After collection, the samples were immediately (within 30 s) frozen in the field by placement in liquid N_2 to quench metabolic and lipid turnover (Wellerdiek et al., 2009). Samples were stored and lyophilised as described as described in section 3.2.3. Post-lyophilisation, roots and other debris (e.g. plant litter) were removed by hand and the samples finely ground using a sterile pestle and mortar, rinsed with distilled water and 70% ethanol between samples, before being transferred to sterile polypropylene 1.5 ml microfuge tubes. Samples were then shipped on dry ice (-78.5°C) to the West Coast Metabolomics Center (UC Davis Genome Center, Davis, CA, USA) for untargeted primary metabolites by automated liner exchange cold injection system gas chromatography time of flight mass spectrometry (ALEX-CIS GCTOF MS) and complex lipid analysis by charged surface hybrid column electrospray ionization quadrupole time of flight tandem mass spectrometry (CSH-ESI QTOF MS/MS).

Untargeted primary metabolite extraction consisted of vortexing a 1:0.025 (w/v) soil-to-3:3:2 (v/v/v) MeCN/IPA/H₂O solution, before shaking for 5 min at 4°C, centrifuging and an aliquot of the supernatant recovered for analysis. Metabolomic analysis was performed on a 6890 GC (Agilent Technologies) coupled to a Pegasus IV TOF MS (Leco Corp., St. Joseph,

MI, USA), injected via a Gerstel CIS4 with dual MPS Injector (Gerstel, Muehlheim, Germany) using the chromatographic parameters described in Fiehn et al. (2008). Briefly, data pre-processing was performed in ChromaTOF vs. 2.32, without smoothing, using 3 s peak width, baseline subtraction just above the noise level, automatic mass spectral deconvolution and peak detection at signal/noise levels of 5:1 throughout the chromatogram. Data were then validated, aligned and filtered using the BinBase algorithm (rtx5) with the following settings: validity of chromatogram (< 10 peaks with intensity $> 10^7$ counts s^{-1}), unbiased retention index marker detection (MS similarity > 800, validity of intensity range for high m/z marker ions), and retention index calculation by 5th order polynomial regression, as described in Fiehn et al. (2008) and Fiehn (2016). Further curation of the data was carried out as described in Fiehn (2016). Final curated results were reported as peak heights. Internal standards were included; however, these were for quality control and peak correction purposes. Data presented are therefore qualitative and compounds are tentatively identified, as commonly accepted for untargeted analysis (Gertsman and Barshop, 2018).

Complex lipid extraction was performed using a modified bi-phasic method of Matyash et al. (2008). The main advantage of Matyash method over the Bligh and Dyer methods is that the lipids are contained in the upper extraction phase (as the Methyl tertiary-butyl ether (MTBE) solvent used in the Matyash method has a lower density than water, compared to the chloroform (CHCl$_3$) solvent used in the Bligh and Dyer methods, which has higher density than water). Thus, the organic phase can be withdrawn directly without risk of contamination from the aqueous phase or the interphase. However, we note that the different methods can reveal different lipid yields (Sostare et al., 2019). Briefly, 225 µl of MeOH (containing internal standards) was added to 40 mg soil sample and vortexed for 20 s, followed by the addition of 750 µl MTBE and vortexed for a further 10 min. Samples were then placed in a bead grinder for 30 s. Subsequently, samples were shaken for 6 min at 4°C, before the addition of 188 µl of MS-grade water and centrifugation (2 min). An aliquot of the supernatant was then removed and evaporated to dryness using a SpeedVac. Dried extracts were re-suspended using a mixture of 9:1 MeOH/toluene (v/v) (containing an internal standard). Sample analysis was performed using an Agilent 1290 Infinity LC system (G4220A binary pump, G4226A autosampler, and G1316C Column Thermostat) coupled to an Agilent 6530 MS in positive ion mode. Lipids were separated on an Acquity UPLC CSH C18 column (100 × 2.1 mm; 1.7 µm). For full instrument parameters see Appendix 1 Table 2. The general workflow for data processing followed the mass spectrometry-data independent analysis (MS-DIAL) software method described in Tsugawa et al. (2015). This was followed by data clean up using the mass spectral

feature list optimizer (MS-FLO), as described in DeFelice et al. (2017). Peaks are annotated in manual comparison of MS/MS spectra and accurate masses of the precursor ion to spectra given in the Fiehn laboratory's LipidBlast spectral library (Kind et al., 2013). MassHunter Quant software was then used to verify peak candidates based on peak shape, peak height reproducibility and retention time reproducibility in replicate samples. Valid and reproducible peaks were analysed by targeted MS/MS with the aim of increasing overall peak annotations. Final curated results were reported as peak heights. Internal standards were included; however, these were for quality control and peak correction purposes only. Hence, data presented are therefore qualitative and compounds are tentatively identified, as is the common with untargeted analysis (Gertsman and Barshop, 2018).

3.3.5. Monitoring grassland CO_2 and N_2O emissions

A mobile automated greenhouse gas (GHG) monitoring system (Queensland University of Technology, Institute for Future Environments, Brisbane, Australia), as previously described in Marsden et al. (2018), was used to monitor emissions from the drought and reference plots. The system can process twelve automated chambers, these were deployed on the 300 kg N ha^{-1} treatments of both grass species under drought and wetted conditions ($n = 3$). Stainless steel chamber bases (0.25 m^2 basal area) were inserted into the plots (10 cm depth) two weeks prior to measurement beginning, and chambers (50 cm $\times$ 50 cm $\times$ 15 cm) were attached to the bases. Emissions were monitored from the 16th July 2018 throughout the remaining drought and subsequent recovery period, to 16th August 2018. Chamber headspace samples were pumped (ca. 200 ml min^{-1}) through Teflon tubing to a LI-COR LI-820 (Licor, St Joseph, MI, USA) to measure CO_2. Samples were then passed through a sodium hydroxide filter to remove remaining CO_2 and moisture before being fed into a GC (SRI 8610C, Torrance, USA) coupled to an ^{63}Ni electron capture detector (ECD) to measure N_2O. Briefly, each block of chambers closed for 1 h, during which time four headspace samples were taken from each chamber (once every 15 min), with a calibration standard analysed every fourth gas sample. This resulted in the continuous measurement of eight fluxes per 24 h period. An SC100 V air compressor (SGS Engineering Ltd., Derby, UK) was used to fill compressed air lines linked to pneumatic actuators on the chambers, to open and close chamber lids automatically. Cumulative fluxes were calculated using the trapezoidal rule (Marsden et al., 2018).

3.3.6. Data processing and statistical analysis

Lipid data were curated by removing lipids for which sample peak heights that were non-statistically different from peak heights of the method blanks (matrix-free negative quality controls). This was performed using a two-sample t-test assuming unequal variance, in Excel 2016. All subsequent analyses were run using R v. 3.6.0 (R Core Team, 2019). Graphical analysis was performed using the package 'ggplot2' (Wickham, 2016). Normality and homogeneity of variance of the chemical and physical soil properties of the Eutric Cambisol were assessed using Shapiro-Wilk test and Bartlett's test, respectively. Not all data conformed to parametric assumptions even after using Log_{10} transformation, therefore, a Kruskall-Wallis test (stats package; R Core Team, 2019) was used to assess the similarities between soil properties in drought and recovery conditions. The 'vegan' package (Oksanen et al., 2019) was used to perform two-way repeated measures ANOVA (analysis of variance) to examine the influence of treatment (drought and reference) and soil moisture on changes in CO_2 flux, with significant differences were further explored using a Tukey post-hoc test. Cumulative CO_2 and N_2O fluxes for drought and watered plots were also calculated through area integration, differences were tested with a t-test. 'vegan' and 'ggplot2' was also used to a construct principal component analysis (PCA) on summed PLFA biomarkers (Appendix 1 Table 3) as a percentage of total PLFA biomass (Frostegård et al., 1993). This was followed by computation of a PERMANOVA (permutational multivariate analysis of variance) on summed PLFA biomarkers as a percentage of total PLFA biomass to identify differences in dispersion between centroids of groups. MetaboAnalyist v4.0 (Chong et al., 2018; Chong and Xia, 2018) was used for normalisation (generalized logarithm transformation (glog) and Pareto scaling) of lipidomic and metabolomic data, as well as ANOVA to identify significant differences in compound concentrations between treatments. Normalised data were also used to create heatmaps (using Euclidean distance and Ward clustering algorithms) of lipidomic and metabolomic compounds using MetaboAnalyst. A significance level of $p < 0.05$ was used for all analysis.

3.4. Results

3.4.1. Complex lipids as affected by drought and N fertilisation regime

Curated complex lipid analysis tentatively identified a total of 239 individual compounds. Of these compounds detected, 134 showed statistically significant differences between treatments ($p < 0.03$). There were three response groups within the complex lipids data (Fig. 3.2); i) compounds that decreased in relative concentration during drought conditions in both reference and drought plots (Response A; $n = 26$), ii) compounds that increased in relative concentration under drought conditions in drought plots only (Response B; $n = 9$), and iii) other compounds with no clear response across treatments (Response C; $n = 15$). Results of the PERMANOVA showed that overall, groupings of water stress ($p < 0.001$) and N loading ($p < 0.001$) had a significant effect on the soil lipid concentrations, while grass species ($p = 0.1$) had no overall impact on the pattern of soil lipids.
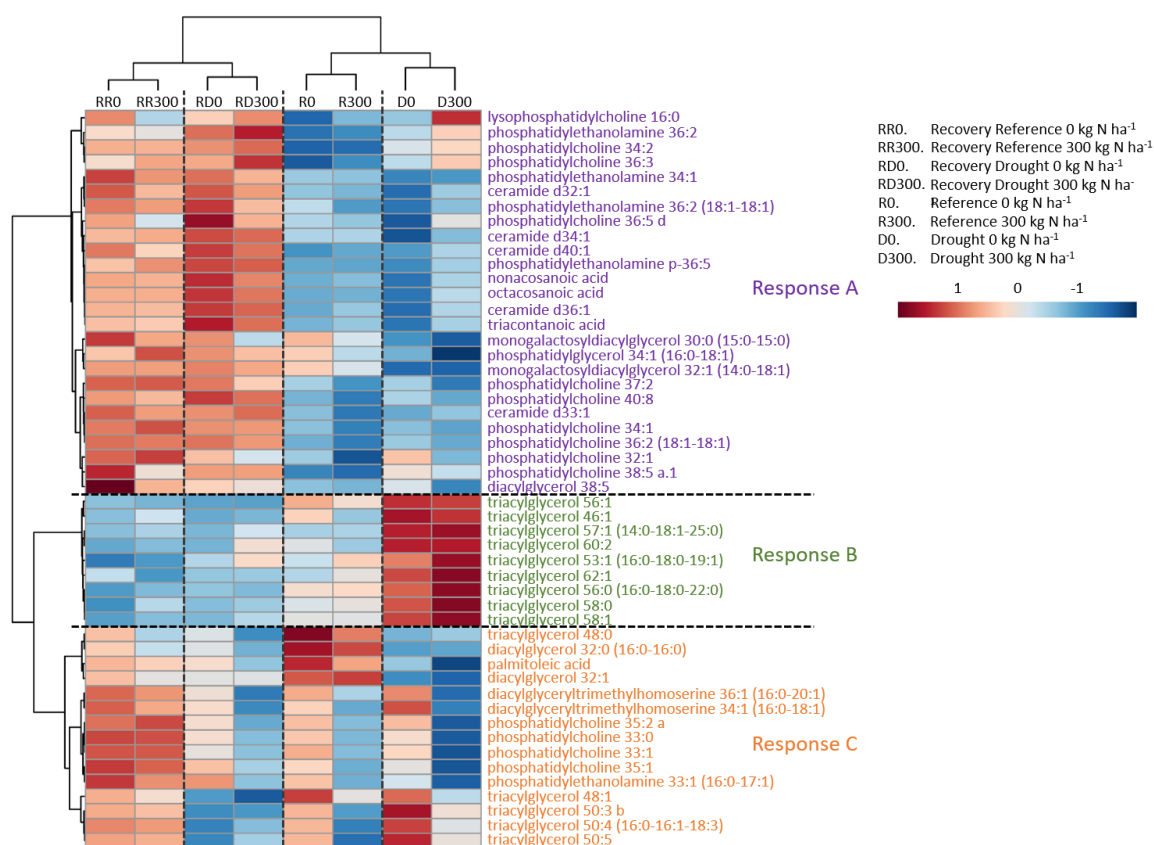
Figure 3.2. *Influence of drought, N fertilisation and grass species on the lipidomic profile of soil. Heatmap showing expression profiles of soil treatment groups (n = 8) based on the 50 most significant tentatively identified lipids as classified by ANOVA p-value (p < 0.03). Lipids*

were subsequently clustered using Euclidean distance and Ward linkage. Data were normalised using a log transformation and Pareto scaling. The colour of samples ranges from red to blue, indicating metabolite concentration z-score; numbers 1.5 to -1.5 on the scale bar indicate the number of standard deviations from the mean.

3.4.2. Primary metabolites as affected by drought and N fertilisation regime

Untargeted primary metabolomic analysis tentatively identified a total of 155 compounds. Of these compounds detected, 118 showed statistically significant differences between treatments ($p < 0.04$). There were two distinct responses within the untargeted metabolome data (Fig. 3.3); i) compounds that increased in relative concentration during drought conditions in the drought plots only (Response A; $n = 4$), and ii) compounds that decreased in relative concentration during drought conditions (Response B; $n = 46$). A heatmap of all metabolites detected in samples, irrespective of p-value can be found in supplementary information (Appendix 1 Fig. S4). Results of the PERMANOVA showed that overall, grouping of water stress ($p < 0.001$) had a significant effect on the soil primary metabolite concentrations, however, both N loading ($p = 0.2$) and grass species ($p = 0.4$) had no overall impact on the dispersion of soil primary metabolite concentrations.
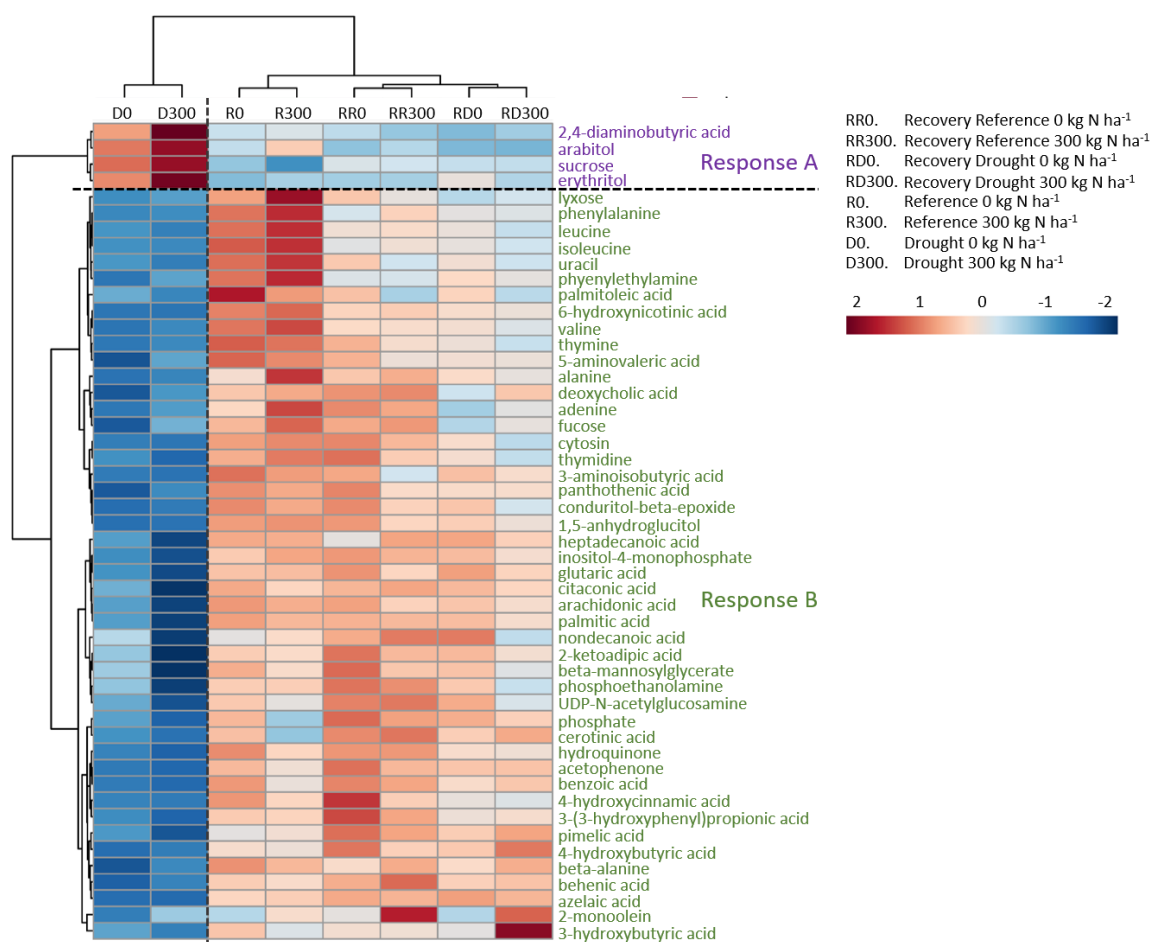
Figure 3.3. *Influence of drought, N fertilisation and grass species on the metabolic profile of soil. Heatmap showing expression profiles of soil treatment groups (n = 8) based on the 50 most significant tentatively identified metabolites as classified by ANOVA p-value (p < 0.04). Metabolites were subsequently clustered using Euclidean distance and Ward linkage. Data were normalised using a log transformation and Pareto scaling. The colour of samples ranges from red to blue, indicating metabolite concentration z-score; numbers 2 to -2 on the scale bar indicate the number of standard deviations from the mean.*

3.4.3. Soil characteristics

Differences in soil characteristics were assessed between drought conditions and recovery conditions, post-rainfall. There were statistical differences ($p < 0.001$) between soil pH, EC, NO_3^-, NH_4^+, K, total PLFA microbial biomass and PLFA fungal-to-bacterial ratio, all of which reduced between drought and recovery sampling dates (Table 3.1). As expected, soil

moisture levels increased significantly during the recovery period ($p < 0.006$) following rainfall in the drought plots. In contrast, there was no difference in the control (irrigated) plots ($p = 0.37$) between the two measurement periods. Soil available P concentrations increased between drought and recovery sampling dates ($p < 0.001$) in all treatments.

3.4.4. PLFA community composition

PCA was used to depict the variation in PLFA-derived microbial community structure between treatments (Fig. 3.4). From this, it can be inferred that the microbial community changed between drought and recovery conditions, and that the effect of water availability was greater than the differences caused by grass species or N fertiliser loading. Changes in the amount of Gram-positive and fungal biomarkers were the vectors predominantly responsible for driving the change in community structure between drought and recovery conditions. However, the results of the PERMANOVA showed that overall, grouping of soil water stress ($p < 0.001$) and N loading ($p < 0.03$) had a significant effect on the soil microbial community, while grass species ($p = 0.9$) had no overall impact on the soil microbial community.
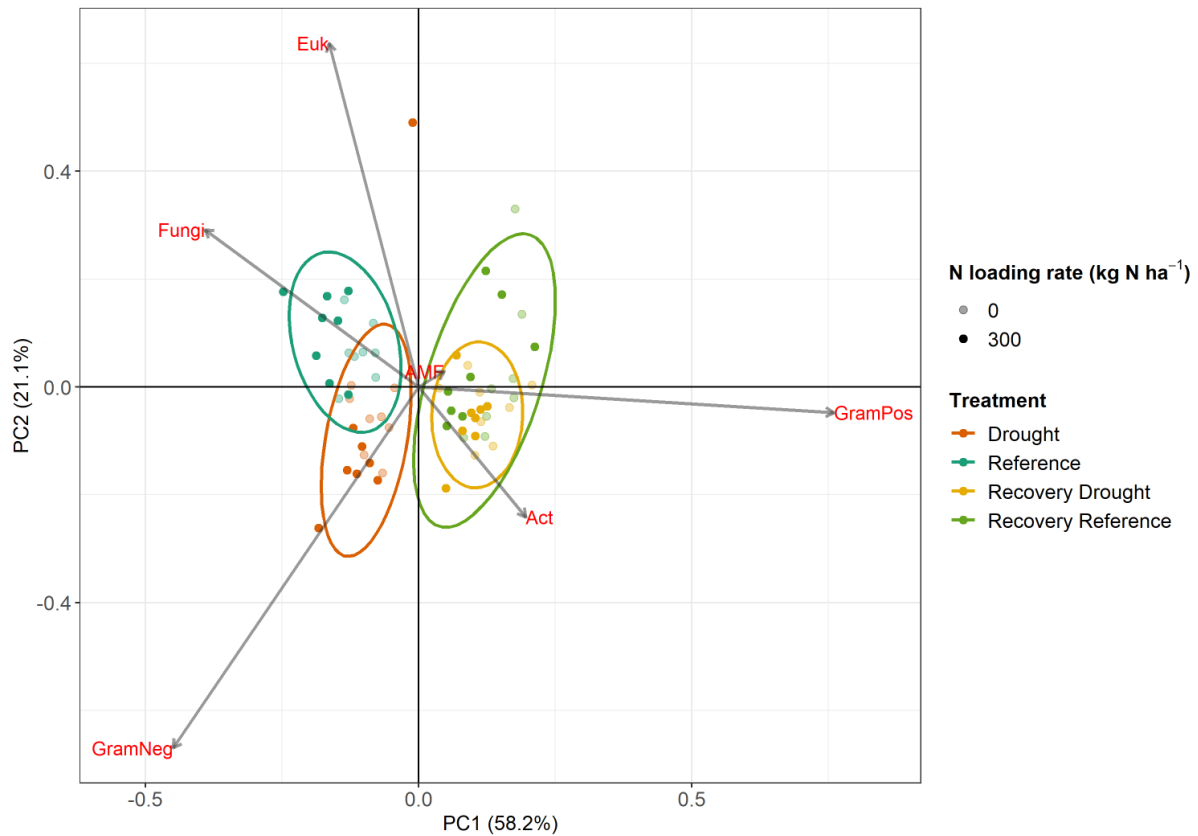
Figure 3.4. *Influence of N and grass species on the PLFA profile of the soil microbial community during and after an extreme drought. 2D principal component analysis (PCA) of PLFA biomarkers as a percentage of total PLFA biomass. Principal component 1 (PC1) explains 58.2 % of total variance, principal component 2 (PC2) explains 21.1 % of total variance. Vector arrows indicate the relationship between variables. Ellipses represent 95% confidence levels of treatment groups.*

3.4.5. Effects of drought and recovery on net ecosystem CO_2 and N_2O flux

A repeated measures ANOVA showed that there were significant differences between CO_2 fluxes by soil water stress ($p < 0.001$), treatment ($p < 0.001$) and day number ($p < 0.001$). Post-hoc analysis showed that there was a significant increase in CO_2 flux between 23rd July (two days after the first considerable rainfall in 29 days) and dates before (20th July) as well as after (26th, 27th and 29th July) ($p < 0.01$). There were no significant differences between dates other than the 23rd July, where CO_2 flux peaked (Fig. 3.1C). However, significant differences

were found between cumulative CO_2 ($p < 0.02$) and N_2O ($p < 0.005$) emissions between the drought and water (reference) treatments (Table 3.2).

Table 3.2*. Influence of drought on the cumulative flux of the greenhouse gases CO_2 and N_2O from 300 kg N ha^{-1} plots, over the sampling period (16th July – 16th August 2018; n = 6). Values represent means ± SEM. Letters denote significant differences between treatments (p < 0.05).*

	Treatment	Cumulative flux
N_2O	Reference	10.6 ± 1.5^a
(mg m$^{-2)}$	Drought	6.6 ± 0.5^b
CO_2	Reference	81.9 ± 7.6^a
(g m^{-2})	Drought	51.1 ± 3.0^b

3.5. Discussion

3.5.1. Effect of drought on soil derived complex lipids

This study aimed to assess the response of novel biological soil quality indicators in response to extreme drought under contrasting N fertiliser loading rates. Current biological indicators (e.g. PLFAs) can be sensitive to changes in environmental conditions, showing shifts in soil microbial community. However, they are unable to relate this change to soil function. Metabolomic and lipidomic data are directly related to the physical state of the soil microbial community (Swenson et al., 2018; Warren, 2018). Identification of specific metabolomic and lipidomic molecules that predominate under extreme conditions, in this case osmotic stress, may begin to elucidate this change in biological function.

Soil complex lipid composition was affected both by drought conditions and soil N loading. Response A (Fig. 3.2) showed a significant decrease in lipid concentrations during drought conditions; the group contained a mix of several types of lipid including phosphatidylethanolamines (PEs), phosphatidylcholines (PCs), fatty acids (FAs) and ceramides, all of which are found in high concentrations within the cell membrane (Dawaliby et al., 2016; Olsen and Jantzen, 2001; Sohlenkamp and Geiger, 2016) and have been reported in soil previously (Ding et al., 2020). PEs are key in the regulation of membrane fluidity, and PCs and ceramides are used in the cell as regulators of osmotic stress (Dawaliby et al., 2016; Hannun and Luberto, 2000; Kiewietdejonge et al., 2006). Many previous studies have shown

that bacteria alter their membrane lipid composition and cell size in response to abiotic stresses (Romantsov et al., 2009; Wang and Levin, 2009; Weijers et al., 2007; Zhang and Rock, 2008). For example, for *Escherichia coli* under osmotic stress, relative concentrations of cardiolipin (CL) increase as phosphatidylethanolamine (PE) decrease (Romantsov et al., 2009). With many bacteria modifying membrane biophysical properties by changing the length of fatty acid chains, or the ratio of saturated:unsaturated fatty acids (Zhang and Rock, 2008). It is highly likely that the long-term osmotic stress induced by a prolonged drought will therefore induce the observed shifts in lipid composition.

Response B (Fig. 3.2) displayed a significant increase of triacylglycerol (TAG) compounds under drought conditions. TAGs are neutral lipids, predominantly found in eukaryotic organisms as storage lipids or forming part of lipid droplets (LDs), as well as a limited number of bacteria mostly of the actinobacteria and cyanobacteria phylum (Lerique et al., 1994; Zhang and Liu, 2017). LDs, organelles formed of a neutral lipid core surrounded by a phospholipid monolayer, are key to eukaryotic and prokaryotic organisms surviving stress conditions (Zhang and Liu, 2017). For example, Zhang et al. (2017) previously reported that LDs have the potential to bind to genomic DNA, increasing bacteria survival rate under stress. Additionally, cytoplasmic LDs are also key in cellular metabolism and homeostasis (Hashemi and Goodman, 2015). This change in TAG concentration under drought conditions is likely to represent a change in soil biological function. If organisms are focusing their energy on storage this is likely to have an impact on normal cell function and metabolism (Fulda et al., 2010).

We can therefore accept hypothesis 2, microbial storage lipid composition did increase during drought conditions, and was probably as a result of water and essential nutrients becoming harder to access. It is likely that TAGs and other storage lipids will only accumulate if nutrient availability decreases more than C availability. In this regard, microbial storage is highly related to the ratio of available nutrients to available C, for instance soil and microbial stoichiometry (Cleveland and Liptzin, 2007; Khan and Joergensen, 2019). However, it must be noted that the increase in storage lipids, particularly TAG, may also have had a role in osmoprotection.

3.5.2. Effect of drought on soil-derived primary metabolites

Compounds that significantly decreased under drought included a range of amino acids (e.g. leucine and valine). Their abundance has previously been used as a proxy for bacterial growth rate (Bastviken and Tranvik, 2001; Pollard and Moriarty, 1984). With a large number

of metabolites from across heterotrophic metabolism decreasing in concentration (Jurtshuk, 1996), many associated directly with microbial growth, it is highly likely that the functioning and growth of the bacterial community was impaired during drought.

Functions of compounds that increased under drought in this study (Response A, Fig. 3.3), were generally all related to osmoprotection. For example, it has also been shown that water soluble carbohydrates (e.g. sucrose) have a key role in continuing growth and development during impaired metabolic activity (Van den Ende and Valluru, 2008) and their accumulation has been shown to occur under osmotic stress, particularly in replacing membrane phospholipids to prevent membrane gelling (Esbelin et al., 2018; Hershkovitz et al., 1991). This is also consistent with the observed decrease in total phospholipid concentrations during the recovery period after alleviation of the drought (Table 3.1). Sugar alcohols (here, arabitol and erythritol), are metabolically interrelated with energy storing carbohydrates under osmotic stress (Hallsworth and Magan, 1995; Kayingo et al., 2001; Kobayashi et al., 2015, 2013). Although these compounds can be secreted out of the cell into the surrounding soil (Zeidler and Muller, 2019), the extraction procedure used here was unable to differentiate between intracellular and extracellular solutes. Also, 2,4-diaminobutyric acid is key in the biosynthesis of ectoine in bacteria; an osmolyte highly associated with organism's potential to survive extreme osmotic stress (Bestvater et al., 2008; Czech et al., 2019). It is likely that ectoine was upregulated alongside 2,4-diaminobutyric acid, however, it was not identified in this study due to limitations of the analytical approaches employed.

Based on these results we can accept hypothesis 1, primary metabolite compounds related to coping with osmotic stress did increase under drought conditions. Metabolites are also responsive to changes over short temporal scales (days). With the soil metabolic profile recovering to a similar level to that of the reference (control) plots after drought alleviation. Although drought plots did not match metabolite profiles of reference plots exactly post-drought, this is likely to be due to the rapid processing of metabolic compounds as more favourable growth conditions occur and the heterogeneous nature of soil (Wang and Levin, 2009). Based on this study we can tentatively accept hypothesis 4, as the microbial community did show some metabolic legacy effect on the alleviation of the drought. However, more work is required to show the persistence of this legacy, and the effect of multiple drought and rewetting cycles on soil metabolic health.

Further examples of metabolites commonly associated with this osmotic adjustment are proline, mannitol, glycine betaine, trehalose and glucose (Giri et al. 2011; Nawaz and Wang, 2020; Warren, 2014). Proline was detected in all samples and was present in significantly

higher concentrations under the drought treatments, irrespective of N loading rate. Trehalose was detected in all samples, with higher concentrations present under the drought treatment under low N compared to reference samples. Glucose was present in all samples with the only significant result observed between the high N loading rate samples under drought and drought-recovery conditions. Glycine betaine was not detected in this study due to the limitations of the GC-MS method, as discussed below. Mannitol, while potentially detectible by the described GC-MS protocol was not identified, possibly due to being below detection limits. Upon rewetting, the microbial recycling of these cellular osmolyte compounds can lead to rapid increases in soil respiration ("the Birch effect") (Slessarev et al., 2020; Warren, 2020a).

While the GC-MS workflow can measure many primary metabolites, it is generally limited by poor resolving power for many N-containing metabolites and highly labile metabolites (e.g. many sugar compounds coelute and have the same *m/z* as other sugar-type compounds). Equally, the samples were only run with the MS in positive ion mode; other compounds may have been detectable in negative mode. Additionally, other compounds (e.g. glycine betaine) are not detectable as they are not amenable to derivatisation. As a result, the compounds detected in this study (summarised in supplementary information) cannot be considered extensive or exhaustive. However, the findings of this study demonstrate that focusing on a targeted range of compounds may simplify the complexity of the biochemical response of the plant-soil system to drought, whilst an untargeted approach to analysis may be beneficial in identifying further biomarkers of stress.

3.5.3. Effect of drought on soil derived PLFA microbial community

PCA clustering of PLFA microbial community shows changes in community structure between treatments, with distinct separation between drought and recovery communities (Fig. 3.4). The shift in community composition was mainly bacterially driven. Conversely, there was little separation between reference and drought groups between the two sampling points. We therefore reject hypothesis 3, as while the soil microbial community did alter their PLFA membrane composition under osmotic stress conditions, this was not significantly different to reference groupings. Additionally, PLFA microbial biomass was significantly reduced during the recovery period compared to the drought period. This is unexpected as a large amount of labile C and other nutrients are likely to become available upon soil rewetting after drought which should drive microbial growth, at least in the short term (Schimel, 2018). For example, it has been shown that a droughted microbial community can respond within seconds of water

addition and that microbial growth can re-establish within 48 h of re-wetting (Jones and Murphy, 2007; Göransson et al., 2013; Meisner et al., 2015; Siebert et al., 2019).

Gram-positive bacteria increased in concentration during the recovery period, probably as a legacy of their greater resistance to osmotic stress owing to their physiology. The lack of increase in the fast-growing Gram-negative community also supports our finding that a large amount of labile C did not become available after rewetting to support microbial growth (Fanin et al., 2019). However, as noted by Naylor and Coleman-Derr (2018), abundance changes in soil microbial community are context dependent and responses are frequently not consistent. Nevertheless, Gram-positive bacteria responded more rapidly than the rest of the biological community to the increase in available moisture and nutrient associated with rewetting, a likely cause of the shift in bacterial-to-fungal ratio (Fanin et al., 2019).

There are a number of limitations of using PLFA analysis to understand changes in microbial community responses to external stress. Firstly, the active, inactive and dead community are sampled all together (Nielsen and Petersen, 2000). Additionally, FA membrane composition may change in response to the prevailing environmental conditions rather than being due to an actual shift in community structure (Bossio and Scow, 1998; Córdova-Kreylos et al., 2006). Equally, the soil microbial community has been shown to substitute phospholipids with betaine lipids under stress conditions, which may not be accounted for in targeted PLFA assays (Warren, 2020b). Therefore, from PLFA data alone it is impossible to separate real community change from the current microbial community adjusting to a new environmental equilibrium. Targeted approaches: measuring active microorganisms (i.e. ^{13}C-labelled PLFA analysis), or combining with a metagenomics approach, may therefore offer additional insights into drought responses of the microbial community, particularly changes of soil microbial activity after rewetting (Willers et al., 2015b).

There was a distinct shift of metabolomic and lipidomic compounds during the drought when compared to the recovery period. For lipidomic and metabolomic compounds it seems that recovery was not purely a reversal of stress responses, with many of the non-drought responsive compounds also increasing during the recovery period (Wedeking et al., 2018). This shows that, in terms of primary metabolic functionality, the microbial community has a resilience to extreme drought and osmotic stress, rapidly reducing metabolic compounds associated with mitigating osmotic stress. However, the change in community structure suggests that the physiology and function of the community is likely to have altered and may be affected for a longer period of time. Although, as discussed, change in PLFA membrane

composition may be due to the prevailing environmental conditions rather than being due to an actual shift in community structure.

3.5.4. Changes in soil and plant respiration

The so called "Birch effect" is generally understood to be caused by the mineralization of either intracellular osmoregulatory substances released by soil microorganisms in response to hypo-osmotic stress and to avoid cell lysis (Unger et al., 2010; Warren, 2014), and/or increased availability (and subsequent consumption) of extracellular organic C and microbial necromass on rewetting (Slessarev et al., 2020). This is typically associated with a sudden, but ephemeral CO_2-efflux from soil upon rewetting, indicating a major shift in function and re-mobilization of internal microbial C stores. Osmolyte hydrolysis and consumption (particularly of trehalose), has been indicated as the intracellular mechanism for rapid increases in microbial respiration (Slessarev et al., 2020; Slessarev and Schimel, 2020).

In this study, the Birch effect was shown to occur immediately after the first day of considerable rainfall in 29 days with elevated levels of CO_2 continuing for 9 subsequent days. The CO_2 flux peaked two days after the first considerable rainfall (Fig. 3.1), and this increase in efflux was evident in both the reference and droughted plots. We may therefore reject hypothesis 5, the post-drought rewetting did result in a flush of CO_2 ("Birch effect") and N_2O emissions but not compared to the reference treatment. However, we note that it is impossible to disentangle root and microbial respiration, with both likely to have increased upon rewetting (Xu et al., 2010).

Overall, the non-droughted (reference) plots had a significantly higher cumulative CO_2 and N_2O flux over the sampling period (+60 % and +61 %, respectively; Table 3.2). We attribute this to the reduced metabolism under severe moisture deficit, reduced rates of nitrification and associated N_2O production, and the lack of NO_3^- substrate and loss of anaerobic microsites available for denitrification (Homyak et al., 2017; Schindlbacher et al., 2012). As we were capitalising on a natural extreme event, we only measured GHG emissions in the latter half of the experiment. Consequently, we were unable to complete a full GHG budget.

3.5.5. Effect of plant species and nitrogen fertilisation rate

This study assumed all lipids and metabolites detected were derived directly from the active soil microbial community. While the majority of compounds detected will be of microbial origin, it is likely that some will have been directly extracted from the soil itself for example from mesofaunal, microbial and plant necromass and humic moieties (Allard, 2006; Swenson et al., 2015).

Despite the *Festulolium* grass type being purported to be more drought tolerant (Ghesquière et al., 2010), surprisingly, grass species had no major effect on the distribution of PLFA, metabolomic or lipidomic concentrations in the soil. This was unexpected given that the two grass species used here have different sugar metabolisms, rates of primary productivity, root architecture and rooting depth (Gallagher et al., 2015; Humphreys et al., 2018). It does, however, support previous work in the same soil that showed that a range of metabolites in soil solution were not affected by grass species (Khalid et al., 2007). It may also imply that root exudates did not contribute to the metabolomic profiles in comparison to those in the microbial biomass.

In contrast to the influence of grass species, PERMANOVA showed that N loading significantly affected the concentration pattern of lipids in soil. While lipids are usually more closely associated with C and energy storage than N, previous studies have indicated that N source can influence the metabolism and concentration of lipids in prokaryotic and eukaryotic cells, with N deficiency leading to TAG accumulation (Anand and Arumugam, 2015; Evans and Ratledge, 1983; Gao et al., 2020). Additionally, bacteria and plants under drought stress have been shown to produce nutrient-rich solutes particularly when N is abundantly available (Liang et al., 2013; Song et al., 2019; Teixidó et al. 2005), creating a more negative osmotic potential and aiding to maintain or improve cell hydration (Ashraf and Foolad, 2007). However, it is also possible that differences in plant growth and rhizodeposition under the higher N regime may have induced changes in microbial metabolism. Further work is required to better understand the complex interaction of cell energy use, metabolic function and storage in response to nutrient availability under drought.

3.6. Conclusions

Changes in soil function in response to moisture scarcity were evident over a number of temporal scales. Most responsive was the CO_2 efflux (likely cause by mineralisation of

drought-related osmolytes), followed by the lipid and metabolites and lastly the PLFAs. Both untargeted primary metabolomic and complex lipid analysis provide high resolution and responsive data on the state of the biological community. In this study we have identified a number of compounds that are responsive to drought stress in grassland soils. However, this analytical approach is still in its infancy and further work is required to i) elucidate the relationship between the stress and the use of microbial storage lipids, ii) further explore whether the soil microbial stress-induced compounds in this study are applicable to other stress conditions, iii) assess other soil microbial communities constituent metabolomic and lipidomic makeup and further relate this to soil quality, iv) complement sequencing and enzymatic studies with metabolomic analysis and other indicators of function e.g. measurement of N cycling rates, and v) test the resilience of the microbial community over several wetting and drying cycles. The results presented here clearly demonstrate that metabolomic and lipidomic analysis can provide good indicators of stress and resilience in the soil biological community. Further, we show that these approaches can be directly applied to field-based studies. However, this increases the inherent complexity of interpretation due to the increased number of variables being considered.

3.7. Acknowledgements

3.8. References

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Chapter 4: Field application of pure polyethylene microplastic has no significant effect on soil biological quality and function

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DJ and DC conceptually conceived and designed the experiment. MM curated the field trial and collected the data. HT performed 16S analysis supervised by MD and SP, RB subsequently performed bioinformatic analysis. SB performed N cycling gene analysis. KM performed data curation and analysis of greenhouse gas data. PC performed SEM analysis of microplastic powder. RWB amalgamated the dataset, performed data analysis and interpretation, and wrote the first draft of the manuscript. RWB, DC, MM, SB, KM, PC, DM, SP and DJ contributed to subsequent revisions.

4.1. Abstract

Plastics are now widespread in the natural environment. Due to their size, microplastics (MPs; defined as particles < 5 mm) in particular, have the potential to cause damage and harm to organisms and may lead to a potential loss of ecosystem services. Research has demonstrated the significant impact of MPs on aquatic systems; however, little is known about their effects on the terrestrial environment, particularly within agroecosystems, the cornerstone of global food production. Soil biology is highly responsive to environmental perturbation and change. Hereby, we investigated the effect of pure low-density polyethylene (LDPE) MP loading (0, 100, 1000, or 10000 kg ha^{-1}) on soil and plant biological health in a field environment over a cropping season. Our results showed that MP loading had no significant effect ($p > 0.05$) on the soil bacterial community (measured by amplicon sequencing of bacterial 16S rRNA gene), the size and structure of the PLFA-derived soil microbial community, or the abundance and biomass of earthworms. In addition, metabolomic profiling revealed no dose-dependent effect of MP loading on soil biogenic amine concentrations. The growth and yield of wheat plants (*Triticum aestivum* L., cv. Mulika) were also unaffected by MP dose, even at extremely high (≥ 1000 kg ha^{-1}) loading levels. Nitrogen (N) cycling gene abundance before and after N fertiliser application showed relatively little change, although further experimentation is suggested, with similar trends evident for soil nitrous oxide (N$_2$O) flux. Overall, we illustrate that MPs themselves may not pose a significant problem in the short term (days to years), due to their recalcitrant and inert nature. We also emphasise that most MPs in the environment are not pure or uncontaminated, containing additives (e.g. plasticisers, pigments and stabilisers) that are generally not chemically bound to the plastic polymer and may be prone to leaching into the soil matrix. Understanding the effect of additives on soil biology as well as the longer-term (years to decades) impact of MPs on soil biological and ecological health in the field environment is recommended.

Keywords: Plastic pollution, Metabolomics, Toxicology, Soil quality, Environmental impact

4.2. Introduction

The use of plastics is globally ubiquitous due to their low cost, malleability and durability. However, inappropriate disposal has led to their progressive accumulation in the environment (Geyer et al., 2017). To date, most plastic and microplastic (MPs; particles < 5 mm in size) pollution research has focused on freshwater and marine systems where the negative effects of plastics on organism health and loss of ecosystem function is now becoming well documented (Avio et al., 2017; Sharma and Chatterjee, 2017). However, plastics are also rapidly being identified as a threat to terrestrial ecosystems, yet their potential effects remain largely unexplored (de Souza Machado et al., 2019).

In agroecosystems, plastic entry may occur through a variety of pathways, with the most common including (i) the use, and incorporation of plastic mulch films to improve plant growth and reduce moisture loss (Huang et al., 2020; Sun et al., 2020; Qi et al., 2020); (ii) the addition of municipally-derived organic fertilisers, digestates or compost (Watteau et al., 2018); (iii) the application of biosolids (van den Berg et al., 2020); (iv) the accumulation of slow-release fertiliser coatings (Katsumi et al., 2021), and (v) atmospheric deposition (Allen et al., 2019). The drive for food security and sustainable intensification has led to an inevitable increase in plastic loading to soils globally. For example, the annual input of plastics into agricultural soils is estimated to be between 63 - 430 and 44 - 300 $\times 10^3$ t in Europe and North America, respectively, and potentially exceeding 1.3×10^6 t annually for China (Jian et al., 2020; Nizzetto et al., 2016a). Globally, this greatly surpasses the extrapolated annual mass discharge of MPs to ocean surface waters, predicted to be $9.3 \times 10^7 - 2.36 \times 10^8$ tonnes (Nizzetto et al., 2016b, 2016a, Sebille et al., 2015). Primary MPs (MPs manufactured for a specific application, e.g. clothing microfibres; de Falco et al., 2019) may be applied through waste streams (i.e. biosolids application) due to their difficulty of removal (Cole et al., 2011). In contrast, secondary MPs are formed through degradation and disintegration of larger plastic pieces (Cole et al., 2011; Rocha-Santos and Duarte, 2015), such as agricultural mulch films (Piehl et al., 2018). Both primary and secondary MPs are likely to influence the ecology, health and function of soils, potentially having similar negative effects to those extensively reported in marine ecosystems, e.g. organismal ingestion leading to oxidative stress and assimilation of endocrine-disrupting chemicals, and subsequent reduced growth and reproduction, as well as bioaccumulation up the food chain (Galloway and Lewis, 2016; Kim et al., 2017). Although, bioaccumulation is likely to be less of an issue comparatively, due to the relatively smaller size of soil-dwelling fauna.

Soil is an extremely valuable and non-renewable resource and provides of range of ecosystem services, not least the provisioning of food resources (Comerford et al., 2013; Kopittke et al., 2019). Maintaining soil health and quality is therefore key for agricultural and anthropogenic sustainability (Hou et al., 2020). Soil quality is often broadly defined as the capacity of a soil to function (Karlen et al., 1997). Traditional measurements of soil quality are based on physical or chemical soil properties, with little exploration of soil biology (Bünemann et al., 2018). However, the fertility and productivity of soil are not simply a function of soil physical and chemical characteristics, and recently a more holistic view has been proposed (Rinot et al., 2019). Soil biology is a crucial mediator and driver of many processes linked to nutrient cycling, plant health, and soil productivity (Lal, 2016). It is highly responsive to changes in management and environmental conditions and is often associated with functional change (Lehman et al., 2015). Research has shown that MPs can have significant negative effects on soil microbial community composition (Guo et al., 2020; Zang et al., 2020; Zhang et al., 2019), enzymatic activities and nutrient cycling (Fei et al., 2020; Huang et al., 2019; Yi et al., 2021), mesofaunal health (Huerta Lwanga et al., 2016; Lahive et al., 2019; Lin et al., 2020), plant health (de Souza Machado et al., 2019; Zang et al., 2020), and greenhouse gas (GHG) emissions (Ren et al., 2020; Sun et al., 2020), all of which will impact the soils ability to function effectively. However, most studies to date have been laboratory or mesocosm based, over relatively short sampling periods (weeks) and in many cases at unrealistic MP doses, which may not accurately reflect processes occurring at the field scale (Fidel et al., 2019).

This field-based study aimed to assess the effect of different quantities (0, 100, 1000, or 10000 kg ha^{-1}) of pure MP loading on the health and function of key soil biological quality indicators over a cropping season, using a range of commonly used biological indicators, as well as the novel use of biogenic amine analysis as indicators of metabolism and N cycling in soil. We hypothesised that i) MP loading will have negative effects on all measured aspects of soil biological quality, ii) higher MP loading rates will increase the detrimental impact on soil biology, and iii) crop biomass and yields will be negatively affected by MP loading.

4.3. Materials and methods

4.3.1. Experimental setup

The experiment took place at the Henfaes Agricultural Research Station, Abergwyngregyn, North Wales (53°14′N, 4°01′W). The soil is classified as a sandy clay loam textured Eutric Cambisol, overlying a glacial till, with a temperate-oceanic climate. The mean annual rainfall is 1060 mm and the mean annual temperature is 10°C. The site has no previous history of plastic pollution or application over the last 50 years (Zang et al., 2020). On 18th April 2019, a randomised plot design was established to create 4 independent replicates ($n = 4$) of each treatment. Each plot (1.4×2.85 m) was then treated with LDPE microplastic powder (RXP1003 natural; Resinex Ltd., High Wycombe, UK), at a rate of 0, 100, 1000, or 10000 kg ha^{-1} by thorough manual mixing with the top 10 cm of soil. This equated to loading rates of 0%, ~0.1%, ~1%, and ~10% (w/w) (soil bulk density = 1040 kg m^{-3}; $n = 4$). These rates were chosen to represent 'existing', 'normal', 'future', and 'extreme' (or 'hotspot') MP loading to soil (Gao et al., 2019; Huang et al., 2020; Qi et al., 2020). The microplastic powder was confirmed to have a very low level of contamination through total carbon (C) and nitrogen (N) analysis using a TruSpec$^{®}$ Analyzer (Leco Corp., Michigan, USA) (Total C, 82.88% ± 0.03%; Total N, 0.03 ± 0.01%; $n = 5$). LDPE was chosen due to its extensive use in agricultural films (Espí et al., 2006; Rong et al., 2021). Plots were subsequently sown with spring wheat (*Triticum aestivum* L., cv. Mulika) at a rate of 400 plants m^{-2}. In line with the fertiliser recommendations for wheat and taking account of the soil's Soil Nitrogen Supply (SNS) (AHDB, 2018), 120 kg N ha^{-1} yr^{-1} was applied to the field as NH_4NO_3 over two applications, 40 kg N ha^{-1} on 3rd June and 80 kg N ha^{-1} on 3rd July (reflecting the late sowing of the crop). For scanning electron microscopy (SEM), LDPE powder was mounted on adhesive tape, coated with gold, and imaged at 10 kV (Tescan Vega3 SEM). These SEM images illustrate the heterogeneous nature of the MP mixture, both in terms of particle size and surface texture (Fig. 4.1).
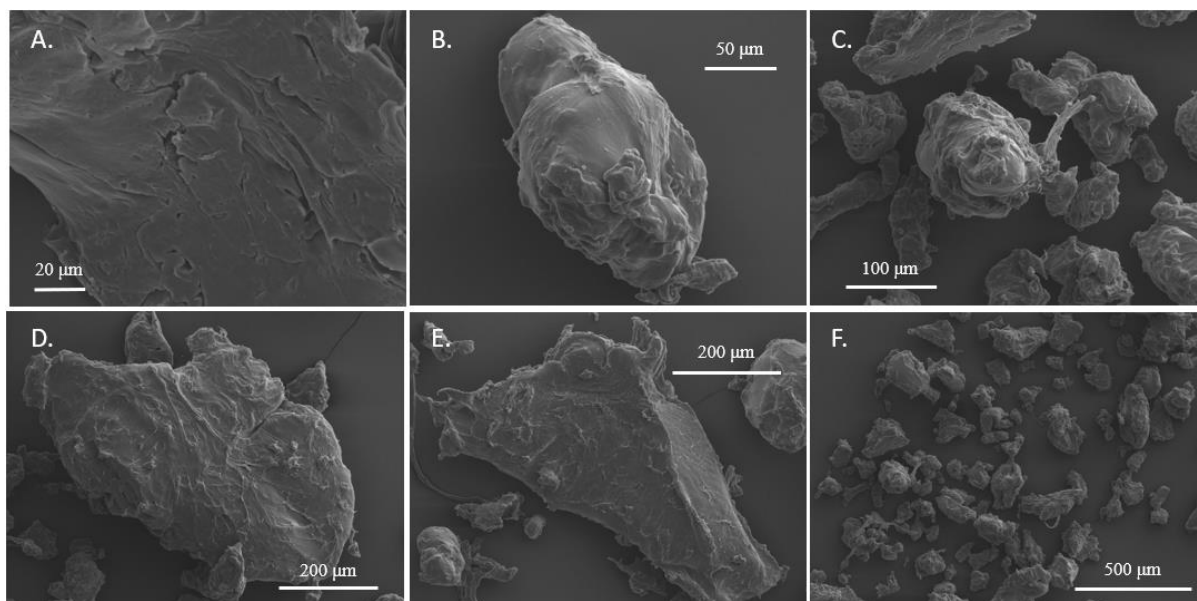
Figure 4.1. *Scanning electron micrographs of microplastic particles before incorporation into the soil. The images were taken across a range of magnifications (A – 20 μm; B – 50 μm; C – 100 μm; D – 200 μm; E – 200 μm; F – 500 μm). Images illustrate the heterogeneous nature of particle size and surface texture within the powder sample.*

4.3.2. Soil sampling and analysis

The soil was sampled one, two, and six months following MP addition. On each sampling occasion, multiple fresh soil cores per plot (n = 12; ø = 1 cm; depth = 0 – 10 cm) were randomly sampled and homogenised by hand to obtain a representative plot soil sample. Soil pH and electrical conductivity (EC) were measured on 1:2.5 (w/v) soil-to-distilled water suspensions by submerging standard electrodes. Within 24 h of soil collection, 1:5 (w/v) soil-to-0.5 M K_2SO_4 extracts were performed. The colorimetric methods of Miranda et al. (2001) and Mulvaney (1996) were used to determine the nitrate (NO_3-N) and ammonium (NH_4-N) concentrations in the K_2SO_4 extracts, respectively. Bulk density cores (0 – 5 cm, 100 cm^3) were also collected oven-dried (105°C, 24 h) before being weighed. Soil characteristics are summarised in Table 4.1. Climatic data from an adjacent weather station for the sampling period and a timeline of sampling are summarised in Appendix 2 Fig. 1.

Table 4.1. *Influence of microplastic (MP) dose and time since application on soil properties. The soil was sampled one, two or six months post microplastic application. Results are expressed on mean dry soil weight basis ± SEM (n = 4). Letters denote significant differences between treatments (p < 0.05). EC – Electrical conductivity.*

MP loading rate (kg ha^{-1})	1 month post-MP application				2 months post MP application			
	0	100	1000	10000	0	100	1000	10000
pH	6.26± 0.04[a]	6.23 ± 0.19[a]	6.26 ± 0.14[a]	6.23 ± 0.10[a]	6.49 ± 0.04[a]	6.34 ± 0.15[a]	6.41 ± 0.12[a]	6.47 ± 0.08[a]
EC (µS cm^{-1})	129 ± 38[a]	91 ± 13[a]	123 ± 24[a]	96 ± 22[a]	37 ± 1.9[a]	36 ± 2.6[a]	31 ± 2.3[a]	31 ± 3.5[a]
NO$_3^-$ (mg N kg^{-1})	67.4 ± 21.7[a]	18.6 ± 4.6[a]	33.4 ± 14.5[a]	38.3 ± 0.70[a]	5.04 ± 2.60[a]	4.96 ± 3.02[a]	1.86 ± 0.09[a]	1.61 ± 0.14[a]
NH$_4^+$ (mg N kg^{-1})	57.5 ± 16.7[a]	11.0 ± 5[a]	22.1± 10.9[a]	45.8 ± 1.6[a]	1.01 ± 0.06[a]	1.11 ± 0.11[a]	1.13 ± 0.05[a]	0.89 ± 0.06[a]
Bulk density (kg m^{-3})					1014 ± 11[a]	1065 ± 27[a]	984 ± 30[a]	977 ± 31[a]
Bacterial/Fungal PLFA ratio					0.11 ± 0.01[ab]	0.11 ± 0.01[ab]	0.11 ± 0.01[ab]	0.14 ± 0.02[a]
Microbial PLFA biomass (µmol PLFA kg^{-1})					174 ± 11[ab]	175 ± 9[ab]	162 ± 3[a]	190 ± 16[ab]

	6 months post MP application			
pH	6.27 ± 0.11[a]	6.16 ± 0.26[a]	6.14 ± 0.11[a]	6.09 ± 0.08[a]
EC (µS cm^{-1})	55 ± 2.4[a]	77 ± 25[a]	55 ± 3.9[a]	51 ± 2.6[a]
NO$_3^-$ (mg N kg^{-1})	10.4 ± 4.30[a]	21.9 ± 9.32[a]	15.5 ± 4.1[a]	10.2 ± 1.08[a]
NH$_4^+$ (mg N kg^{-1})	2.64 ± 0.30[a]	5.36 ± 2.09[a]	3.28 ± 0.88[a]	3.00 ± 1.05[a]
Bulk density (kg m^{-3})	1065 ± 22[a]	1106 ± 48[a]	1092 ± 44[a]	1062 ± 61[a]
Bacterial/Fungal PLFA ratio	0.09 ± 0.00[b]	0.10 ± 0.00[ab]	0.11 ± 0.01[ab]	0.10 ± 0.01[ab]
Microbial PLFA biomass (µmol PLFA kg^{-1})	199 ± 6[ab]	201 ± 8[ab]	197 ± 6[ab]	218 ± 12[b]
Earthworm biomass (g m^{-2})	92 ± 9[a]	54 ± 6[a]	71 ± 24[a]	79 ± 22[a]
Earthworm abundance (individuals m^{-2})	26 ± 5[a]	13 ± 2[a]	24 ± 13[a]	20 ± 6[a]

4.3.3. Phospholipid fatty acid (PLFA) profiling of the microbial community

Soil sampling for PLFA analysis was performed after 2 and 6 months of MP addition. Fresh homogenised soil samples, collected as described in section 4.2.2, were subsampled for PLFA analysis. The subsampled soil was subsequently stored at -80°C to prevent lipid turnover. Lyophilisation was performed using a Modulyo Freeze Dryer (Thermo Electron Corporation, Waltham, MA, USA) attached to a rotary vane pump (Edwards Ltd., Crawley, UK). Samples were shipped on dry ice (-78.5°C) to Microbial ID Inc. (Newark, DE, USA) for analysis. The method of Buyer and Sasser (2012) was used for extraction, fractionation and transesterification of samples. Analysis was performed on a 6890 gas chromatograph (GC) (Agilent Technologies, Wilmington, DE, USA) equipped with an autosampler, split–splitless inlet, and flame ionization detector. The system was controlled with MIS Sherlock® (MIDI, Inc., Newark, DE, USA) and Agilent ChemStation software. GC-FID specification, analysis parameters and standards are as described in Buyer and Sasser (2012).

4.3.4. Biogenic amine extraction and analysis

Biogenic amine extraction was performed 6 months after microplastic addition. They are a subset of the metabolome, key in the processing and cycling of N, which has previously been shown to be sensitive to changes in biological quality (Brown et al., 2021; Withers et al., 2020). On this sampling occasion, additional multiple soil cores ($n = 5$; ø = 1 cm; depth = $0 - 10$ cm) were taken across each plot and homogenised by hand to obtain a representative soil sample. After collection, samples were transferred (< 1 h) to a -80°C freezer to quench metabolic amine turnover. Samples were stored and lyophilised as described in section 4.2.3. Post-lyophilisation, roots and other debris (e.g. plant litter) were removed and the samples were then ground using a stainless-steel ball mill (MM200, Retsch GmbH, Haan, Germany), to aid in the recovery of biogenic amines. The mill was sterilised between samples by rinsing with deionised water followed by a 70% ethanol solution. Ground soil was transferred to sterile polypropylene 1.5 ml microfuge tubes and shipped, on dry ice (-78.5°C), to the West Coast Metabolomics Center (UC Davis Genome Center, Davis, CA, USA) for untargeted biogenic amine analysis using hydrophilic interaction chromatography electrospray quadrupole time of flight tandem mass spectrometry (HILIC-ESI QTOF MS/MS).

Briefly, extraction consisted of vortexing (~15 s) a 0.4:1 (w/v) soil-to-3:3:2 (v/v/v) MeCN/IPA/H$_2$O solution, before shaking for 5 min at 4°C, centrifuging (2 min, 14000 g) and recovering an aliquot of the supernatant for analysis. LC/QTOFMS analysis of extracted aliquots was performed on an Agilent 1290 Infinity LC system (G4220A binary pump, G4226A autosampler, and G1316C Column Thermostat) coupled to a SCIEX Triple TOF mass spectrometer, total runtime was 16.8 min. Polar compounds are separated on an Acquity UPLC BEH Amide Column, 13 nm (pore size), 1.7 µm (particle size), 2.1 mm × 150 mm maintained at 45°C at a flowrate of 0.4 ml min^{-1}. Solvent pre-heating (Agilent G1316) was used. The mobile phases consist of: (A) Water, 10 mM ammonium formate, 0.125% formic acid and (b) acetonitrile: water (95/5, v/v), 10 mM ammonium formate, 0.125% formic acid. The gradient was: 0 min 100% (B), 0-2 min 100% (B), 2-7 min 70% (B), 7.7-9 min 40% (B), 9.5-10.25 min 30% (B), 10.25-12.75 min 100% (B), 16.75 min 100% (B).

A sample volume of 1 µl for positive mode and 3 µl for negative mode was used for the injection. Sample temperature was maintained at 4°C in the autosampler. The mass spectrometer was operated with gas temperatures set to 300°C and pressure to 345 kPa (curtain gas (CUR) – 2.4 bar; IonSpray Voltage Floating (ISFV) – 4500 V; declustering potential (DP) – 10 V; capillary electrophoresis (CE) – 100V). Electrospray ionization (ESI) performed full scans in the mass range m/z 50–1200. The number of cycles in MS1 was 1667 with a cycle time of 500 ms and an accumulation time of 475 ms. Data were collected in both positive and negative ion mode and analysed using MS DIAL, open software for metabolome analysis, as described in Tsugawa et al. (2015). Final curated results were reported as peak heights, internal standards were included for quality control and peak correction purposes only. Therefore, data presented are qualitative and compounds are tentatively identified, as is routine for untargeted analysis (Gertsman and Barshop, 2018). A full compound list is presented in supplementary information with standardised reference nomenclature being generated using RefMet (Fahy and Subramaniam, 2020).

4.3.5 Soil N$_2$O flux

A mobile, automated GHG monitoring system, utilising a GC-Electron Capture Detector (8610C, SRI Instruments, CA, USA), as previously described in Marsden et al., (2018), was used to monitor nitrous oxide (N$_2$O) fluxes from three of the four replicates for each treatment. Stainless steel chamber bases (50 × 50 cm; 0.25 m^2) were installed into plots

two weeks before MP application, to which chambers (0.0625 m^3) were tightly secured. A foam strip on the base of each chamber ensured a tight seal. Briefly, the automated sampling system provided eight greenhouse gas flux measurements per 24 h period, per chamber during uninterrupted measurement. Emissions were monitored for 6 months from installation. However, this manuscript focuses on the 2-week periods following initial MP loading, to test whether the background emissions from the soil were perturbed by MP incorporation and the two subsequent N fertiliser application events, respectively, as these periods were likely to produce the greatest fluxes (Bell et al., 2015; Cardenas et al., 2019).

4.3.6. High-throughput sequencing and quantitative PCR analysis

4.3.6.1. 16S rRNA gene sequencing

Soil samples for 16S rRNA gene sequencing were collected after 6 months of MP incorporation. Five soil cores ($n = 5$; $\phi = 1$ cm; depth $= 0 – 10$ cm) were taken from each plot and homogenised by hand to obtain a representative sample. After collection, samples were passed through a 2 mm sieve and subsequently transferred (< 1 h) to a -80°C freezer for pre-extraction storage. Genomic DNA was extracted by mechanical lysis from 0.25 g soil per sample using a DNA Soil Fecal/Soil Microbiome Kit (ZymoResearch, CA, USA). Quality and concentration of extracted DNA were assessed by agarose gel electrophoresis (AGE) using a Qubit 4.0 Fluorometer dsDNA BR Assay Kit (Life Technologies, United States). Libraries of 16S rRNA gene amplicons were created using primers for rRNA marker genes, specifically for the V4 region of the 16S rDNA targeting bacteria and archaea (515F/806R), as previously described in Fadrosh et al. (2014). PCR was performed using a ViiA7 qPCR system (Applied Biosystems, MA USA). Thermocycling conditions were: initial denaturation at 95°C for 3 min, followed by 25 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s with a final elongation at 72°C for 5 min. Purified amplicons were then quantified using the aforementioned Qubit 4.0 Fluorometer, pooled in equimolar amounts and the final pool was run on the Illumina MiSeq platform (Illumina Inc., CA).

4.3.6.2. Bioinformatic analysis

The previously described protocols of Fadrosh et al. (2014) and Distaso et al. (2020) were used to process raw sequencing reads. Briefly, data pre-processing extracted the barcodes from sequences, and subsequently cleaned primer sequences using tagcleaner. Barcodes and sequences were then re-matched using in-house python scripts and the resulting filtered reads analysed using QIIME v1.3.1. Firstly, the libraries were demultiplexed based on the different barcodes. Then, the sequences were classified into operational taxonomic units (OTUs) combining *de novo* and reference-based methods (open-reference OTU generation algorithm) using the SILVA reference database version 132 (Yilmaz et al., 2014).

4.3.6.3. Quantitative PCR of N cycling functional genes

Samples for quantitative PCR (qPCR) of N cycling functional genes were collected on the 3rd July (pre-N fertiliser application) and on the 15th July (12 days post-N fertiliser application). On each occasion five soil cores ($n = 5$; ø $= 1$ cm; depth $= 0 - 10$ cm) were taken per plot and homogenised by hand to obtain a representative sample. After collection, samples were passed through a 2 mm sieve and subsequently transferred (<1 h) to a -80°C freezer for pre-extraction storage. Samples were extracted for NO_3-N and NH_4-N, as described in section 4.2.2. DNA was extracted by mechanical lysis from 0.25 g soil per sample using a DNEASY Powersoil kit (Qiagen, Hilden, Germany). The quality and concentration of extracted DNA were assessed by AGE.

To obtain the standard curves for qPCR assays, functional genes (urease (*ureC*)*,* archaeal ammonia oxidation (AOA-*amoA*), bacterial ammonia oxidation (AOB-*amoA*), complete nitrification (*comammox*), nitrite reductase (*nirK*; *nirS*), nitrous oxide reductase (*nosZ*) and nitrogenase iron protein (*nifH*)) were amplified using the primers listed in Appendix 2 Table 1. The concentration of plasmid was determined on a Qubit 4.0 Fluorometer dsDNA BR Assay Kit (Life Technologies, United States), and used for the calculation of standard copy numbers. qPCR was performed using a QuantStudio 7 System (Applied Biosystems, Waltham, United States). The thermocycling conditions are for each gene are summarised in Appendix 2 Table 1. For each gene, a high amplification efficiency of 92 – 105% was obtained, the R^2 values were > 0.99 and no signal was observed in the negative controls. The copy numbers for each sample of soil DNA were calculated based on comparison with the standard curve.

qPCR was performed using a QuantStudio 7 System (Applied Biosystems, Waltham, United States). Results were subsequently normalised by the extracted DNA concentration for each sample to account for differences in microbial biomass within samples.

4.3.7. Earthworm abundance and biomass

Earthworm abundance and weight were assessed after 6 months. Briefly, a 0.018 m^3 ($0.3 \times 0.3 \times 0.2$ m) pit was dug in a randomly selected location in each experimental plot. Soil from the pit was placed into a tray and thoroughly manually sorted, and earthworms collected. All earthworms were counted (abundance) and weighed (biomass). Abundance is expressed as individuals m^{-2} and biomass as fresh weight biomass m^{-2}.

4.3.8. Wheat harvest data

Spring wheat was harvested at full maturity, 5 months after sowing. The harvest protocol consisted of hand cutting, with shears, 1×2.85 m strip, through the centre of each experimental plot, to remove edge effects. Samples were then dried (85°C, 48 h). For each harvested sample, ears were removed from stems and each were weighed. Ear and stem weight were subsequently added to calculate a total wheat biomass dry weight per plot or biomass yield. Biomass yield was used as it is highly related to grain yield and gives an overall indicator of plant health (Damisch and Wiberg, 1991). After drying, harvested wheat seeds were separated, weighed and ground, and subsequently analysed for total C and N using a TruSpec® Analyser (Leco Corp., St. Joseph, MI, USA) and a C:N ratio calculated.

4.3.9. Statistical analysis

All statistical analysis was run using R v 4.0.3 (R Core Team, 2021) unless otherwise stated. With all graphical analysis being constructed in '*ggplot2*' (Wickham, 2016) unless otherwise stated. A significance level of $p < 0.05$ was used for all analyses.

Normality and homogeneity of variance of the chemical and physical soil properties of the treated Eutric Cambisol were assessed using Shapiro-Wilk's test and Levene's test, respectively. For data that did not conform to parametric assumptions even after using log$_{10}$

transformation (NO$_3$-N, NH$_4$-N, EC and PLFA Fungal:Bacterial ratio) a Kruskal-Wallis test (stats package; R Core Team, 2021) was used to assess the similarities between MP treatments and sampling dates, otherwise a one-way ANOVA (Analysis of variance) was used (for pH, bulk density and total PLFA biomass). The results for this are summarised in Table 4.1. A one-way ANOVA was also used to assess treatment variations in wheat biomass data (total aboveground biomass, stem and leaf biomass, ear biomass and harvested wheat seed C:N ratio) and earthworm data (abundance and biomass).

The '*vegan*' (Oksanen et al., 2020) and '*ggplot2*' (Wickham, 2016) packages were used to construct NMDS (Non-metric multidimensional scaling) analysis of the PLFA community based on Bray–Curtis dissimilarities. All PLFAs detected were used in the analysis, to represent the whole microbial community. This was followed by computation of an ANOSIM (Analysis of similarities) to identify differences in dispersion between centroids of groups as determined by MP loading rate, or time of sampling. Fungal-bacterial ratios and Gram positive to Gram negative ratios were calculated by summing the FA biomarkers for the respective groups (summarised in Appendix 2 Table 2). Total biomass was calculated by summing the concentration of PLFAs recovered.

Fluxes of N$_2$O for each chamber were calculated using the methods described in Scheer et al., (2014). The linear slope of N$_2$O concentrations over time included either three or four data points. N$_2$O fluxes for each two-week period (post-MP and fertiliser application, respectively) were graphically analysed. Trapezoidal integration was used to calculate cumulative N$_2$O emissions for each treatment, these were tested for significance using for Kruskal-Wallis tests, after failing parametric assumptions.

Bacterial observed OTU richness was tested for significant differences using ANOVA. The evenness of the 16S community was also calculated using Pielou's evenness (Jost, 2010) and tested for significant differences using ANOVA. NDMS, followed by an ANOSIM (Analysis of similarities) was used to test statistically whether there was a significant difference between groups of sampling units between treatments (β-diversity).

N cycling gene abundance, before and after a N fertilisation event was analysed using mixed effect models with the '*lme4*' package (Bates et al., 2015). We considered MP loading rate and sampling time and their interaction as fixed effects and individual plots as temporal random effects. For each variable, residuals from each model were tested for normality, autocorrelation and heteroscedasticity using graphical tools. For all genes, a log$_{10}$ conversion

was found to improve the fitness of all models. An ANOVA was then run on each model to test treatment effects, significant results were further explored using a Tukey adjusted post-hoc test using the '*emmeans*' package (Lenth, 2021). Pre- and post- fertilisation soil NO_3-N and NH_4-N concentrations were analysed by ANOVA.

MetaboAnalyst v5.0 (Chong et al., 2018; Pang et al., 2020) was used for the analysis of biogenic amine data. First, normalisation was performed using generalised logarithm transformation (glog) and Pareto scaling. Normalised data was subsequently used for heatmap creation (using Euclidean distance and Ward clustering algorithms). One-way ANOVA was also performed to identify significant differences in compound concentrations between treatments.

4.4. Results

4.4.1. 16S bacterial community

In total, 7179 bacterial operational taxonomic units (OTUs) were identified across all 16S rRNA gene reads. There was little variation in the proportional abundance of OTUs between the different MP treatments with Proteobacteria (Gram-negative) and Actinobacteria (Gram-positive) being the most abundant phyla (Fig. 4.2A). There were no significant differences between bacterial OTU richness ($F_{(3,12)} = 0.32$, $p > 0.8$) (Fig. 4.2B) or evenness ($F_{(3,12)} = 1.74$, $p > 0.2$) (Fig. 4.2C) across the different treatments, as tested by ANOVA. Equally, the NMDS ordination shows no clear separation or divergence in soil bacterial communities between the MP treatments and the unamended control (Fig. 4.2D). Lastly, we found no significant differences in bacterial β-diversity between the treatments, as confirmed by ANOSIM analysis ($p > 0.8$).
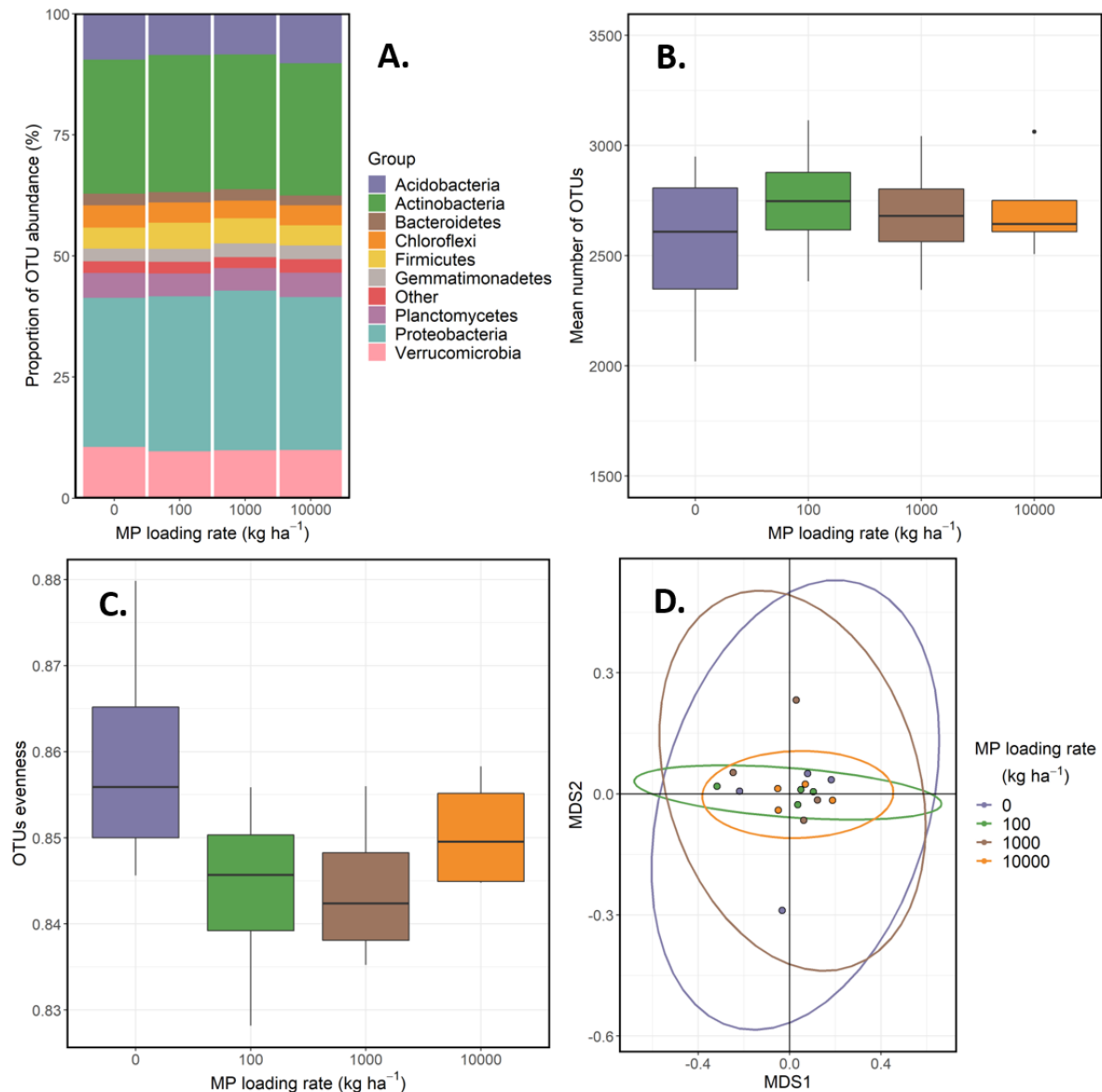
Figure 4.2 16S sequencing bacterial community in response to different microplastic doses (n = 4). A) Proportionate abundances of major phyla within each microplastic loading rate. B) Boxplot of observed bacterial OTU richness against microplastic loading rate (n = 4). C) Boxplot of bacterial OTU evenness against microplastic loading rate (n = 4). D) Non-metric multidimensional scaling (NMDS) ordination plot of bacterial OTU community composition across microplastic loading rates.

4.4.2. PLFA-derived community

The fungal-bacterial ratio of PLFAs remained similar across all treatments, there was a significant difference between the 2 months post-application 10000 kg ha^{-1} and the 6 months post-application 0 kg ha^{-1} MP loading rates, with the latter having a higher prevalence of bacteria (Table 4.1). Total PLFA biomass was also similar across all treatments, with a significant difference between the 2 months post-application 1000 kg ha^{-1} and the 6 months post-application 10000 kg ha^{-1} MP loading rates, the latter having a higher PLFA biomass yield. NMDS analysis was used to show the clustering of all soil-derived PLFA compounds, under MP treatments, 2 and 6 months after initial MP application (Fig. 4.3). Overall, the different MP treatments separated by sampling date, with a clear separation between the 2 and 6-month points. The PLFA derived community was also more closely grouped at the 6-month sampling point. Results of the PERMANOVA confirmed that there was no significant difference in group dispersion between MP loading treatments ($p > 0.2$). However, there was a significant difference in group dispersion between sampling times ($p < 0.001$). Additionally, there was no interaction effect between MP loading and sampling time ($p > 0.9$).
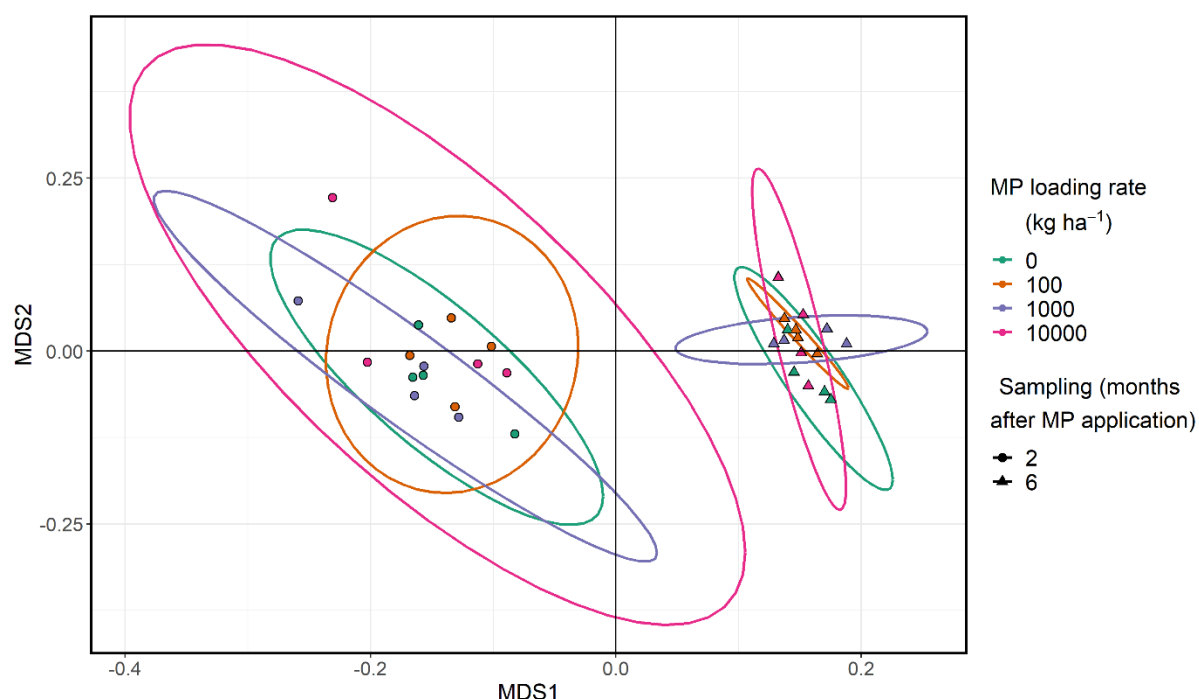
Figure 4.3. *NMDS plot of the PLFA profile for each microplastic soil treatment. Ellipses represent 95% confidence intervals for each treatment.*

4.4.3. N cycling genes

The presence and abundances of eight genes involved in the N cycle, specifically *ureC*, *amoA* (AOA, AOB, and comammox), *nirK*, *nirS*, *nosZ* and *nifH*, (functions are summarized in Appendix 2 Fig. 2), were assayed by qPCR before and after an N fertilisation event. We acknowledge that the primers used to amplify the functional genes (e.g. *ureC*) do not target all of the community. In most cases, gene abundance was not greatly affected by either MP loading rate or sampling time (i.e. pre- and post-N fertilisation) (Fig. 4.4, Appendix 2 Table 3). However, ANOVA showed that there were significant differences for *nirK* ($F_{(3,12)} = 4.6$, $p < 0.05$) and *nosZ* ($F_{(3,24)} = 3.2$, $p < 0.05$) abundance, respectively, by MP loading. For both *nirk* and *nosZ* gene abundance, LMS post-hoc analysis showed a significant difference between 100 kg ha^{-1} and 1000 kg ha^{-1} MP loading ($p < 0.05$). For AOB, ANOVA also showed a significant interaction effect between MP loading rate and sampling time ($F_{(3,24)} = 3.5$, $p < 0.05$). LMS post-hoc analysis showed that there were significant differences between 0 kg ha^{-1} and 1000 kg ha^{-1} MP loading, pre-fertilisation ($p < 0.05$) and between 0 kg ha^{-1} MP loading, pre-fertilisation, and 10000 kg ha^{-1} MP loading post-fertilisation ($p < 0.05$). Concentrations of soil NO_3-N ($F_{(1,12)} = 16.6$, $p < 0.01$) and NH_4-N ($F_{(1,12)} = 22.0$, $p < 0.01$) were significantly higher post-fertilisation (Fig. 4.4 E, F).
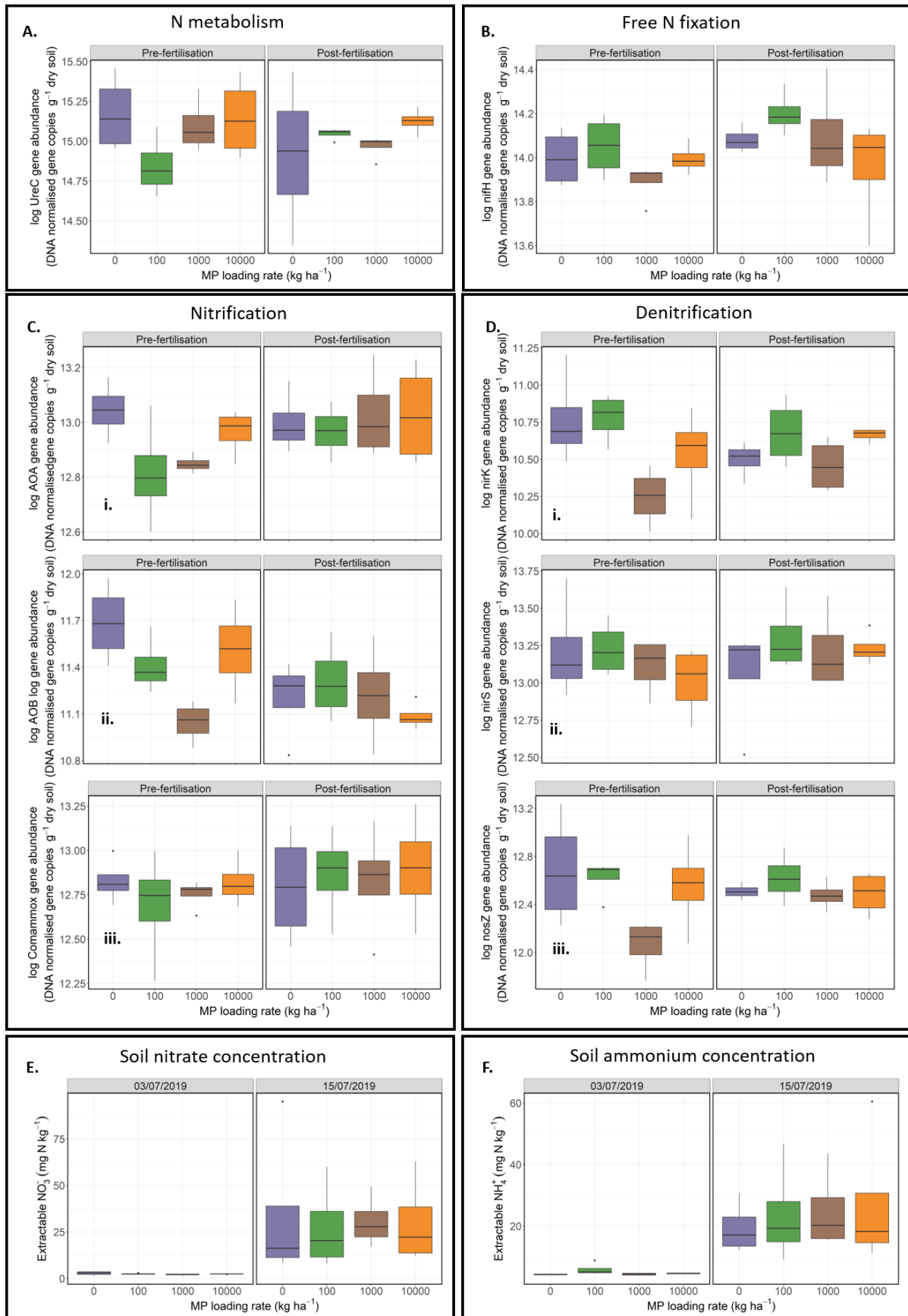
***Figure 4.4.** N cycling gene soil abundances pre- and post-N fertiliser application (n = 4). A) Urease-associated gene UreC, B) Free N fixation associated gene nifH, C) Nitrification-associated genes, the amoA gene of; i) AOA, ii) AOB, iii) comammox, D) Denitrification-associated genes; i) nirK, ii) nirS, iii) nosZ, E) Soil nitrate, F) Soil ammonium. All gene abundances were normalised by extracted DNA quantities to account for differences in microbial biomass and transformed by $\log_{10}$. Soil nitrate and ammonium are reported by dry soil weight (n = 4).*

4.4.4 N₂O flux

Kruskal-Wallis analysis showed that there were no significant differences between cumulative N₂O fluxes for the 2 week period following initial MP application ($H_{(3)} = 0.74$, $p = 0.9$), or the first ($H_{(3)} = 4.6$, $p = 0.2$) and second fertiliser ($H_{(3)} = 3.6$, $p = 0.3$) application events. Fluxes over each period are summarised in Fig. 4.5.
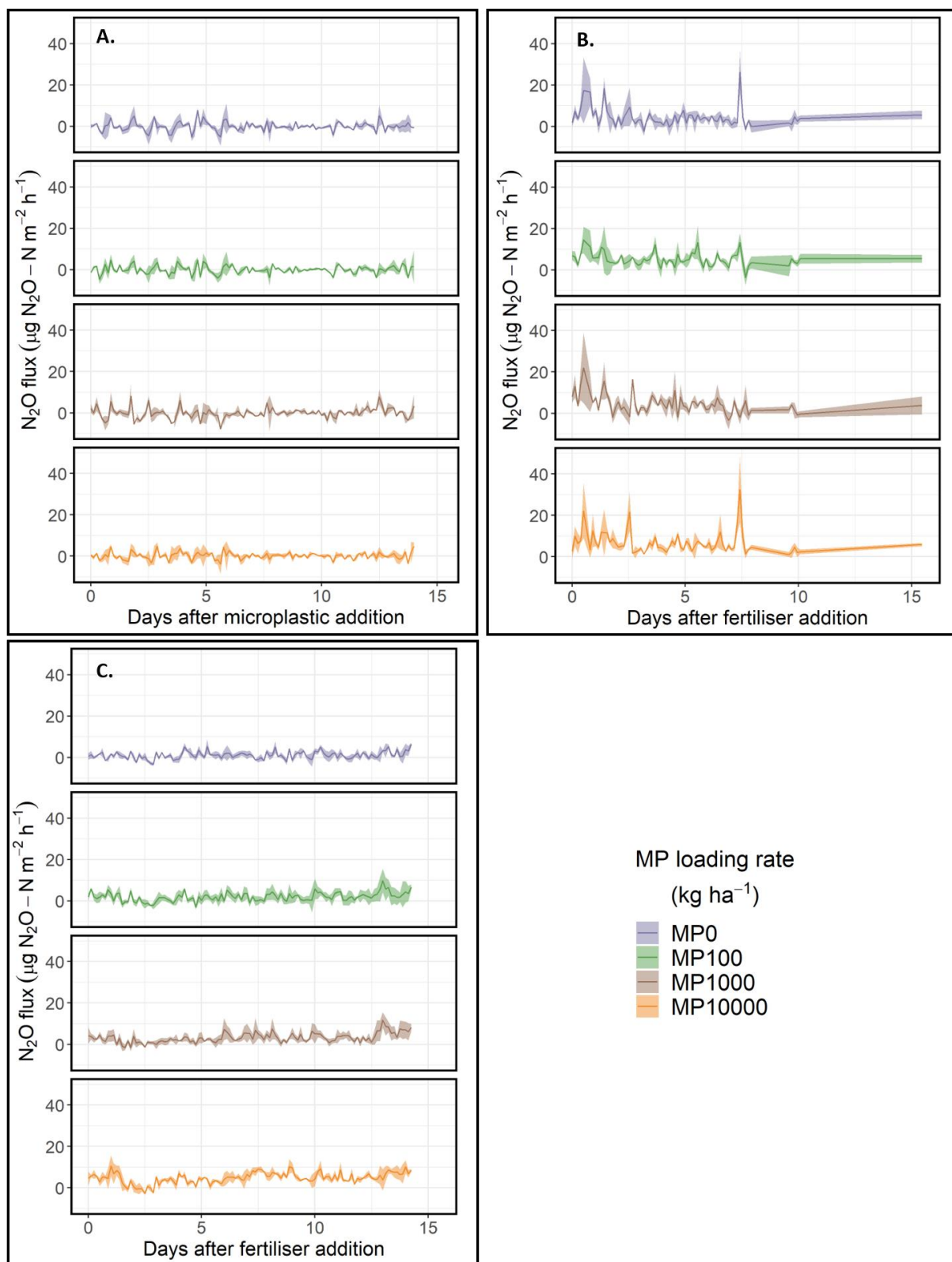
Figure 4.5. *N₂O fluxes from soil upon; A) initial MP loading, B) N fertilisation event one (40 kg N ha⁻¹ equivalent), C) N fertilisation event two (80 kg N ha⁻¹), by MP loading treatment. In each panel, the line represents the mean flux (n = 3) and the shaded area represents the upper and lower bounds of the SEM.*

4.4.5. Biogenic amines

Untargeted biogenic amine analysis identified a total of 112 tentatively identified compounds. Of these known compounds detected, none showed statistically significant differences between treatments. There were no clear grouping or responses within the biogenic amine data (Fig. 4.6). The samples were characterised by a wide range of compounds (Appendix 2 Fig. 3) but predominated by amino acids and peptides.
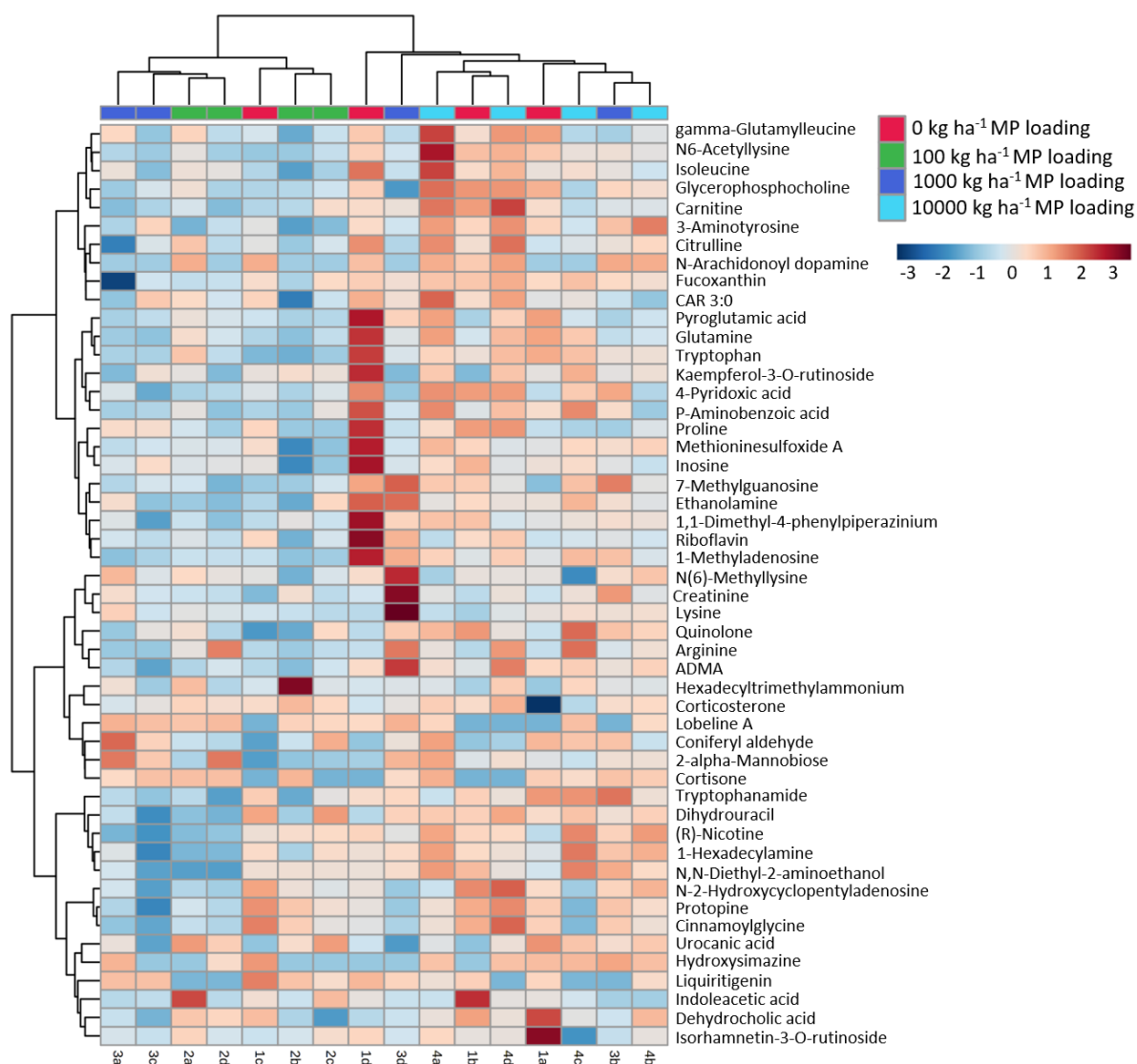
Figure 4.6. *Influence of microplastic application rate on the biogenic amine (BA) concentration in soil. Heatmap showing expression profiles of soil treatments based on the top 50 most significant known BAs identified by ANOVA (p < 0.03). BAs are clustered using Euclidean distance and Ward linkage. Data was normalised using a log$_{10}$ transformation and Pareto scaling. The colour of samples ranges from red to blue, indicating metabolite*

concentration z-score; numbers 3 to -3 on the scale bar indicate the number of standard deviations from the mean.

4.4.6. Soil properties including inorganic N

Overall, there were no significant differences in soil chemical properties (pH, EC, NO_3-N and NH_4-N) associated with the MP treatment as tested by ANOVA or Kruskal Wallis ($p > 0.1$). Trends in the data show some natural variation in all soil properties measured throughout the season (summarised in Table 4.1).

4.4.7. Earthworms abundance and biomass

Earthworm abundance and biomass was not significantly affected by MP loading. All earthworms identified in the samples were endogenic. Overall, there were no significant differences between total earthworm biomass ($F_{(3,12)} = 0.63$, $p > 0.6$) or earthworm abundance ($F_{(3,12)} = 0.85$, $p > 0.4$; Table 4.1).

4.4.8. Plant biomass

Plant biomass was not significantly affected by MP loading, however, yields of this field trail were lower than the typical wheat yields for the year (DEFRA, 2019). There were no significant differences between total above ground plant biomass ($F_{(3,12)} = 0.09$, $p > 0.9$), stem and leaf biomass ($F_{(3,12)} = 0.08$, $p > 0.9$), ear biomass ($F_{(3,12)} = 0.09$, $p > 0.9$), or harvested seed C:N ratio ($F_{(3,11)} = 0.03$, $p > 0.9$; Fig. 4.7).
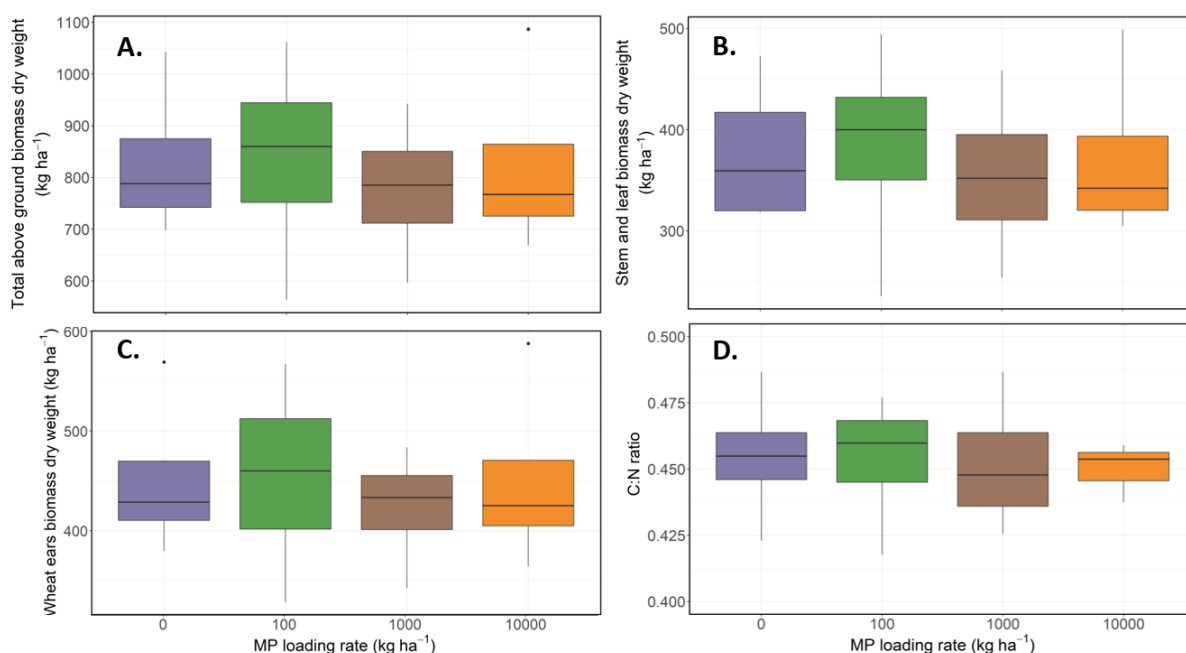
Figure 4.7. *Effect of microplastic application rate on above-ground wheat biomass (n = 4). A) Total above-ground biomass, B) Stem and leaf biomass, C) Ear biomass and D) Seed C:N ratio.*

4.5. Discussion

4.5.1. 16S bacterial community response to MP addition

Soil microorganisms are vital to soil functioning and are considered the most sensitive indicator of soil quality, due to their ability to rapidly respond to changing environmental conditions (Bünemann et al., 2018; Lau and Lennon, 2012; Schimel, 2018). Therefore, despite a significant amount of functional redundancy (Jia and Whalen, 2020), substantial shifts in the microbial community are likely to represent a change in soil function (Lehman et al., 2015). This study showed that after 6 months of pure microplastic addition to previously uncontaminated soil, there was no significant change in the proportional abundance of the bacterial community (Fig 4.2A), bacterial richness (Fig 4.2B), evenness, or bacterial community compositional divergence (β-diversity) (Fig 4.2C). To contextualise this, a previous study at the same site, showed significant changes in the microbial community under biochar application over similar time scales (Jones et al., 2012).

Currently, the effect of MPs loading on soil microorganisms is unclear. Our findings are contradictory to several studies, which observed significant effects of microplastic (e.g.

LDPE; Huang et al., 2019), polyvinyl chloride (PVC; Yan et al., 2020), and combined PE and PVC (Fei et al., 2020; Seeley et al., 2020)) addition on the soil bacterial community, particularly richness, evenness, and diversity. However, H. Chen et al. (2020) and Judy et al. (2019) showed various microplastic additions had no significant effects on the microbial community over short time periods (70 d and 9 months, respectively). Additionally, Ren et al. (2020) reported mixed but largely positive effects of MP on the microbial community (increase in richness and diversity) in a fertilised soil over a 30 d period, although the microorganisms may have reacted to the fertiliser addition and not the MPs. Based on these studies it is evident that the type of plastic incorporated into the soil will dictate the biological and ecological effects exhibited. Therefore, further studies of the effects of different types of plastic, and combinations of plastics are required to fully understand any impact on soil health.

4.5.2. Effect of MP loading on soil PLFAs

PLFAs give a representation of the living soil microbial biomass and provide a snapshot of soil community structure and abundance at the time of sampling. NMDS clustering of PLFA microbial community shows a large amount of overlap between MP loading rates implying community structure had not changed significantly (Fig. 4.3). This is contrary to previous microcosm studies that have shown significant shifts in PLFA derived microbial community even under relatively low levels of MP loading (Zang et al., 2020). MPs are a recalcitrant C pool and are only likely to become bioavailable as a viable C source over long time periods (years to decades) with the aid of natural abiotic degradation (hydrolysis, photo-oxidation or thermal oxidation) (Ángeles-López et al., 2017; Chamas et al., 2020) and to a lesser extent biological degradation (e.g. earthworms) (Huerta Lwanga et al., 2016). This biochemical inertness in the short to medium term is unlikely to cause major shifts in microbial communities. In terms of soil physical properties, MPs have been suggested as a new and distinct microbial habitat, for example for biofilm colonisation and formation (McCormick et al., 2014; Zhang et al., 2019), potentially leading to a change in the microbial community. However, this was not observed in this study as there was no significant community divergence in MP treatments from control plots in either 16S bacterial community or PLFA derived microbial community. The SEM (Fig. 4.1) illustrates that the MP powder used here is not porous or cavity-containing and therefore may not offer an attractive habitat for microbial colonisation (Or et al., 2006). Additionally, we would dispute this theory, as studies with

biochar, a similarly recalcitrant C source, have shown that microbial colonisation is very sparse, concluding that even after several years biochar did not provide a substantial habitat for soil microbes (Quilliam et al., 2013). However, this requires confirmation with experimental evidence for MPs.

Separation between all MP loading treatments groups between the two sampling points (2 months and 6 months post MP addition) illustrated a distinct temporal shift in the structure of the microbial community. Seasonal, as well as cropping-associated, shifts in the PLFA composition in soil have been observed (Duncan et al., 2016; Ferrari et al., 2015; Moore-Kucera and Dick, 2008). These shifts are generally associated with membrane adaptation to changing environmental stress levels (for example, temperature, moisture or nutrient availability), resulting in physiological community change (Blagodatskaya and Kuzyakov, 2013; Bossio and Scow, 1998). It is likely the observed change in the soil PLFA community between sampling points may be due to natural seasonal changes (for example the difference in soil moisture, illustrated in Appendix 2 Fig. 1).

4.5.3. Effect of N cycling gene abundance pre- and post- N fertilisation

Within agroecosystems, N availability is often considered the predominant limiting factor in plant growth (Vitousek and Howarth, 1991) and the second most limiting factor after C in microbial growth (Kuypers et al., 2018; Buchkowski et al, 2015). Microbial uptake, assimilation, and cycling of mineral and organic N is key to soil function, and as such N cycling processes (mineralisation, nitrification, and denitrification) have been used as sensitive and ecologically relevant indicators of soil quality and ecological stability (Bünemann et al., 2018; Iqbal et al., 2020). Changes in the abundance of the key regulatory functional genes involved in these processes are likely to indicate changes in soil function. However, there is little evidence of how MPs could affect soil N cycling (Iqbal et al., 2020). Overall, this study showed little change in the abundance of N cycling functional genes between pre- and post- inorganic N addition under all MP loading rates. Genes that did differ significantly in abundances between treatments were denitrification associated (*nirK* and *nosZ*) and nitrification associated (AOB *amoA*). For both denitrification associated genes, lower abundances were displayed within the 1000 kg ha^{-1} treatment compared to the 100 kg ha^{-1} treatment (Fig. 4.4C), with no effects on abundances at either higher or lower MP loading rates. AOB *amoA* gene abundance was significantly lower than control levels in the 100 kg ha^{-1} treatment pre-fertilisation and

10000 kg ha^{-1} treatment post-fertilisation. The general trend in N cycling gene abundances showed variability pre-fertilisation. Post-fertilisation this variability was reduced and gene abundances were more even across all MP loading treatments, while soil inorganic N was significantly increased post-fertilisation (Fig. 4.4).

N fertilisation has been shown to have a mixed effect on N cycling genes (Tosi et al., 2020). Effects are highly dependent on the nature of the N source applied (inorganic or organic), with inorganic sources of N having a much weaker effect than organic sources of N, as well as the fertilizer duration, crop rotation, and pH (Ouyang et al., 2018). The results of this study show that there were no large changes in soil N cycling functional genes in the presence of MP loading. Although there may have been several further factors influencing N gene abundance, for example when fertiliser was applied when the soil was very dry (Appendix 2 Fig. 1), preventing soil biology from accessing the additional N. Equally, as alluded to above, C is the primary limiting factor for soil microbiology, if the community was already C limited then it is unlikely that there would be significant growth or change stimulated by N addition. Studies have shown that MPs have the potential to affect N cycling processes, for example repression of key N cycling enzymes (e.g. leucine-aminopeptidase and N-acetyl-β-glucosaminidase (Awet et al., 2018; Bandopadhyay et al., 2020)) and N hydrolysis (Huang et al., 2019). However, N cycling is a key soil function, particularly in agricultural soil, and the longer-term impacts of MPs on should be explored in more detail.

4.5.4 Effect of MP loading on soil N$_2$O flux

N$_2$O is a potent greenhouse gas, with a global warming potential (GWP) 298 times larger than carbon dioxide (CO$_2$) and it is a stratospheric ozone-depleting substance (Stocker, 2014). In soil, it is primarily produced by the biological pathways of nitrification and denitrification. As such it can be used as a functional indicator of soil biological quality at an ecosystem processes scale (Bünemann et al., 2018). Therefore, understanding whether MP addition influences soil N$_2$O fluxes will be key to understanding their overall environmental impact. It has been shown that MPs may reduce soil N$_2$O emissions by inhibiting the microbial phyla associated with N cycling genes (Ren et al., 2020; Rillig et al., 2021), although results vary depending on the type of MP applied and environmental conditions (Shen et al., 2020; Sun et al., 2020).

While chambers in this study included plant and soil, the plant contribution of N_2O is minimal (Chang et al., 1998), therefore we focussed on the soil contribution. Here, N_2O flux from the soil after MP and fertiliser applications, respectively, were very low (Fig. 4.5). N_2O fluxes are commonly observed after fertiliser application (up to 250 μg N_2O-N $m^{-2} h^{-1}$; Carswell et al., 2018), however, we observed none. Equally, there were no differences between fluxes between MP loading levels (Appendix 2 Table 4). However, it is difficult to attribute this low flux directly to the microplastic application, particularly as control plots also exhibited small fluxes. Notably, much of the sampling period was dry (Appendix 2 Fig. 1), this is likely to have suppressed N_2O emission, as water filled pore space (WFPS) was too low to allow the development of the anaerobic 'hotspots' required for N_2O production (via denitrification) and emission (Barrat et al., 2020; Dobbie and Smith, 2001). We therefore recommend further field-based measurement of MPs effect on N_2O and other GHGs (particularly CO_2 and methane (CH_4)), under a range of climatic conditions and soil types.

4.5.5. Biogenic amines as effected by MP loading

BAs are low molecular weight organic bases synthesised by prokaryotes and eukaryotes in the soil, mainly through decarboxylation of amino acids or amination and transamination of aldehydes and ketones. In a food context, BAs are often seen as undesirable due to their potentially toxic properties (Mah et al., 2019), in this sense they are potential food quality indicators (Ruiz-Capillas and Herrero, 2019). However, there is also evidence that BAs have a role in quorum sensing in the gut between bacteria and host organisms (Hughes and Sperandio, 2008; Sudo, 2019).

There has been little exploration of BAs in the soil system specifically. But it is generally understood that increased N availability in the soil will increase the number of BAs synthesised both by soil biota and plants (Pérez-Álvarez et al., 2017). Equally, homospermidine biosynthesis has been proposed as a stress regulator in rhizobia (Fujihara, 2009). In this study, one of the first to profile the soil BAs, we found no significant change in the BA amine profile of soil applied with MPs compared to control values, 6 months after initial MP application (Fig 4.5, Appendix 2 Fig. 3). A large range of compounds were extracted, many of which have putative functions including 5′-methylthioadenosine, an inhibitory by-product of methionine metabolism, which can be processed to salvage biogenically available sulphur (North et al., 2017). As well as abscisic acid, a plant hormone that regulates many aspects of plant growth,

including development, maturation, and stress response (Nambara, 2016) and CcpA, which is a core transcriptional regulator in the control of catabolism in Gram-positive bacteria (Carvalho et al., 2011). However, due to the variability in response to MP loading and between replicates (Fig. 4.6), further research is required to understand the role BAs may play in both quorum sensing and stress regulation in the soil system, as well as their spatial homogeneity.

4.5.6. Effect of MP on earthworms

Earthworms are key representatives of soil fauna in relation to soil health, performing an important role in the formation and maintenance of soil fertility and structure, as well as being a major contributor to invertebrate biomass in soil (Blouin et al., 2013). Therefore, understanding the risks that MPs may pose to their health, abundance, and functioning within the agroecosystem is a priority. Earthworms have been shown to transport MPs throughout the soil profile either through adhesion to the exterior of the earthworm body (Rillig et al., 2017b) or egestion of smaller MP particles (Huerta Lwanga et al., 2016). Our study found that there were no significant differences in earthworm abundance or biomass after 6 months of MP incorporation into the soil (Table 4.1); however, we did not measure egestion or adhesion. This result is inconsistent with much of the existing literature on earthworm exposure to MPs in soil, with several studies reporting negative effects on earthworm physiology (e.g. skin damage, induction of oxidative stress, loss of body weight, reduction in growth, mortality), although experiments were all laboratory or mesocosm based, over short time periods (< 60 days) (Boots et al., 2019; Cao et al., 2017; Y. Chen et al., 2020; Huerta Lwanga et al., 2016; Judy et al., 2019; Rodríguez-Seijo et al., 2019). MP loading rates in the aforementioned experiments ranged from 0.01% to 2% (w/w). Here we added MPs at the rates of 0%, ~0.1%, ~1% and ~10% (w/w), while earthworm health was not directly measured, a lack of change in earthworm abundance or biomass suggests that earthworm health had not diminished significantly, even at high MP loading. By proxy, this also suggests that earthworms do not actively avoid areas of microplastic contamination in the field, as in this study there were no barriers to earthworms leaving the MP loaded plots.

With this, it must be noted that this study only incorporated MPs into the top 10 cm of soil, therefore exposure of earthworms to MPs will likely depend on their ecotype, with endogenic earthworms likely to have higher exposure rates than the deeper dwelling anecic earthworms. As MPs are moved through the soil profile over time it is likely that the full extent

of the impact on earthworms will be clearer. Equally, the longer-term (years to decades) impact of MPs is likely to be more severe than the short term. As MP particles degrade and fragment, they will become more ingestible to macrofauna and microfauna, although it is likely that the MP powder added in this study was already small enough to be digestible, possibly leading to greater mortality in soil-dwelling fauna (Lahive et al., 2019). Likewise, earthworms live several years, therefore it is likely that this study captures only a snapshot of the earthworm lifecycle. Longer term monitoring is required to establish trends in earthworm health.

4.5.7. Crop health as affected by MP loading

The ability to effectively grow healthy crop plants is a key ecosystem service provided by the soil in an agroecosystem context, underpinning human health and nutrition (Power, 2010). However, data on the effect of MP loading on crop yield and health is limited. MPs have the potential to affect plants in several ways; altering the soil structure, immobilising nutrients, contaminant transport, or adsorption and direct toxicity (Rillig et al., 2019). Several short-term laboratory studies have shown the negative effect of MPs on plant health and biomass (de Souza Machado et al., 2019; Qi et al., 2020; Zang et al., 2020). The results of this field study are contradictory to these studies, suggesting that MPs, even at extremely high loading rates, have no significant effects on the aboveground, ear biomass, or C:N ratio of the harvested seed of *T. aestivum* over one cropping season. However, the effect of MPs on root biomass and rooting structure was not measured in this study, though it is likely that the aboveground biomass would be affected if root growth characteristics were altered by MPs, as a high proportion of wheat roots are found within the top 10 cm of soil (Li et al., 2011).

4.5.8. Implications and future research direction

Most existing data on MPs is based on laboratory or mesocosm based experiments. While these data are useful, field studies better represent real-world conditions. Longer-term (years to decades) datasets are required to obtain a more comprehensive understanding of the effect of MPs on soil physiochemistry as well as soil biology and plant health. The study of extremely high MP loading rates may also be useful to understand future effects of MP on soil, if continuous loading occurs (e.g. repeated use of plastic mulch films). Generally, it is recommended that loading rates for future MP studies should reflect realistic loading rates in

soil to accurately reflect a perturbed system. Even in heavily mulched soil MP loading rarely exceeds 325 kg ha^{-1}, although this is likely to increase as MPs continue to be added to the soil (Huang et al., 2020); additionally, little data explicitly reporting loading rates is available, with many studies choosing to report as items kg^{-1} (Büks and Kaupenjohann, 2020).

It must also be noted that the potential negative impacts of (particularly conventional) MPs on soil and ecosystem health are likely to increase over time as their decomposition rates are extremely slow relative to the rate of entry to the system, leading to a progressive accumulation within soil (Rillig, 2012; Rillig et al., 2017a), potentially becoming persistent organic pollutants. Equally, while biodegradation is possible to a small extent, it is likely MPs relative recalcitrance means that microbes will prefer less energetically expensive C sources, and therefore, biological, co-metabolic, break-down of plastic is unlikely to occur to any great extent in field soils (Ng et al., 2018). That is what our data suggests, i.e. that if there are no additives, once a biofilm has formed on the outside, pure MPs are no different from an inert sand particle.

This study applied pure MP LDPE powder, with very low levels of contaminants and additives present. The chemical formulation of MP entering agricultural soils, however, is expected to vary widely due to their origin (e.g. mulch film, biosolids) giving rise to variable amounts of additives (co-pollutants) such as plasticisers (generally low-volatility, insoluble and chemically stable; Campanale et al., 2020), colourants and pigments (inorganic pigments containing heavy metals or organic pigments including various chromophoric families that are potentially carcinogenic and mutagenic; Gičević et al., 2020; Völz, 2009), ultraviolet (UV) stabilisers (inorganic or organic cadmium, barium, or lead salts; Stenmarck et al., 2017) or other polymers (Steinmetz et al., 2016). Generally, additives are not chemically bound to the plastic polymer and subsequent leaching of these additives may pose more of a hazard to soil ecology (particularly microorganisms) than the relatively recalcitrant MP themselves, particularly in the short term (days to years). The exchange and effects of additives or contaminants between plastic particles and the surrounding soil environment and the subsequent effect on soil function (e.g. enzyme inhibition) is a key area for future terrestrial plastics research.

It is also important to state that the majority of published literature on MPs does not state the purity of the plastics, MP used and the type (and concentration) of aforementioned additives incorporated. Reporting of this information is highly recommended in future

literature, due to the potential varying effects on the soil environment as well as toxicity to soil ecology, which may significantly affect the results, particularly of biological studies.

4.6. Conclusions

This study demonstrated that the application of pure LDPE MP powder to a field site with no previous history of plastic pollution or application had no significant effect on soil biological health or function over one growing season (6 months). In this regard, we reject hypotheses i, ii and iii, as there were no significant changes in biological quality, crop biomass, or yield with MP loading; equally no effect of loading rate was observed. In conclusion, MPs themselves may not pose a significant problem, at least in the short term (days to years) due to their recalcitrant nature. Further work should be undertaken focusing on the effect of additives and contaminants on soil function and plant health, as well as the longer-term (years to decades) effects of MP incorporation to soil, in a field context.

4.7. Acknowledgements

4.8. References

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Chapter 5: Nutrient (C, N and P) enrichment induces significant changes in the soil metabolite profile and microbial carbon partitioning

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5.1 Abstract

The cycling of soil organic matter (SOM) and carbon (C) within the soil is governed by the presence of key macronutrients, particularly nitrogen (N) and phosphorus (P). The relative ratio of these nutrients has a direct effect on the potential rates of microbial growth and nutrient processing in soil and thus is fundamental to ecosystem functioning. However, the effect of changing soil nutrient stoichiometry on the small organic molecule (i.e., metabolite) composition and cycling by the microbial community remains poorly understood. Here, we aimed to disentangle the effect of stoichiometrically balanced nutrient addition on the soil metabolomic profile and microbial carbon use efficiency by adding a labile C source (glucose) in combination with N and/or P. After incorporation of the added glucose into the microbial biomass (48 h), metabolite profiling was undertaken by UPLC-MS/MS. 494 metabolites were identified across all treatments mainly consisting of lipids ($n = 199$), amino acids ($n = 118$), carbohydrates ($n = 43$), > 97% of which showed significant changes in concentration between at least one treatment. Overall, glucose-C addition generally increased the synthesis of other carbohydrates in soil, while addition of C and N together increased peptide synthesis, indicative of protein formation and turnover. The combination of C and P significantly increased the number of fatty acids synthesised. There was no significant change in the PLFA-derived microbial community structure or microbial biomass following C, N and P addition. Further, N addition led to an increase in glucose-C partitioning into catabolic processes (i.e., reduced C use efficiency, determined by ^{14}C-labelling) suggesting the biomass was N, but not P limited. Based on the metabolomic profiles observed here, we conclude that inorganic nutrient enrichment causes substantial shifts in both primary and secondary metabolism within the microbial community leading to changes in resource flow and thus soil functioning.

Keywords: Metabolic profiling, Soil organic carbon, Stoichiometry, Nutrient cycling, Carbon mineralisation

5.2. Introduction

A major portion of terrestrial carbon (C) cycling is mediated and driven through soil microorganisms (Gougoulias et al., 2014). Soil microbes and their ability to metabolise (i.e. catabolise and anabolise small molecules fundamental to biological function) is inherently governed by the stoichiometry of bioavailable nutrients present in the soil (Cleveland and Liptzin, 2007). In most soils, available C is the primary factor limiting microbial growth (Heuck et al., 2015), with the availability of inorganic nutrients (N and P) being secondary regulators once C limitation is overcome (Creamer et al., 2016). It has been suggested that as the soil stoichiometric balance, here referring to the ratio of C:N:P, reaches the optimum for microbial cells, growth will lead to C storage, with no additional limitations (i.e., pH, oxygen and moisture status) (de Sosa et al., 2018; Mason-Jones et al., 2021). However, there is limited information on how the availability of these compounds will affect soil metabolomic processing and function, particularly at the individual metabolite level. Ultimately, it is the relative balance of these metabolites that determines key soil processes (e.g., the amount of C stored in the microbial biomass, the release of organic acids and mineral weathering rates, secondary metabolite production).

The metabolome is defined as the entirety of small molecules (< 1500 Da) found within a biological sample (Klassen et al., 2017). Primary metabolism, concerning the small molecules directly involved in the growth, development and reproduction of an organism, is key to normal organismal function (Rojas et al., 2014). Glucose, being a simple sugar, is considered a ubiquitous, labile, C substrate and is key in glycolysis, the key energy production pathway in most microorganisms (Sanchez and Demain, 2008). N and P are also potentially rate limiting nutrients, important in protein and amino acid synthesis, and energy metabolism and formation of various organic acids and phospholipids, respectively (Vitousek and Howarth, 1991; Kornberg, 1995; Kuypers et al., 2018; Y. Zhang et al., 2019).

It has been suggested that N and P cycling rates are intrinsically linked due to the potential for P limitation to develop under high N availability; as well as in terms of their impact on SOC processing under different stochiometric balances (Brailsford et al., 2019). Thus, nutrient inputs will shift the underlying stoichiometry of SOC and ultimately the soil organic matter (SOM) pools, which are key in both soil health and ecosystem service provision (i.e. climate regulation, crop production and water management (Garratt et al., 2018)). However, based on the current literature, it is unclear how changes to soil nutrient stoichiometry impact

the soil biological community's metabolism and SOC cycling. Generally, inference of changes in SOM/C cycling have been made through direct measurement of soil chemistry (Abrar et al., 2020), soil processes (e.g. CO_2 flux, exoenzyme activity; Hartman and Richardson, 2013), shifts in microbial community structure (Aanderud et al., 2018) or functional gene assays (Schleuss et al., 2019). However, high-resolution metabolomic approaches to C cycling have been shown to be very sensitive to changes in soil conditions (Withers et al., 2020; Overy et al., 2021), yet are rarely applied.

This laboratory-based mesocosm study investigates the effect of changing nutrient stoichiometry on primary and secondary metabolism of soil microorganisms, with the aim of providing a mechanistic understanding of the microbial breakdown and metabolomic processing of labile C, N and P substrates. We used a combination of (i) liquid chromatography/ mass spectrometry (LC/MS) based untargeted primary metabolomics assay, (ii) a phospholipid fatty acid (PLFA) assay to assess cell growth and structural community change, and (iii) a ^{14}C-labelled glucose assay to assess the temporal uptake and transformation of labile nutrient substrates.

Within the context of a typical agricultural soil this study aims to provide a better mechanistic understanding of biogenic nutrient processing. We hypothesise that: 1) nutrient (C:N:P) addition will cause a significant shift in the whole (intercellular and extracellular) metabolic profile of soil, 2) nutrient addition will not have an impact on the size and structure of the microbial community in the short-term (48 h), 3) glucose addition would lead to an increase in the Gram-negative-to-Gram-positive bacterial ratio and a decrease in the fungal-to-bacterial ratio due to preferential bacterial growth, and 4) eliminating nutrient limitation will increase the microbial removal of low-molecular weight C from a high C, low inorganic N and P environment.

5.3. Materials and methods

5.3.1. Soil characteristics and analysis

Independent replicate samples (0 - 10 cm, $n = 5$) of a sandy clay loam textured Eutric Cambisol soil were collected from a postharvest maize (*Zea mays* L.) field located at the Henfaes Agricultural Research Station, Abergwyngregyn, North Wales (53°14′N, 4°01′W). Following collection, the field-moist soil was sieved through a 2 mm mesh to remove stones

and plant material and ensure sample homogeneity. Soil characteristics are summarised in Table 5.1. Briefly, gravimetric soil moisture was determined by oven drying (105°C, 24 h), organic matter was ascertained by loss-on-ignition in a muffle furnace (450°C, 16 h) (Ball, 1964). C:N ratio was determined on oven-dried, ground soil using a TruSpec® Analyzer (Leco Corp., St. Joseph, MI, USA). pH and electrical conductivity were determined on 1:5 (w/v) soil-to-DI H_2O extracts using standard electrodes. Bioavailable N and P levels in soil were determined using 1:5 (w/v) soil-to-0.5 M K_2SO_4 and 1:5 (w/v) soil-to-0.5 M AcOH (acetic acid) extracts, respectively. Dissolved organic carbon (DOC) and total nitrogen (TN) were determined in the K_2SO_4 extracts using a Multi N/C 2100S Analyzer (AnalytikJena, Jena, Germany). Nitrate (NO_3-N) and ammonium (NH_4-N) in the K_2SO_4 extracts were measured by the colorimetric methods of Miranda et al. (2001) and Mulvaney, (1996), respectively. Phosphate (PO_4-P) was measured on the AcOH extracts using the colorimetric method of Murphy and Riley (1962). Cations (Na, K and Ca) were measured on the AcOH extracts using a Sherwood Model 410 Flame Photometer (Sherwood Scientific Ltd, Cambridge, UK).

Table 5.1. *Characteristics of the soil used in the study. Values represent mean ± SEM (n = 4), reported on a dry weight basis.*

Soil characteristics	
Gravimetric moisture content (%)	32.6 ± 1.5
Organic matter (%)	6.57 ± 0.26
pH	5.7 ± 0.1
EC ($\mu S\ cm^{-1}$)	64 ± 4
Total C (%)	2.62 ± 0.06
Total N (%)	0.30 ± 0.01
C:N ratio	8.7 ± 0.1
Dissolved organic C ($mg\ C\ kg^{-1}$)	54.7 ± 3.0
Total N ($mg\ N\ kg^{-1}$)	39.0 ± 3.8
Extractable NO_3^- ($mg\ N\ kg^{-1}$)	6.3 ± 0.3
Extractable NH_4^+ ($mg\ N\ kg^{-1}$)	1.0 ± 0.2
Extractable PO_4^+ ($mg\ P\ kg^{-1}$)	14.1 ± 0.6
Exchangeable Na ($mg\ kg^{-1}$)	13.5 ± 1.3
Exchangeable K ($mg\ kg^{-1}$)	219.2 ± 63.9
Exchangeable Ca ($mg\ kg^{-1}$)	134.9 ± 12.7

5.3.2. Soil treatment

To stimulate microbial metabolism and metabolite production, a nutrient solution was added to the soil. In general, the most common factor limiting microbial activity is the availability and quality of C (Demoling et al., 2007). As such, glucose was chosen as the primary nutrient to be added, as it represents a major input of C, in both monomeric and polymeric form, into soil systems and is utilised by almost all organisms within the microbial community (Gunina and Kuzyakov, 2015; Reischke et al., 2015). As N and P also have the potential to be microbial growth limiting, glucose was either added alone or in the presence of N, or P, or N + P at a stoichiometric ratio of 60:7:1 (C:N:P) based on the ratio of the microbial biomass (Cleveland and Liptzin, 2007; Brailsford et al., 2019). The concentration at which glucose was added to the soil was based on the likely amount released on plant cell death (50 mM; Jones and Darrah, 1996; Teusink et al., 1998). This concentration was chosen as it provided an excess of C (30 mmol C kg^{-1}) relative to the size of the native microbial biomass (18 mmol C kg^{-1}, of which ca. 10% is active; Wang et al., 2014) and DOC pool (4.6 mmol C kg^{-1}) and would therefore promote microbial growth, but remain realistic. Preliminary experiments showed that this level of glucose induces exponential microbial growth after ca. 16 h (data not presented). The N was added as NH_4NO_3 (3.5 mmol N kg^{-1}) and P was added as NaH_2PO_4 (0.5 mmol P kg^{-1}). Following treatment addition, soil samples were incubated at 25 °C for 48 h (reflecting a summer period), in the dark to stimulate microbial growth and substrate utilisation.

5.3.3. Untargeted soil metabolomic sample preparation, extraction, and analysis

Prior to use, all glassware was acid-washed (5% HCl, > 24 h) to remove chemical residues, rinsed in HPLC-grade water three times, and subsequently dry heat sterilised (150 °C, 2.5 h) (Jain et al., 2020). All equipment used to process the soil (e.g., tweezers and spatulas) were thoroughly cleaned both before and between samples with deionised water and 70 % industrial methylated spirit (IMS) to prevent cross-contamination. Nutrients in the concentrations described in section 5.3.2 were added by pipette in 1 ml of HPLC-plus grade water (Sigma-Aldrich, Munich, Germany) evenly across the surface of 10 g fresh weight of soil in 240 ml polypropylene sample containers (Snap-Seal®; Corning, NY, USA). This larger container was initially used to ensure even coverage of the soil surface with the nutrient solution. Five independent soil replicates ($n = 5$) were used per treatment. Samples were

subsequently incubated as described in section 5.3.2. At the end of the incubation period, samples were immediately transferred to 20 ml glass vials and fumigated with ethanol-free chloroform for 24 h to lyse microbial cells, in order to increase metabolite yield (Swenson et al., 2015) and limit microbial processing of metabolites, using the method of Vance et al. (1987). After fumigation, samples were immediately frozen (-80 °C) to quench any residual metabolic and enzymatic activity (Wellerdiek et al., 2009). From this point onwards, all samples were stored at -80 °C unless otherwise stated and while being processed (i.e. out of the freezer) samples were kept on ice (4 °C). Lyophilisation of samples (> 24 h) was then performed using a Modulyo Freeze Dryer (ThermoFisher Corp, Waltham, MA) equipped with an RV vacuum pump (Edwards Ltd., Crawley, UK). Samples were then mechanically ground using a Retsch MM200 stainless steel ball mill (Retsch GmbH, Haan, Germany) for 60 s at a frequency of 20 Hz to aid in cell lysis and metabolite recovery and sample homogeneity (Wang et al., 2015; Withers et al., 2020). The ball mill was cleaned thoroughly between samples with HPLC-grade water followed by 70% IMS, to avoid cross-contamination.

The following extraction method is based on the hybridised methods of Swenson et al. (2015) and Fiehn et al. (2008). Briefly, we used the extraction method from Swenson et al. (2015) and the solvent makeup (3:3:2) from Fiehn et al. (2008), to ensure broad metabolite coverage, with the recovery of both polar and non-polar metabolite classes. Further, the weight of soil extracted was increased in order to maximise the volume of supernatant available for preconcentration by lyophilisation, and subsequent analysis. A pre-experiment was used to compare the efficiency of the metabolite extraction method proposed here to that of the Swenson et al. (2015) method (the results are summarised in Appendix 3 Section S1).

Upon extraction, 6 g of each fumigated, lyophilised, ground soil sample was weighed into a 35 ml glass centrifuge tube (Kimax®; DWK Life Sciences, Stoke-on-Trent, UK), to which 24 ml of pre-cooled (-20°C) acetonitrile (MeCN)/isopropyl alcohol (IPA)/HPLC-plus grade water (H_2O) (3:3:2 v/v/v) extractant was added, using a glass pipette. Samples were then horizontally shaken on ice (4°C) at a frequency of 200 min^{-1} for 1 h, then centrifuged at 3320 $\times$ g for 15 min (Swenson et al., 2015). Supernatants were then pipetted, using glass pipettes, into 20 ml glass vials and stored at -20 °C (to ensure metabolic activity was quenched but the supernatant was not frozen). Samples were left unfiltered due to the potential of contamination from dissolution of the filter paper and plastic housing. The supernatant was lyophilised in 2 ml glass vials using a Modulyo Freeze Dryer with RV pump attached to a SpeedVac vacuum concentrator (Savant; ThermoFisher Corp.). The vials were periodically topped up with the

supernatant, taking note of the quantity added (~15 ml total) and lyophilised to complete dryness. Samples were then shipped on dry ice (-78.5 °C) to Metabolon Inc. (Morrisville, North Carolina, USA) for untargeted LC/MS metabolomic analysis. Upon analysis, samples were dissolved in methanol:water (4:1 v/v) and subjected to the standard Metabolon sample preparation procedure. Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectroscopy (UPLC-MS/MS) analysis parameters, bioinformatics, compound ID and data curation are summarised in Appendix 3 Section S2.

5.3.4. ^{14}C-glucose labelled nutrient metabolism assays

Soil metabolism was measured by nutrient depletion following a protocol similar to that described for freshwater sediments in Brailsford et al. (2019). Briefly, 2 g fresh weight of soil was added to a sterile 50 ml polypropylene centrifuge tube (Falcon®, Corning Inc., Corning, NY), and 200 μl of ^{14}C-[U]-glucose (Lot 3,632,475; PerkinElmer Inc., Waltham, MA) was added to the soil surface to a final C concentration of 38 μM (3.7 kBq ml^{-1}). This level of glucose in the unamended control reflects the natural background level of glucose in soil solution in this soil (Boddy et al., 2007). Five independent soil replicates ($n = 5$) were used per treatment. To measure glucose depletion in each of the C, N and P amended treatments (as described in section 5.3.2), ^{14}C-labelled glucose was added alone or in the presence of N, or P, or N + P in the concentrations described in Section 5.3.2.

After addition of the ^{14}C-labelled substrate, a 1 M NaOH trap (1 ml) was suspended above the soil to catch any respired ^{14}CO$_2$. The tubes were then hermetically sealed and incubated at room temperature (20 ± 1°C) in the dark. The NaOH traps were replaced after 0, 1, 3, 6, 9, 24, 36, 48, 60, 72, 80, 96, 103, 122 148 and 168 h, post glucose application. The efficiency of the NaOH traps was > 98% (as determined by collecting ^{14}CO$_2$ generated from adding excess 0.1 M HCl to 0.001 M NaH^{14}CO$_3$). The amount of ^{14}C in the NaOH traps was measured by mixing with Optiphase HiSafe 3 liquid scintillation cocktail (PerkinElmer Inc., Waltham, MA, USA) and placing on a Wallac 1404 scintillation counter (Wallac EG&G, Milton Keynes, UK) with automated quench correction.

The amount of ^{14}C-labelled glucose remaining in the soil was determined after 7 d (168 h) by extracting each sample with 1:5 (w/v) ice-cold (4°C) 1 M KCl to halt any further glucose turnover (Rousk and Jones, 2010). Samples were shaken (200 rev min^{-1}, 30 min) and centrifuged (33,000 g, 5 min). Subsequently, 1 ml of supernatant was mixed with Optiphase

HiSafe 3 liquid scintillation cocktail (PerkinElmer Inc., Waltham, MA, USA) and their ^{14}C content measured by liquid scintillation counting as described above.

5.3.5. Soil PLFA analysis

Nutrients in the concentrations described in section 5.3.2 were added in 2 ml of HPLC-plus grade water (Sigma-Aldrich, Munich, Germany) to 20 g fresh weight of soil in 240 ml polypropylene sample containers (Snap-Seal®; Corning, NY, USA). Four independent soil replicates ($n = 4$) were used per treatment. Samples were subsequently incubated as described in section 5.3.2. At the end of the incubation period, samples were immediately transferred to sterile 50 ml polypropylene centrifuge tubes (Falcon®, Corning, NY, USA) and frozen (-80 °C) to quench lipid turnover. Lyophilisation (> 24 h) was then performed using a Modulyo Freeze Dryer with RV pump. Samples were then shipped on dry ice (-78.5 °C) to Microbial ID (Newark, DE, USA) for extraction, fractionation and transesterification using the high throughput method of Buyer and Sasser (2012). Subsequently, samples were analysed using an Agilent (Agilent Technologies, Wilmington, DE, USA) 6890 gas chromatograph (GC) equipped with autosampler, split–splitless inlet, and flame ionization detector. The system was controlled by MIS Sherlock® (MIDI, Inc., Newark, DE, USA) and Agilent ChemStation software. GC-FID specification, analysis parameters and standards can be found in Buyer and Sasser (2012). Microbial biomass was calculated as the sum of all PLFAs detected in the sample. The PLFAs detected are summarised in Appendix 3 Table S1.

5.3.6. Statistical analysis

All statistical and graphical analysis was performed in the R environment (v 4.1.1; R Core Team, 2021), and graphical analysis was constructed using the '*ggplot2*' package (Wickham, 2016), unless otherwise stated. Analysis was deemed significant if $p < 0.05$. All metabolomic statistical analysis was performed using, natural log (ln) transformed median scaled imputed data. A principal component analysis (PCA) was constructed using the '*vegan*' package (Oksanen et al., 2020), in order to reduce the dimensionality of the dataset and give a visual representation of data variance. An analysis of similarity (ANOSIM) was subsequently performed using '*vegan*' to test treatment separation statistically.

To measure the magnitude of the effect of nutrient (C, N and P) addition, the number of compounds with significant differences using pairwise treatment comparisons were assessed using the '*stats*' package (R Core Team, 2021), and for compounds deemed significantly different between treatments the direction of change was summarised. Fatty acids (FAs) were also examined in greater detail; a statistical heatmap of a number of exemplary short chain, medium and long chain saturated and unsaturated FAs, with pairwise treatment comparisons was carried out as above and then graphically represented.

Specific examples of representative metabolites (carbohydrates, amino acids, peptides and FAs) and compounds associated with specific metabolic pathways (TCA cycle and glutamate pathway) were further explored graphically and statistically using ANOVA models in the '*stats*' package, significant results were additionally tested using a Tukey posthoc test using the '*agricolae*' package (de Mendiburi, 2019).

A non-metric dimensional scaling (NMDS) approach was used to condense the multivariate PLFA data in a comprehensible number of dimensions and visualize the relative degree of similarity among samples using the whole PLFA dataset, this was performed using the '*vegan*' package. All PLFAs detected were used in the analysis, to represent the structure of the whole microbial community. An analysis of similarity (ANOSIM) was subsequently performed using '*vegan*' to test separation statistically. PLFA derived microbial biomass was also tested using ANOVA, as above.

The cumulative mineralisation of ^{14}C-labelled glucose over time was calculated for each replicate. The final percentage (7 d post glucose application) of respired $^{14}CO_2$ was used to calculate microbial C use efficiency (Jones et al., 2018). The final concentration of ^{14}C labelled compounds in the soil (as determined by ice-cold 1 M KCl extraction on day 7) was not subtracted from this, as it was assumed that these compounds were the result of glucose turnover in the soil, i.e., either metabolic by-products or end-products (Glanville et al., 2016). Differences in total ^{14}C-CO_2 respiration was assessed using a Kruskall-Wallis test, followed by a pairwise Wilcox posthoc test, as data did not conform to parametric assumptions. The final concentration of ^{14}C-labelled compounds in the soil was assessed by ANOVA, as above.

5.4. Results

5.4.1. Soil primary metabolite profile

In total, 494 individual metabolite compounds were identified across all treatments. This included, 199 fatty acids, 118 amino acids, 43 carbohydrates, 41 nucleotides, 21 peptides and 72 other compounds including xenobiotics, secondary metabolites and cofactors and election carriers.

PCA (Fig. 5.1) was used to gain a high-level overview of data variance and sampling grouping. The ANOSIM, confirmed significant similarity between treatment groups (R = 0.147, $p = 0.002$). However, qualitative interpretation of the PCA showed that the Glu treatment was not strongly separated from the no-addition and control treatment groups, possibly because these groups appeared to exhibit more variation between replicates than the mineral (+ N/ + P/ + N + P) treated groups (Appendix 3 Fig. S1). The addition of Glu + N and Glu + P led to extensive shifts in the soil metabolome, however, the changes in the overall metabolic profile of the soil were distinctly different for the two elements. As noted above, P addition in the absence of N resulted in a dramatic accumulation of many lipids, but if N was also present, this accumulation was much less, or reversed, as evidenced by the proximity of the Glu + N and Glu + N + P groups in Figure 5.1 and shown in Table 5.2.
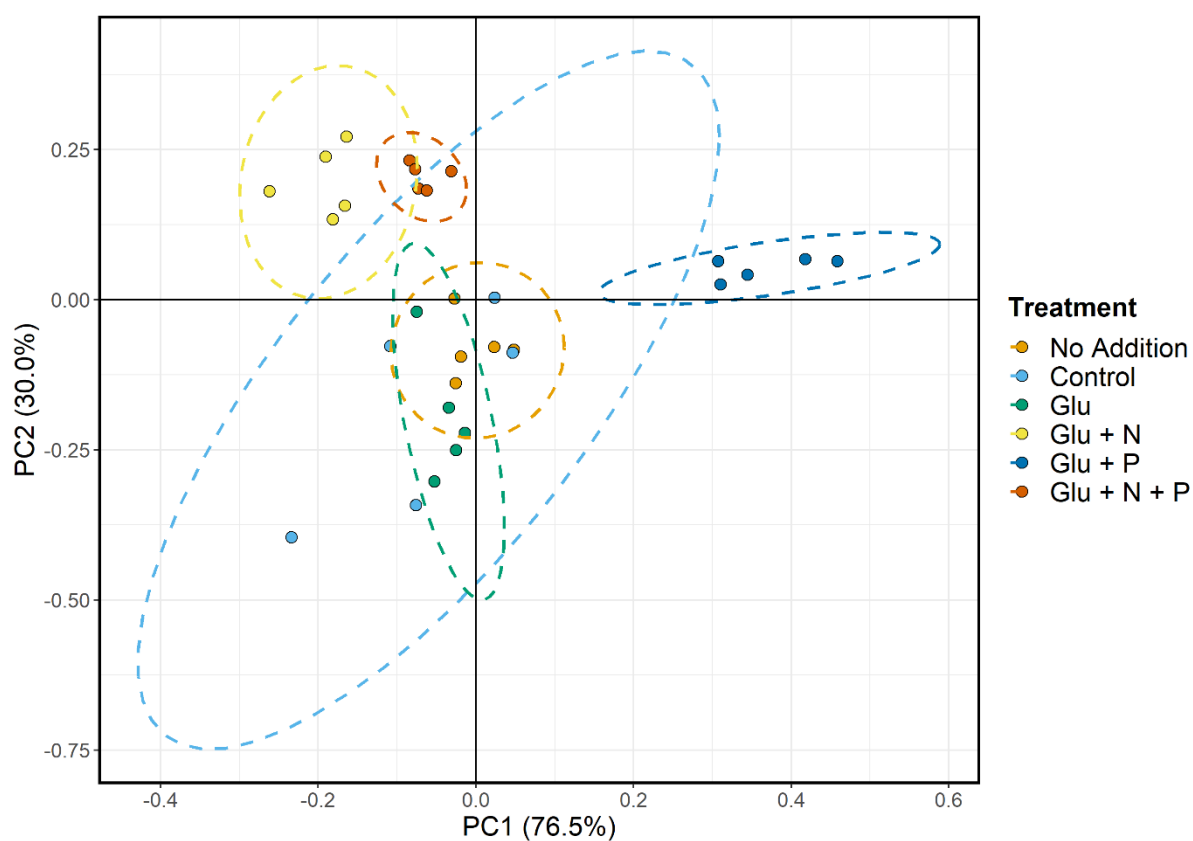
Figure 5.1. *Influence of nutrient (C, N and P) addition on the primary metabolite profile of soil. 2D principal component analysis (PCA) of soil metabolite composition. Principal component 1 (PC1) explains 76.5% of total variance, principal component 2 (PC2) explains 30.0% of total variance. Plotting was performed on natural log (ln) transformed median scaled data.*

***Table* 5.2.** *Summary of changes in fatty acids (FAs) between nutrient addition treatments. A tabulated statistical heatmap; numbers in the table indicate the ratio of the mean scaled intensity for a metabolite between two experimental groups being compared. Red and green filled cells indicate a significant decrease in metabolite concentration and a significant increase in metabolite concentration, respectively, using a Welch's two sample t-test ($p \leq 0.05$). Statistical analysis was performed on natural log-transformed data.*

		Effect of N			Effect of P				Effect of N + P				
	Biochemical name	**Glu + N / No addition**	**Glu + N / Control**	**Glu + N / Glu**	**Glu + P / No Addition**	**Glu + P / Control**	**Glu + P / Glu**	**Glu + P / Glu + N**	**Glu + N + P / No addition**	**Glu + N + P / Control**	**Glu + N + P / Glu**	**Glu + N + P / Glu + N**	**Glu + N + P / Glu + P**
Short chain	**butyrate/isobutyrate (4:0)**	**0.48**	0.8	0.83	**0.12**	**0.19**	**0.2**	**0.24**	**0.19**	**0.31**	**0.32**	**0.39**	1.61
	isovalerate (i5:0)	0.74	0.69	0.84	0.42	0.4	0.48	0.57	**0.28**	**0.26**	**0.32**	**0.38**	0.66
	Valerate (5:0)	0.66	0.85	0.87	**0.21**	**0.27**	**0.27**	**0.31**	**0.25**	**0.32**	**0.33**	**0.37**	1.2
	Isocaproate (i6:0)	0.81	0.52	1.03	**0.15**	**0.1**	**0.19**	**0.18**	**0.23**	**0.15**	**0.29**	**0.28**	1.53
	Caproate (6:0)	1.06	1.03	1.44	**0.33**	**0.33**	**0.45**	**0.32**	**0.59**	**0.57**	0.8	0.56	**1.76**
Medium and long chain saturated	**Caprate (10:0)**	0.99	1.02	1.12	1.13	**1.16**	**1.28**	1.14	1	1.03	**1.13**	1.01	**0.89**
	Myristate (14:0)	**0.68**	0.64	0.76	**4**	**3.8**	**4.5**	**5.92**	0.84	0.8	0.95	1.25	**0.21**
	(12 or 13)-methylmyristate (a15:0 or i15:0)	**0.38**	**0.34**	**0.44**	**7.87**	**7.05**	**9.11**	**20.81**	**0.56**	0.51	**0.65**	1.49	**0.07**
	Palmitate (16:0)	0.82	0.7	0.88	**4.81**	**4.09**	**5.13**	**5.85**	0.97	0.83	1.04	1.18	**0.2**
	Margarate (17:0)	0.52	0.42	0.6	**5.71**	**4.62**	**6.59**	**10.98**	0.8	0.65	0.92	1.54	**0.14**

		C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
	Stearate (18:0)	0.84	0.78	0.86	2.98	2.65	2.96	3.42	1	0.92	1.02	1.18	0.35
	(16 or 17)-methylstearate (a19:0 or i19:0)	0.28	0.24	0.31	6.72	5.8	7.51	24.08	0.49	0.43	0.55	1.77	0.07
	Arachidate (20:0)	0.67	0.48	0.76	5	3.58	5.67	7.46	1.19	0.85	1.35	1.78	0.24
Unsaturated	Palmitoleate (16:1n7)	0.34	0.31	0.34	9.38	8.58	9.29	27.57	0.51	0.47	0.51	1.5	0.05
	Oleate/vaccinate (18:1)	0.36	0.31	0.34	12.65	11.01	12.05	35.55	0.57	0.5	0.54	1.6	0.05
	Eicosenoate (20:1)	0.26	0.25	0.29	5.84	5.5	6.42	22.09	0.5	0.47	0.55	1.9	0.09
	Erucate (22:1n9)	0.28	0.25	0.3	5.62	5.09	6.15	20.22	0.52	0.47	0.57	1.86	0.09
	Hexadecadienoate (16:2n6)	0.44	0.43	0.35	1.69	4.67	3.76	10.76	0.51	0.51	0.41	1.18	0.11
	Linoleate (18:2n6)	0.23	0.31	0.26	9.14	12.64	10.36	40.43	0.37	0.51	0.42	1.63	0.04
	Dihomo-linoleate (20:2n6)	0.36	0.34	0.4	8.23	7.75	9.15	23.06	0.61	0.57	0.67	1.7	0.07
	Hexadecatrienoate (16:3n3)	0.6	0.61	0.48	2.57	2.65	2.08	4.32	0.64	0.66	0.52	1.07	0.25
	Linolenate (alpha or gamma; 18:3n3 or 6)	0.43	0.5	0.33	11.09	13.1	8.46	25.94	0.63	0.74	0.48	1.47	0.06
	Stearidonate (18:4n3)	0.52	0.56	0.38	5.26	5.69	3.83	10.2	0.64	0.69	0.46	1.24	0.12
	Dihomo-linolenate (20:3n3 or 6)	0.23	0.24	0.26	11.77	12.76	13.42	52.25	0.49	0.53	0.56	2.17	0.04

Arachidonate (20:4n6)	0.2	0.21	0.21	15.35	16.48	16.49	78.46	0.46	0.5	0.5	2.37	0.03
Eicosapentaenoate (20:5n3)	0.8	0.82	0.67	10.48	10.65	8.66	13.02	0.72	0.73	0.59	0.89	0.07
Docosapentaenoate (22:5n3)	0.6	0.81	0.72	11.64	15.82	13.97	19.51	0.6	0.81	0.72	1	0.05
Docosapentaenoate (22:5n6)	0.11	0.12	0.13	12.04	13.31	14.46	113.56	0.37	0.41	0.44	3.49	0.03
Docosahexaenoate (22:6n3)	0.23	0.22	0.3	16.75	16.38	21.59	73.01	0.48	0.47	0.61	2.08	0.03

Treatment pairwise comparison (number of compounds significant and direction of change) is summarised in Table 5.3. Overall, the number of metabolites significantly affected and/or produced in the soil following the addition of water (Control) to the untreated soil (No addition) was minimal ($n = 25$; 5% of the total number of metabolites detected). However, all the compounds affected were lower in Control treatment, possibly representing a dilution effect compared to the No addition treatment. Glu addition alone (relative to the water-only Control) produced a number of significantly different compounds ($n = 55$; 11% of total detected), generally resulting in higher concentrations of carbohydrate molecules. This effect was noted in all comparisons involving Glu relative to the Control group, whether or not N and/or P was present. The additional effects of N and/or P caused the generation of a large number of further compounds (in some treatments up to half of all compounds), when compared to the Control or the Glu treatment groups. Additionally, N and/or P addition led to a larger number of changes relative to the Glu group than were seen relative to the Control group.

Table 5.3. Magnitude of metabolic change between treatments. Summary of the number of compounds significantly different between treatments, as measured by pair-wise comparison ($p \leq 0.05$), and the direction of change.

Effect	Comparison	Compounds significant	Increase | Decrease
Effect of H_2O (control)	Control / No addition	25	0 | 25
Effect of Glu	Glu / No addition	142	51 | 91
	Glu / Control	55	46 | 9
Effect of N	Glu + N / No addition	173	82 | 91
	Glu + N / Control	137	90 | 47
	Glu + N / Glu	175	95 | 80
Effect of P	Glu + P / No addition	233	202 | 31
	Glu + P / Control	223	204 | 19
	Glu + P / Glu	246	222 | 24
	Glu + P / Glu + N	215	170 | 45
Effect of N + P	Glu + N + P / No addition	237	131 | 106
	Glu + N + P / Control	142	117 | 25
	Glu + N + P / Glu	215	147 | 68
	Glu + N + P / Glu + N	85	67 | 18
	Glu + N + P / Glu + P	240	80 | 180

In terms of specific molecules, glucose itself was similar in all treated groups, and significantly higher than No addition and the Control, as would be expected (Figs. 5.2 and 5.6). With regard to compounds associated with the TCA cycle, the presence of P was a key factor in compound (and intermediate) synthesis, with notable increases in alpha-ketoglutarate, succinate and fumarate, under Glu + P (Fig. 5.3). Relative N deficit (and C excess) led to lower levels of amino acids and other nitrogenous compounds, while the restoration of the C:N balance generally increased the concentration of these compounds. However, 14 (52 %) proteinogenic aromatics compounds (e.g. phenylalinine and tryptophan) were found at similar levels in all groups, regardless of treatment with the exception of phenyllactate and kynurenate (Fig. 5.4A).
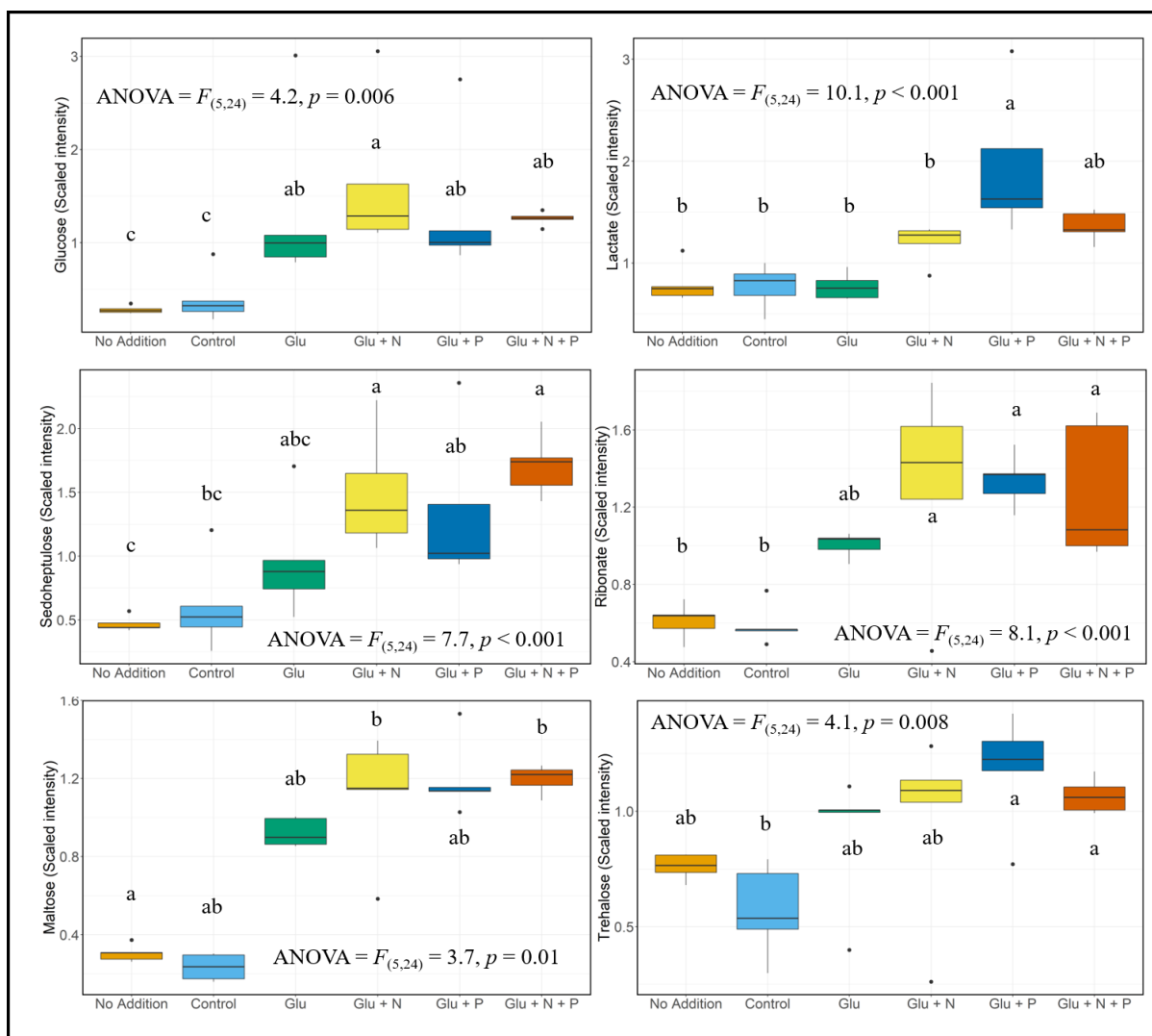
Figure 5.2. Response of exemplar carbohydrate molecules (glucose, lactate, sedoheptulose, ribonate, maltose and trehalose) within the soil in response to nutrient (C, N and P) addition. Letters indicate statistical differences between treatment groups (p < 0.05).
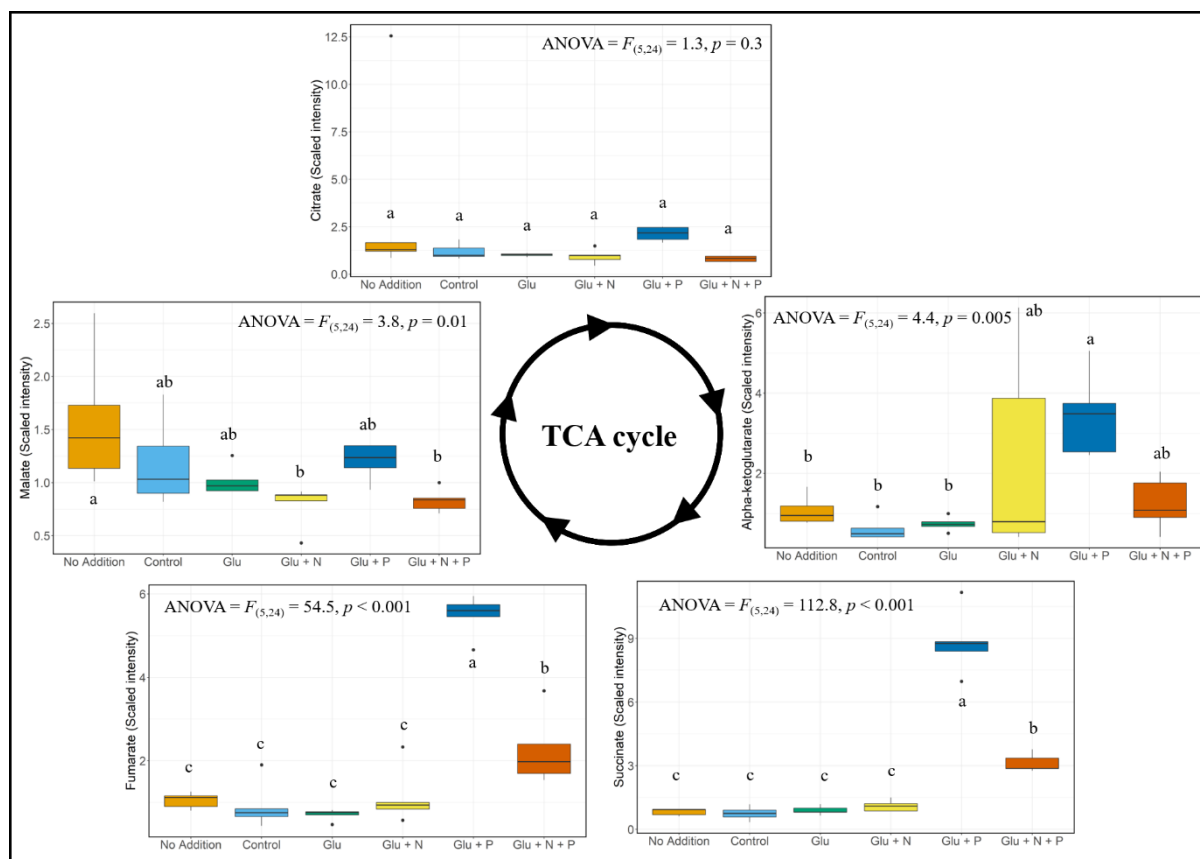
Figure 5.3. *Response of compounds related to the tricarboxylic acid (TCA) cycle (citrate, alpha-ketoglutarate, succinate, fumarate, malate) within the soil in response to nutrient (C, N and P) addition. Letters indicate statistical differences between treatment groups (p < 0.05).*
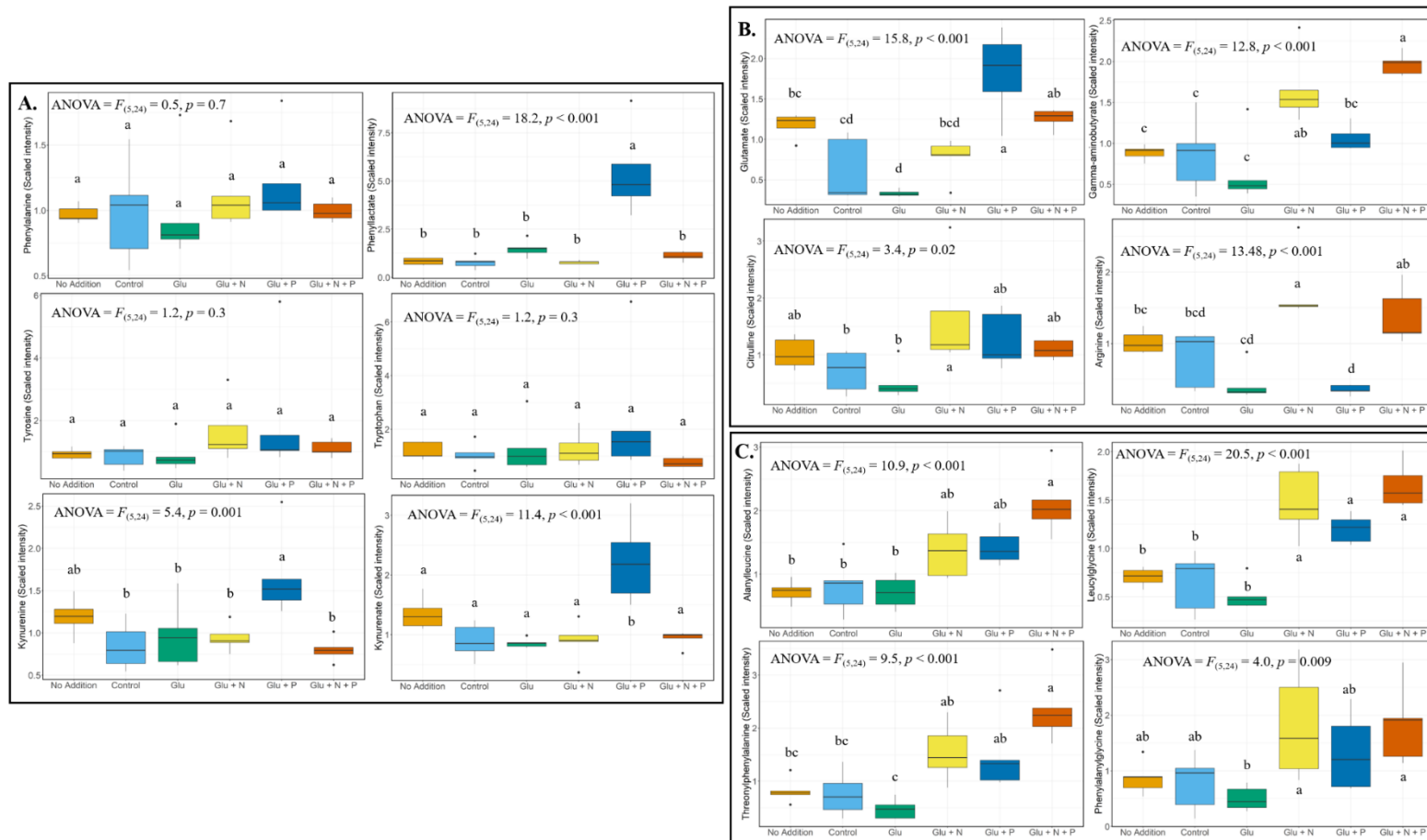
Figure 5.4. *Response of proteinogenic and peptide compounds within the soil in response to nutrient (C, N, and P) addition. Panel A summarises exemplar amino acid compounds related to the aromatic family (phenylalanine, phenyllactate, tyrosine, tryptophan, kynurenine and kynurenate), Panel B summarises exemplar amino acid compounds related to the glutamate pathway (glutamate, gamma-aminobutyrate, citrulline and arginine) and Panel C summarises exemplar oligopeptides (alanylleucine, leucylglycine, threonylphenylalanine and phenylalanylglycine). Letters indicate statistical differences between treatment groups ($p < 0.05$).*

Lipid metabolism was dramatically and consistently altered by supplementation of just Glu + P, but not by the combination of Glu + N or Glu + N + P (Table 5.2). This was particularly characterised by strong accumulation of long chain FAs in Glu + P treated samples. This effect was reversed for the short chain fatty acids (C4-C8; SCFAs). While medium chain length FAs were found at increased levels, the observed increases were less that seen for long chain FAs, especially polyunsaturated fatty acids (PUFAs), as illustrated in Table 5.2.

5.4.2 PLFA profile

The size of the microbial biomass showed significant differences between treatments, as tested by ANOVA ($F_{(5, 18)}$ = 2.82, p = 0.04) (Appendix 3 Fig. S2). However, further exploration using a Tukey HSD posthoc test showed no significant pairwise differences. NMDS ordination analysis was used to visually explore the clustering of the PLFA compounds produced under soil treatments (Fig. 5.5). Generally, there was little separation, with all 95% confidence intervals showing significant overlap, suggesting the PLFA derived microbial community structure was similar across all samples. This was further tested using an ANOSIM which, while significant, confirmed similarity between groups (R = 0.192, p = 0.02).
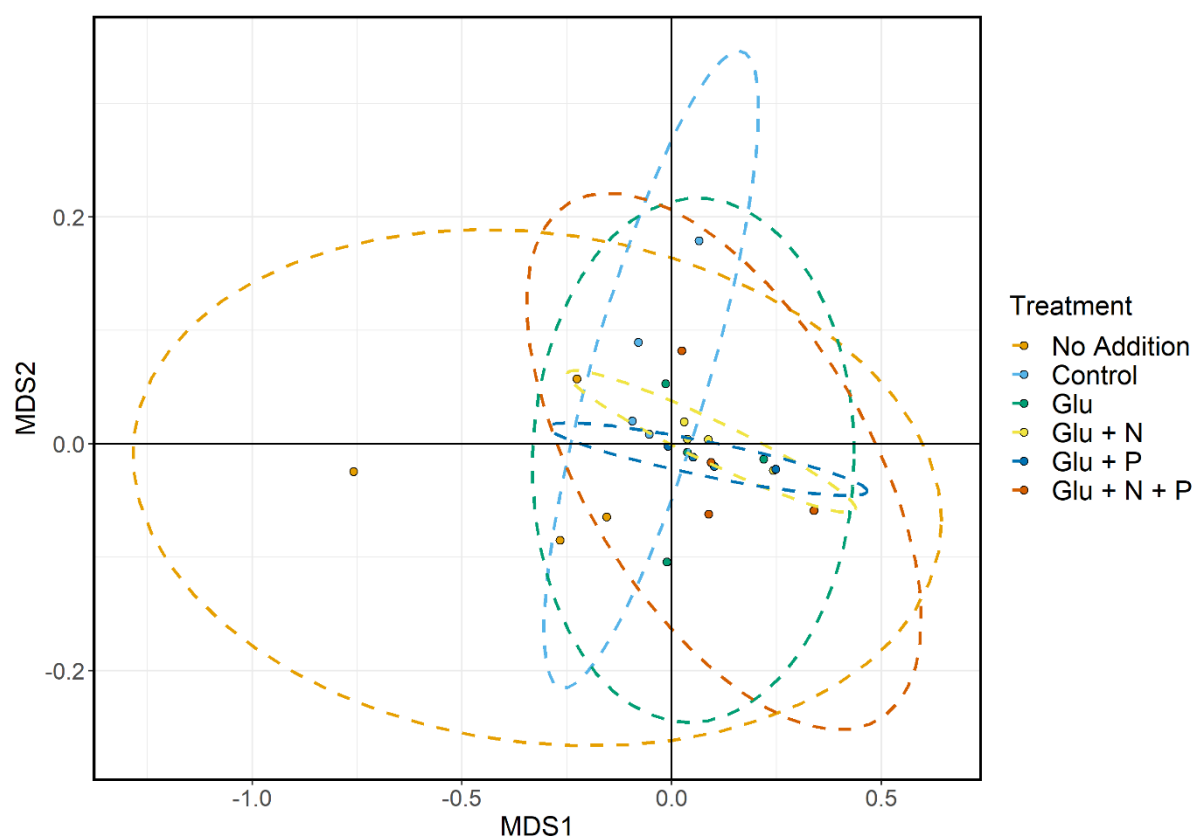
***Figure** 5.5. Influence of nutrient (C, N or P) addition on the PLFA derived microbial community structure of soil. NMDS plot of PLFA profiles of each soil treatment. Ellipses represent 95% confidence intervals for each treatment.*

5.4.3. ^{14}C-glucose mineralisation

After incubating the soil with ^{14}C-labelled glucose for 48 h, the microbial biomass had entered a phase of exponential growth, with a significant amount (greater than half of the final (7 d) total of respired ^{14}C) having been taken up by the microbial biomass and respired as CO_2 (Fig. 5.6). During this first 36 h period following ^{14}C-Glu addition Glu alone showed the most rapid mineralisation rate, with Glu + N and Glu + P closely grouped and Glu + N + P substantially slower. However, by 48 h Glu-alone had the lowest total $^{14}CO_2$ production, while Glu + N + P had the second highest behind Glu + N. The water-only Control treatment showed no lag phase, likely due to the significantly lower amounts of C being applied in the other treatments (glucose was only added at a trace amount in this treatment).
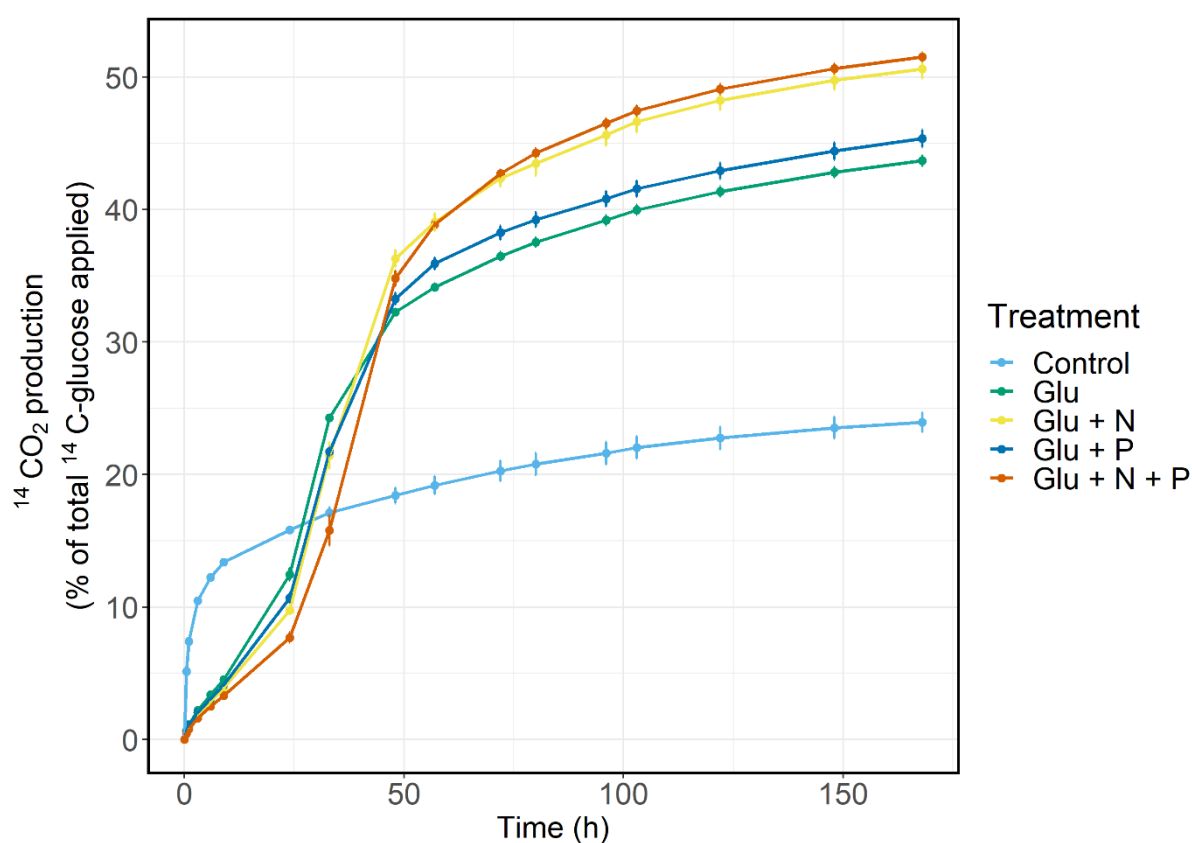
Figure 5.6. *Microbial mineralisation of ^{14}C-labelled glucose to $^{14}CO_2$ in soil over 1 week (168 h) either in the presence or absence of additional C, N or P. Treatments were replicated in quintuplicate (n = 5), and error bars indicate the SEM.*

By 168 h (7 d) the mineralisation rates in all treatments had slowed and had appeared to enter a quasi-stationary growth phase (Fig. 6). Kruskall-Wallis followed by pairwise Wilcox test showed there was a significant difference between treatments, specifically, between Control and all treatments ($p \leq 0.02$), Glu and Glu + N ($p = 0.02$) and Glu + N + P ($p = 0.016$), Glu + N and Glu + P ($p = 0.02$), and Glu + P and Glu + N + P ($p = 0.016$). There were also significant differences in the amount of ^{14}C labelled compounds remaining in the soil ($F_{(4,19)}$, $p < 0.001$), with Glu and Glu + P treatments having a higher percentage of ^{14}C compounds compared to the other treatments (Fig. 5.7).
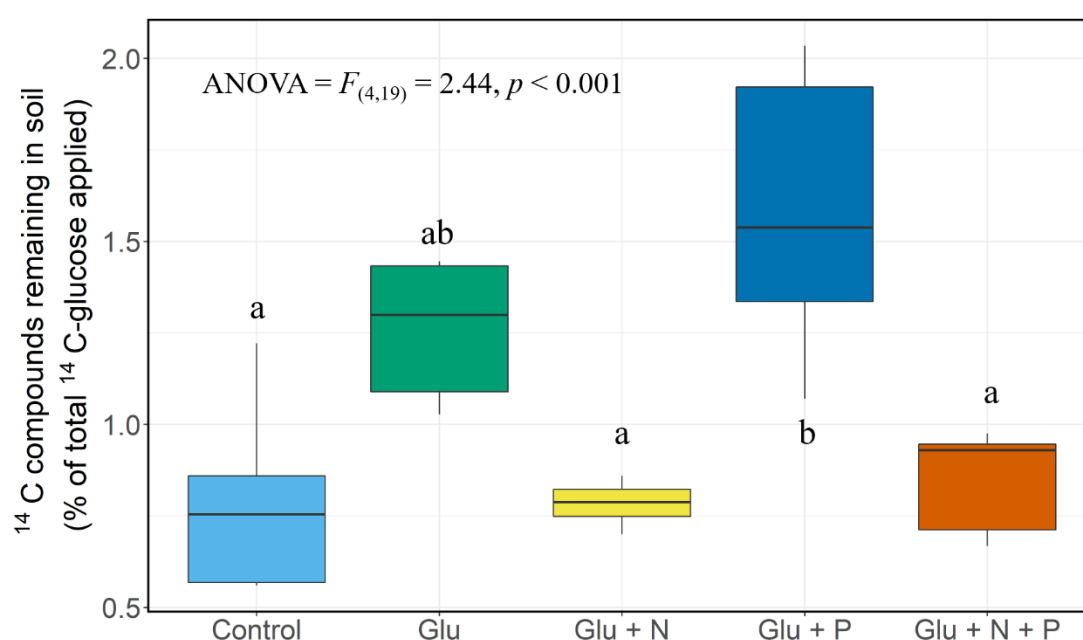
Figure 5.7. *Percentage of ^{14}C-labelled material remaining in the soil after 1 week following the addition of ^{14}C-labelled glucose either in the presence or absence of additional C, N or P. Letters indicate statistical differences between treatment groups (p < 0.05).*

5.5. Discussion

5.5.1. Primary metabolite changes induced by nutrient addition

PCA ordination of the metabolite data (Fig. 5.1) illustrate the general clustering of the No addition, Control and Glu treatments, suggesting that these treatments caused little change in the overall metabolite profile of the soil. Although this response is not surprising in the water-only (Control) treatment, we were expecting a large metabolic shifts in the glucose-only

treatment based on the observed exponential growth response in the ^{14}C mineralization profile and increase in microbial biomass. Generally, microbial growth in soil is limited by the stochiometric ratio of C to macronutrients (i.e., N or P; Griffiths et al., 2012; Hobbie and Hobbie 2013), therefore the addition of a labile C substrate (here glucose) is likely to have a significant impact on the growth of the microbial community and the metabolite profile. The Control treatment showed little difference to the No addition treatment, likely representing the negligible biological effect of water addition and a microbial biomass that is primarily limited by C. Equally, all treatments receiving glucose had statistically significantly higher levels remaining in soil solution compared to the control and no addition treatments, suggesting that there was still a considerable amount of residual substrate that had not been metabolised within the 48 h incubation period, as we aimed to add sufficient glucose so C was not limiting for the duration of the incubation. On average, after 1 week, 99% of the applied glucose had been consumed or respired from the soil (Fig. 5.7). N and/or P addition led to more changes relative to the Glu group than were seen relative to the Control treatment (Table 5.3), consistent with the theory that limiting C may restrict pathways that provide substrates for N and P interactions (Griffiths et al., 2012). In the subsequent sections we will discuss the changes in metabolites within several major molecular groups, namely, carbohydrates, compounds related to the TCA cycle, amino acids and FAs.

5.5.1.1 Carbohydrates

Carbohydrates (sugar, starch and cellulose) contribute significantly to the makeup of SOM (Ratnayake et al., 2013; Reardon et al., 2018). They are also key metabolites in soil microorganisms, functioning as metabolic substrates, as well as structural and intra- and exo-cellular components (Lowe, 1978). As stated in section 5.5.1, glucose was found in significantly higher concentrations in all treatments relative to the control and no addition. The conversion of glucose to lactate by anaerobic glycolysis is often used by cells that cannot produce enough energy (adenosine 5′-triphosphate (ATP)) through oxidative phosphorylation, to meet cellular demand (i.e. under anaerobic conditions) (Melkonian and Schury, 2021). Unicellular organisms undergoing exponential growth have been shown to grow by glucose fermentation, producing a range of small organic molecules such as ethanol, lactate or other organic acids (Vander Heiden et al., 2009). Here, while the addition of glucose alone did not stimulate lactate production, lactate was increased in combination with the element additions (particular under P treatments; Fig, 5.2). Glucose also led to elevations of several other sugars,

sugar acids, and sugar alcohols, as well as di- and tri-saccharides (Fig. 5.2). In most cases, the carbohydrate compounds were higher under the nutrient treatments relative to the glucose alone group, consistent with generally elevated metabolic activity (Fuhrer et al., 2005; Vénica et al., 2018). Equally, storage polysaccharide compounds (e.g., glycogen) are too large to be detected by the methods employed here, these compounds may have been an important store of excess C (Mason-Jones et al., 2021).

5.5.1.2. TCA cycle related compounds

The tricarboxylic acid cycle (TCA cycle) is the series of chemical reactions that release stored energy through the oxidation of organic molecules. Compounds related to the TCA cycle were the exception to the pattern described in section 5.5.1.1 (Fig. 5.3). In this case it is possible that P was limiting for maximum oxidative phosphorylation. On P addition this limitation was removed, leading to significant increases in key compounds related to TCA, namely succinate and fumarate. Inorganic phosphate is the key compound in the synthesis of adenosine triphosphate (ATP) from adenosine diphosphate (ADP) (Berg et al., 2002a; Phillips et al., 2009).

5.5.1.3. Amino acids

Amino acids are the structural units required for protein (and enzyme) synthesis, therefore are extremely important for cell function, as well as providing substrates for biochemical reactions. (Moe, 2013). The Glu treatment contained the lowest levels for these amino acids, even below the pre-existing levels in the control soil, while Glu + N and Glu + N + P in most cases had significantly higher levels. It is typical that relative N deficit (and C excess) leads to lower levels of amino acids and other nitrogenous compounds as the microbial community scavenge nitrogenous compounds from the soil (Geisseler and Horwath, 2014; Hicks et al., 2021), and that restoration of the C:N ratio results in increases in these compounds. We observed this scenario for many amino acids and their derivatives, especially in the class of amino acids derived through the glutamate pathways (arginine, glutamine, proline, histamine) (Fig. 5.4B). Glutamate is key to a number of metabolic processes in cells, including protein synthesis and glycolysis and the TCA cycle (Helling, 1998; Feehily and Karatzas,

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2013), and is one of the most ubiquitous amino acids in soil, as a component of root exudates and the dissolved organic nitrogen (DON) pool (Paynel et al., 2001; Forde and Lea, 2007).

An exception to this pattern in amino acid concentration was the pathway for aromatic amino acids (Fig. 5.4A). The proteinogenic aromatics were found at similar levels in all groups, regardless of treatment. Amino acids possessing aromatic ring structures are generally less attractive to soil microbes as a source of organic N, due to their higher C:N ratio and complexity (Sauheitl et al., 2009). However, the Glu + P treatment did cause increases in several aromatic amino acid derivatives, for example phenyllactate, kynurenine, and kynurenate, presumably through stimulation of catabolic pathway elements. Aromatic amino acids are often the metabolic starting point for the production of a variety of secondary metabolite (Parthasarathy et al., 2018). And, while secondary metabolism was not specifically examined in this study, future research is recommended due to its importance in organismal interaction and sensing (Karlovsky, 2008).

While whole proteins are too large to be considered in a metabolic analysis, peptides and tripeptides are considered markers for protein turnover (Doherty and Beynon, 2006). In this analysis the addition of glucose alone (Glu) did not lead to changes in peptide levels relative to the residual amounts in untreated soil, but both N and P (and their combination) did however increase these markers (Fig. 5.4C). These results are consistent with increased metabolic activity (protein production and growth) resulting from relief of the nutrient limitations (Hartman and Richardson, 2013).

5.5.1.4. Fatty acids

FAs are key to cellular function, contributing to a number of roles, including as membrane lipids (i.e. PLFAs) as well as storage and cell signalling (Carvalho and Caramujo, 2018). FA biosynthesis pathways are highly conserved across the kingdoms of life (Berg et al., 2002b). Here we illustrate that soil microbial lipid metabolism was dramatically and consistently altered by the addition of Glu + P to the soil, but not by the combined treatment, Glu + N + P (Table 5.2, Appendix 3 Fig S1). P is an essential component of lipid metabolism, particularly in the synthesis of PLs, which under unstressed conditions are the dominant polar membrane lipid class. P is liberated and solubilised from inorganic P (applied here) by phosphatase enzymes (Jones and Oburger, 2011; Alori et al., 2017), and it has previously been

suggested that P mineralization and P solubilization are constrained by soil stoichiometry, because N and organic C are required for the synthesis of phosphatase (Widdig et al., 2019).

Equally, an absence of P has also been shown to induce lipid accumulation (Yang et al., 2018). This study suggested the opposite, with P surplus, alongside a carbon substrate (Glu + P) causing lipid accumulation, particularly among long chain FAs, which are generally involved in cell structure (i.e., PLs) and storage (e.g., triacyclglycerols (TAGs)) (Salati and Goodridge, 1996; Brown et al., 2021).

Short chain fatty acids (SCFAs) decreased significantly under the Glu + P and Glu + N + P treatments (Table 5.2). It is likely that this is a result of the increase in the ratio of labile C and P substrate, which was used preferentially to the soil's inherent more recalcitrant organic matter derived substrates. As SCFAs are the metabolic end products of the anaerobic fermentation of recalcitrant polymeric carbohydrates (e.g., cellulose, starch, chitin) (Silva et al., 2020). SCFAs are functionally important metabolites, serving as electron donors for other functional microorganisms (e.g. fermentative Fe(III)-reducing microorganisms) and may also act as a substrate for the SCFA-utilising bacterial population (He and Qu, 2008; Awasthi et al., 2018). Although the soils here were kept generally aerobic and therefore anaerobic processes were likely not dominant.

N deprivation has also been shown to induce storage lipid accumulation (Weng et al., 2014), while N deprivation was not a direct treatment here, it may have been induced as a result of the unbalanced soil stoichiometry (i.e. C:N:P ratio) particularly under Glu + P treatments. However, here, N provision without P (Glu + N) led to decreased levels of longer chain fatty acids, but not for short chains. This potentially illustrates the partitioning of microbial resources; with N addition leading to the metabolism of a greater number of nitrogenous compounds (i.e. amino acids and proteins), while P addition led to the metabolism of a greater number of P-reliant compounds (i.e. FAs and compounds dependent on oxidative phosphorylation).

In terms of lipid metabolism, we can speculate that plentiful P (but a deficit of N) led to increased oxidative phosphorylation, which provided sufficient ATP for robust fatty acid synthesis, but the enzymes and regulatory loops for lipid metabolism and utilization could not be generated under N deficit conditions. The combination of P and N may have either alleviated this bottleneck to allow normal metabolism to utilize the fatty acids or may have allowed for alternative pathways that preclude the generation of the fatty acids in the first place.

5.5.2. Soil biological community response

The size and structure of the soil biological community underpins soil function (Wagg et al., 2014), driving SOM turnover and biogeochemical cycling (Rousk and Bengtson, 2014). Thus, C-induced shifts in soil microbial community structure may result in changes in soil function, notwithstanding the functional redundancy which exists within the community. Surprisingly, we observed no significant change in the ratio of key microbial taxa in response to C addition when assayed using the conventional PLFA biomarker approach (Fig. 5.5; Frostegård et al., 2011). In accordance with other studies, we did show an initial lag phase in ^{14}C-glucose use (ca. 10-16 h) followed by a short-lived exponential mineralization phase, a pattern consistent with microbial growth (Hill et al., 2008; Rousk and Baath, 2007). From the mineralization response and the total PLFA data, we conclude that glucose addition did stimulate *de novo* biomass production. This suggests that all components of the biomass grew equally, or that the conventional PLFA-biomarker approach failed to capture rapid changes in the active microbial community. We hypothesized that glucose addition would lead to an increase in the Gram-negative-to-Gram-positive bacterial ratio and a decrease in the fungal-to-bacterial ratio due to preferential bacterial growth (Fanin et al., 2019). However, this was not observed, at the 48 h point of sampling. Overall, our results suggest that metabolite extraction and analysis by metabolomic methods may have greater sensitivity than conventional GC-MS based analysis of PLFAs.

5.5.3 Use of LMW carbon

The soil microbial community is expected to experience large pulse inputs of C, N and P in response to rhizodeposition, fertilisers and abiotic stress events (e.g. dry-rewet, freeze thaw) (Göransson et al., 2013; Jones et al., 2009; Warren, 2014). The treatments used here were chosen to reflect these. Although the depletion of ^{14}C-labelled glucose (50 mM) occurred rapidly in all treatments (implied by the $^{14}CO_2$ emission), an initial lag phase was apparent (Fig. 5.6). We ascribe this slow initial response to a low microbial biomass and an initial saturation of internal metabolic pathways, rather than an overloading of the membrane transport systems (Hill et al., 2008). Note that no lag phase was observed when only a natural trace amount of glucose (38 µM) was added and that it was also catabolised more rapidly. At the time our metabolomic measurements were made (48 h), the amount of glucose-C recovered as $^{14}CO_2$ in

the 50 mM glucose treatments was much larger (ca. 50%) than that in the trace glucose addition (38 µM, ca 18%; Fig. 5.6). This is consistent with a major shift in microbial C partitioning and thus C use efficiency, with more glucose-derived C being channelled into energy intensive growth rather than maintenance metabolism. The addition of extra nutrients also induced changes in internal C partitioning, with the presence of N leading to a reduction in C use efficiency, while P addition generally had no observable effect. We ascribe this response to the removal of N limitation allowing slightly more glucose-C (ca. 5%) to be channelled into catabolic rather than anabolic processes (Mooshammer et al., 2014; S. Zhang et al., 2019). The higher concentrations of ^{14}C labelled compounds remaining in the soil in the Glu and Glu + P treatments after 7 d (Fig. 5.7) may also have been a result of this N limitation, reflecting the *de novo* production of exoenzymes (proteases) required to mine extra N from SOM to sustain further growth. It is also possible that some of the glucose-derived C in the N-free treatments was allocated to storage C pools, a phenomenon that is induced by nutrient imbalance (Mazoni et al., 2021).

5.5.4 Implications for SOC cycling

As plants develop, they have been shown to influence and select their soil microbial community through rhizosphere engineering (Sasse et al., 2018). Root exudates are nutrient rich solutions, provide the substrate for soil microbial growth and hotspots of interspecies interactions and biochemical cycling (Nadarajah, 2016; Canarini et al., 2019). While this experiment examined the fundamental response of the soil microbial community to relatively low concentrations of nutrient inputs, the field environment is vastly more complex than the microcosms examined here. Metabolomics has already been identified as a nascent field with potential for the study of the complex relationships within the rhizosphere (Mhlongo et al., 2018). However, further work must establish the effect of nutrient addition on the competition and interactions between plants and the soil microbial community. These experiments must also consider the extraction method used, e.g. water, where the water-soluble fraction is most likely to yield the most representative of compounds readily accessible to the biological community (exo-metabolites), or a solvent extraction, which will yield more extensive intra- and exo-metabolites (Swenson et al., 2015).

Finally, here we used glucose as a simple, labile substrate, however, in the natural environment glucose would typically be accompanied by the release of other monomers (e.g.

organic acids, amino acids) and oligomers (e.g. oligopeptides, oligosaccharides). For example, cellobiose, as a product of cellulose degradation, is one of the most ubiquitous and abundant disaccharides in soil, which can then be further broken down to glucose (Schellenberger et al., 2011; Chmolowska et al., 2016). Further study of the soil microbial community metabolic response to complex mixtures of organic compounds is therefore recommended.

5.6. Conclusions

Addition of labile nutrients in stoichiometrically balanced (and unbalanced) ratios led to significant, rapid (< 48 h) changes within the soil metabolome as well as a difference in cumulative soil respiration rates over 7 days. Treatments with a combination of glucose and minerals elements tended to have a greater effect on the soil metabolism than glucose alone, in most cases this is attributed to an elevated microbial activity as nutrient limitations are alleviated. The most profound of these changes was the significant increase in FAs under Glu + P treatment likely attributed to increased oxidative phosphorylation, while the relative N deficit prevented lipid metabolism and utilisation. Treatments without N addition had significantly lower cumulative soil respiration rates over 7 days, while P substrate addition had no significant impact on respiration, suggesting N was the main nutrient limiting microbial growth in this soil (after C). Inorganic nutrient enrichment of soils is likely to have substantial implications for labile and recalcitrant C cycling and microbial resource partitioning within the soil system. Understanding the fundamental changes in small molecule cycling is therefore likely to improve knowledge of both chemical ecology and soil and microbial function. Further research is suggested to further understand metabolic changes in the soil with regard to C input from plants (particularly in the rhizosphere) and under more complex substrate mixtures.

5.7 Acknowledgements

5.8 References

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Chapter 6: Volatile organic compounds (VOCs) allow sensitive differentiation of biological soil quality

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6.1. Abstract

Understanding the change in function of the biological community under different soil conditions is key to effective soil quality monitoring and mitigation of soil degradation. Current measures of biological soil quality suffer from drawbacks with most techniques having high expense, low throughput or a narrow focus on one component of the community. The aim of this study was to assess the use of volatilomics as a method to profile the soil microbial community and compare the technique to phospholipid fatty acid (PLFA) profiling as a measure of biological soil quality. An agricultural grassland soil (Eutric Cambisol) was subjected to a range of stresses in replicate laboratory mesocosms. Treatments included the imposition of hypoxia/anoxia by flooding with freshwater or saltwater in the presence or absence of plant residues. The volatile organic compound (VOC) and PLFA profile of each treatment was then compared to unamended mesocosms. We hypothesized that the VOC fingerprint of soil would be highly responsive to changes in microbial metabolic status/functioning and thus provide a complementary approach to PLFAs for evaluating soil biological health. We also hypothesized that the VOC profile would have greater discriminatory power than PLFAs for determining differences between soil treatments. A headspace solid phase microextraction (HSSPME) method coupled with gas chromatography quadrupole-time of flight mass spectrometry (GC/Q-TOFMS) was used to analyse the broad spectrum of VOCs produced by each soil. Across all soil treatments 514 unique VOC peaks were detected. Overall, VOCs showed greater sensitivity than the PLFA analysis in separating soil quality treatments. Eighteen individual VOCs were identified which were primarily responsible for this separation (e.g. indole, α-ionone, isophorone, 3-octanone, *p*-cresol, 2-ethyl-phenol). Anaerobic soils amended with residues showed the greatest separation from other treatments, with most of this differentiation associated with ten individual VOCs. The anaerobic soils also showed a significant reduction in the number of VOCs emitted but an increase in total VOC emissions. In conclusion, our findings provide evidence that soil VOCs rapidly respond to changes in soil quality and therefore hold great potential as a novel, functionally relevant diagnostic measure of biological soil quality.

Keywords*: soil function, metabolomics, method, microbial communities, soil quality indicator

6.2. Introduction

Soils are key to providing a wide range of ecosystem services crucial for earth system function and stability (Adhikari and Hartemink, 2016). However, most of the ice-free soils on the planet have been exploited either directly or indirectly by humans. Anthropogenic activity has subsequently contributed to the global-scale degradation of around 6 million ha of agricultural land (UNFAO and ITPS, 2015). Projections estimate that 95% of the land area on Earth could become degraded by 2050 (Montanarella et al., 2018). Therefore, it is essential that we continually assess the quality of our soils so that the provision of ecosystem services (e.g. nutrient cycling, water purification, food provisioning, climate regulation) can be sustained. Effective soil monitoring is fundamental to understanding the causes of degradation, which in turn could decrease the economic burden of soil degradation, which is estimated to be $231 bn globally and $2 bn in the UK (Graves et al., 2015; Nkonya et al., 2016).

Soil quality is often broadly defined as the capacity of a soil to function (Karlen et al., 1997). Although a range of soil quality indicators have been proposed, these mainly focus on the measurement of chemical attributes of the soil (e.g. pH, plant macronutrients, organic matter) and the physical characteristics of the soil (e.g. texture, bulk density, aggregate stability, hydrophobicity) (Bünemann et al., 2018; Schloter et al., 2018). However, soil fertility and productivity are not merely a function of soil physical and chemical characteristics. Soil biology is a crucial mediator in many processes linked to nutrient cycling, plant health and soil productivity, and is highly responsive to changes in management and environmental conditions, often being correlated to functional change (Lal, 2016; Lemanceau et al., 2015). Common soil biological indicators include measures of microbial activity (e.g. basal or substrate-induced respiration, enzyme activity) and the size and composition of the microbial community (e.g. metagenomics-metabarcoding, mesofaunal counts, $CHCl_3$-fumigation-extraction) (Bending et al., 2004). However, these methods all suffer from major drawbacks, including: (i) problems defining critical thresholds of 'good' or 'bad' soil quality, (ii) low sample throughput, (iii) high labour or equipment costs, (iv) narrow focus on one component of the community, and (v) limited spatial resolution/integration.

Volatile organic compounds (VOCs) are relatively low-molecular weight organic compounds (typically <250 amu) with high vapour pressures that give the soil its odour and can be produced via both biotic and abiotic processes (though biological production of soil VOCs far exceeds the production of VOCs by abiotic processes) (Insam and Seewald, 2010).

Farmers and land managers have, for centuries, used soil odour to infer soil quality (Semple, 1928). Study of soil odour first began to develop with the documentation of geosmin, the odour of moist soil in 1891 (Berthelot and André, 1891). Since then, studies have identified a large number of VOCs produced from soil but the full extent of the functional significance of these compounds still remains largely unknown (Peñuelas et al., 2014). Biological VOCs are secondary metabolites, therefore not directly involved in organismal growth, development or reproduction. However, soil VOC emissions are highly dynamic, responding rapidly to changes in soil conditions and thus giving the soil system a unique VOC emission profile depending on soil conditions, the taxa present in soil, and their metabolic activities (Insam and Seewald, 2010). The type and amount of VOC compounds emitted from soil are dependent on a range of factors including; nutrient availability (Wheatley et al., 1996), oxygen status (McNeal and Herbert, 2009), moisture availability (Asensio et al., 2007), organic matter inputs (Seewald et al., 2010), temperature (Schade and Custer, 2004), pH (Insam and Seewald, 2010) and interactions (sorption) with the solid phase (Serrano and Gallego, 2006). This sensitivity of VOCs to soil conditions makes them a good candidate as an indicator of soil biological quality.

Phospholipid fatty acid (PLFA) analysis has become a standard method for profiling the soil microbial community; giving a quantitative description of the microbial community within a sample (Frostegård et al., 2011). PLFA analysis provides information of the size of the microbial biomass, biomarkers of bacterial and fungal community structure, and an insight into the functional composition of the community (Willers et al., 2015). Microbial community composition and structure is responsive to management or naturally induced changes driven by soil physico-chemistry (Chang et al., 2017; Cobb et al., 2017; Hardy et al., 2019). For example, soil pH and organic matter content can greatly affect the PLFA composition of the microbial community, particularly in regard to fungal:bacterial ratios (Rousk et al., 2009; Welc et al., 2012). Despite the development of alternative methods in soil biological analysis, for example metabarcoding (Orwin et al., 2018) or community level physiological profiling (CLPP; Ramsey et al., 2006), PLFA analysis remains a rapid, sensitive and reproducible method of detecting differences in community composition between treatments (Frostegård et al., 2011).

This laboratory mesocosm study aims to critically test the relationship between microbial community structure (PLFAs) and VOC production under a range of soil stresses (anoxia/waterlogging, salinity) and organic matter regimes (e.g. urine, plant residue addition). It tests the hypothesis that there will be significant differences in both the PLFA profile of the microbial community and the number and amount of VOCs emitted between treatments.

However, as VOC production largely reflects the metabolism of the active microbial community, we hypothesize that it will have greater discriminatory power to resolve differences between treatments in comparison to PLFAs which reflects both the active, inactive and dead microbial biomass pools.

6.3. Materials and methods

6.3.1. Experimental setup

A Eutric Cambisol ($n = 5$, depth = 0 - 10 cm, Ah horizon) was collected from a post-harvest maize field located at the Henfaes Agricultural Research Station, Abergwyngregyn, North Wales (53°14′N, 4°01′W). The site is characterised by a temperate-oceanic climate regime with a mean annual temperature of 11 °C and annual rainfall of 960 mm. On collection, the soil was sieved to pass 2 mm to remove stones and plant material and to ensure sample homogeneity. Field-moist soil (200 g) was then placed in 300 cm^3 polypropylene sample containers and treatments applied. Treatment consisted of the following, i) aerobic - control, ii) aerobic – amended with lysed grass residue (5 % w/w), iii) aerobic – amended with sheep urine (equivalent of 5 L m^{-2}), iv) anaerobic – with river water, v) anaerobic – with river water, amended with lysed grass residue (5 % w/w), vi) anaerobic – with sea water, and vii) anaerobic – with sea water, amended with lysed grass residue (5 % w/w) (Fig. 6.1). There were five independent replicates of each treatment. Grass residues were lysed by freezing for 1 h at -80 °C before being incorporated. This ensured that the plant material was metabolically inactive prior to addition (i.e. no *de novo* biotic plant emissions). Sheep urine was collected from Welsh Mountain ewes (*Ovis aries* L.) as described in Marsden et al. (2018) and was loaded onto soil at rates equivalent to a typical sheep urination event (Selbie et al., 2015). To induce anaerobiosis, treatments were saturated with either freshwater or saline water and the containers hermetically sealed. These conditions reflected recent coastal and river flooding events which have occurred close to the sampling site (Sánchez-Rodríguez et al., 2018). Aerobic treatments remained unsealed and kept at constant weight throughout the 10-day incubation through the daily addition of deionised water. All mesocosms were subsequently incubated at 24 °C to stimulate the soil microbial community. This reflected summer soil temperatures at the field site. Duplicate mesocosms of each treatment replicate were created to

allow for half of the mesocosms to be destructively sampled for soil properties and PLFAs, and half to be analysed for VOC's.
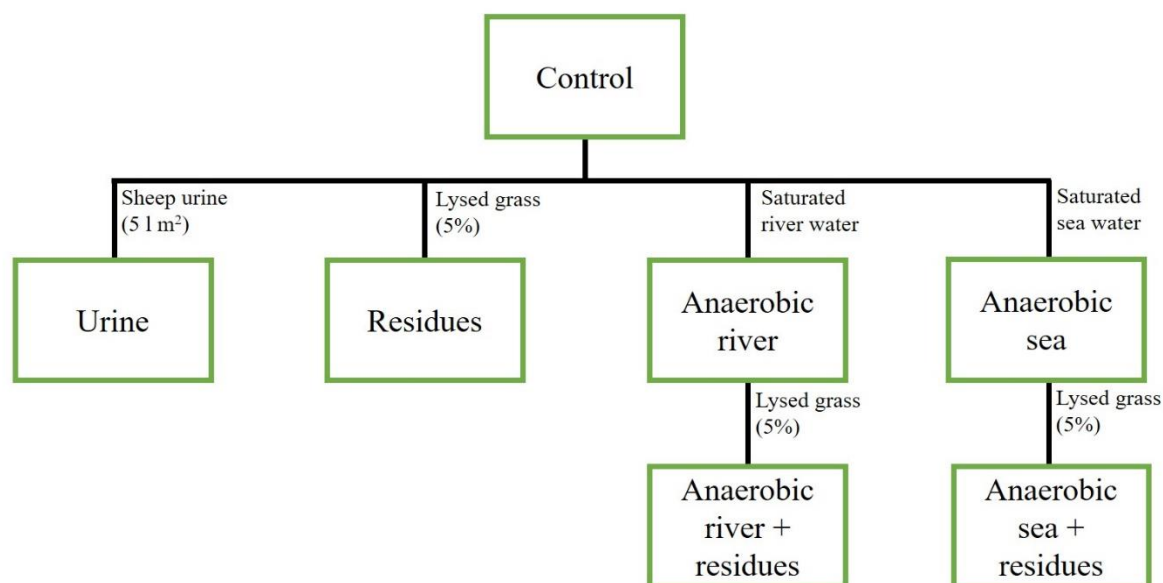
Figure 6.1. *Flow diagram illustrating the seven treatments applied in the study.*

6.3.2. Soil sampling and analysis

At the end of the 10-day incubation, excess water was drained from the anaerobic mesocosms and soil was homogenised thoroughly by hand with a spatula and analysis undertaken immediately. Soil moisture content was determined gravimetrically by oven drying (105 °C, 24 h) and soil organic matter was determined by loss-on-ignition (450 °C, 16 h) (Ball, 1964). Soil pH and electrical conductivity (EC) were measured using standard electrodes submerged in 1:5 (w/v) soil-to-deionised water suspensions. The oxidation–reduction potential (ORP) was measured directly in the soil using a SenTix® ORP-T 900 combination electrode (Xylem Analytics, Weilheim, Germany) connected to a mV reader. Total C and N was determined on oven-dried, ground soil using a TruSpec® Analyzer (Leco Corp., St. Joseph, MI).

At the end of the incubation, 1:5 (w/v) soil-to-0.5 M K_2SO_4, 1:5 (w/v) soil-to-0.5 M AcOH (acetic acid) and 1:5 (w/v) soil-to-deionised (DI) H_2O extractions were performed. TOC (total organic carbon) and TN (total nitrogen) were determined on K_2SO_4 extracts using a Multi N/C 2100S Analyzer (AnalytikJena, Jena, Germany). Nitrate (NO_3-N) and ammonium (NH_4-

N) concentrations within the K_2SO_4 extracts were determined by the colorimetric VCl_3 method of Miranda et al. (2001) and the salicylic acid method of Mulvaney (1996), respectively. Available P was measured on the DI H_2O extracts using the molybdate blue colorimetric method of Murphy and Riley (1962). Cations (Na, K and Ca) were determined in the AcOH extracts using a Sherwood Model 410 Flame Photometer (Sherwood Scientific Ltd, Cambridge, UK). Soil characteristics are summarised in Table 6.1.

Table 6.1. *Characteristics of treated soils used in this study. Values are expressed on a mean dry soil weight basis ± SEM (n = 5). Letters denote significant differences between treatments using a Kruskall-Wallis with Dunn Post-hoc test and Bonferroni correction (p < 0.05).*

	Aerobic			Anaerobic			
	Control	**Residues**	**Urine**	**River water**	**River water + residues**	**Sea water**	**Sea water + residues**
Texture				Sandy clay loam			
Soil moisture (%)	$29.3^A \pm 1.2$	36.2 ± 1.3	$29.8^{B,C} \pm 0.5$	74.1 ± 1.8	$83.1^{A,B} \pm 1.5$	68.2 ± 1.1	$86.5^C \pm 1.3$
pH	$6.14^A \pm 0.15$	$5.71^{B,C} \pm 0.06$	$7.53^{A,B,D} \pm 0.11$	6.51 ± 0.08	$6.90^C \pm 0.05$	$6.23^D \pm 0.09$	6.88 ± 0.06
EC (μS cm^{-1})	27.1 ± 11.7	54.0 ± 6.3	72.3 ± 2.0	$3.3^{A,B} \pm 0.1$	21.0 ± 1.8	$563.6^A \pm 7.1$	$661.6^B \pm 18.9$
ORP (mV)	$413.5^{A,C} \pm 4.9$	325.6 ± 9.8	216.5 ± 2.8	$24.5^{B,D} \pm 4.7$	$-73.7^{A,B} \pm 3.6$	141.5 ± 11.3	$-144.9^{C,D} \pm 16.2$
Total C (%)	2.63 ± 0.05	2.97 ± 0.18	2.44 ± 0.05	2.44 ± 0.11	2.90 ± 0.12	2.59 ± 0.08	2.6 ± 0.10
Total N (%)	0.28 ± 0.01	$0.36^{A,B} \pm 0.02$	$0.32^C \pm 0.01$	$0.25^A \pm 0.01$	0.3 ± 0.01	$0.25^{B,C} \pm 0.01$	0.3 ± 0.01
C:N ratio	9.40 ± 0.31	$8.22^C \pm 0.15$	$7.74^{A,B,D} \pm 0.17$	$9.21^A \pm 0.57$	$9.53^B \pm 0.16$	$9.97^{C,D,E} \pm 0.22$	$9.42^E \pm 0.44$
Dissolved organic C (mg C kg^{-1})	$51.0^{A,C} \pm 2.1$	87.3 ± 14.3	171.6 ± 10.5	67.1 ± 4.8	$240.3^{A,B} \pm 8.6$	$58.4^{B,D} \pm 4.7$	$303.3^{C,D} \pm 45.1$
Extractable NO$_3^-$ (mg N kg^{-1})	36.3 ± 1.7	$162.8^{A,B,D} \pm 17.0$	$70.8^C \pm 6.4$	$0.65^A \pm 0.16$	1.02 ± 0.23	$0.37^{B,C} \pm 0.24$	$0.77^D \pm 0.18$
Extractable NH$_4^+$ (mg N kg^{-1})	$3.33^{A,C} \pm 1.24$	51.32 ± 6.57	$487.04^{A,B} \pm 31.59$	$2.70^B \pm 0.50$	81.97 ± 4.66	3.28 ± 0.56	$98.72^C \pm 5.78$
Extractable P (mg P kg^{-1})	2.61 ± 0.33	2.47 ± 0.24	$3.12^C \pm 0.11$	$4.21^{A,B,D} \pm 0.27$	$1.90^A \pm 0.08$	$2.05^B \pm 0.16$	$1.74^{C,D} \pm 0.04$
Exchangeable Na (mg Na kg^{-1})	$13.8^{A,C} \pm 1.5$	28.3 ± 2.2	80.2 ± 5.9	$17.9^{B,D} \pm 1.1$	22.3 ± 2.5	$5477^{A,B} \pm 344$	$6490^{C,D} \pm 99$
Exchangeable K (mg K kg^{-1})	$89.5^{A,C} \pm 11.1$	406.8 ± 33.9	$646.9^{A,B} \pm 35.9$	$121.2^{B,D} \pm 8.6$	321.7 ± 74.3	376.2 ± 25.1	$671.8^{C,D} \pm 20.0$
Exchangeable Ca (mg Ca kg^{-1})	$1099^A \pm 77$	1083 ± 90	$1169^B \pm 133$	1455 ± 117	$1689^{A,B} \pm 116$	1262 ± 61	1587 ± 1028
Bacterial/Fungal PLFA ratio	0.06 ± 0.00	0.10 ± 0.01	$0.07^A \pm 0.01$	0.07 ± 0.00	0.07 ± 0.01	$0.13^B \pm 0.06$	$0.04^{A,B} \pm 0.01$
Microbial biomass (μmol PLFA kg^{-1})	$110.2^{A,B,D} \pm 2.6$	$177.6^A \pm 11.0$	145.0 ± 6.1	$128.7^C \pm 3.6$	$215.6^{B,C} \pm 14.1$	158.7 ± 20.0	$209.4^D \pm 27.3$

EC - electrical conductivity, ORP - redox potential.

Characterisation of the soil microbial community was performed by PLFA analysis. Homogenised soil was stored at -20 °C prior to, and post lyophilisation using a Modulyo Freeze Dryer with RV pump (Edwards, Crawley, UK). 15 g samples were shipped, on dry ice, to Microbial ID Inc. (Newark, DE, USA), extracted, fractionated, and transesterified using the high throughput method of Buyer and Sasser (2012). Subsequently, samples were analysed using an Agilent (Agilent Technologies, Wilmington, DE, USA) 6890 gas chromatograph (GC) equipped with autosampler, split–splitless inlet, and flame ionization detector. The system was controlled by MIS Sherlock® (MIDI, Inc., Newark, DE, USA) and Agilent ChemStation software. GC-FID specification, analysis parameters and standards can be found in Buyer and Sasser (2012).

6.3.3. VOC extraction, collection, analysis and data processing

Duplicate soil samples, as aforementioned in section 6.2.1, were stored at 4 °C before analysis. VOCs were collected using headspace solid-phase microextraction (HSSPME) due to the method's suitability for evaluating complex sample matrices. A multi-component solid-phase microextraction (SPME) fibre (50/30 µm DVB/CAR/PDMS; Supelco, Bellefonte, USA), was selected due to both its sensitivity to a wide range of non-polar compounds and the thickness of the fibre (Cserháti 2010), which allowed transfer of the fibre from the extraction vial into the GC without the likelihood of significant loss of adsorbed volatile compounds. This was attached to a manual SPME holder (Supelco, Bellefonte, USA) for sampling.

Briefly, the SPME fibre was conditioned in the GC injection port at 270 °C prior to each soil being sampled, until no interfering peaks were obtained in blank injections. Fresh soil (3.5 g) was deposited into a glass vial (10 mL) (Supelco, Bellefonte, USA) alongside a magnetic stirrer bar for agitation, and subsequently capped with a SPME compatible PTFE/silicone septum (Supelco, Bellefonte, USA). To equilibrate the samples, they were agitated in a thermostat bath (80 °C) for 60 min, to decrease the partition coefficient between the sample and the headspace, and increase the vapour pressure (Zhang and Pawliszyn, 1993). A preconditioned SPME fibre was then manually inserted through the septum into the vial and exposed to the headspace for 20 min. The SPME fibre was then withdrawn into the holding sheath, removed from the vial and directly (< 30 s) desorbed into a GC-MS injection port at 250 °C in split mode (1:10 split ratio) for 2 min. A gas chromatograph (7890B; Agilent Technologies, Palo Alto, USA) interfaced to a quadrupole time-of-flight mass spectrometer

(7200B; Agilent Technologies, Palo Alto, USA) (GC/Q-TOFMS) was used for compound identification. Chromatographic separation was obtained on a non-polar column (HP-1 50 m length x 0.32 mm id x 0.17 µm D_f; Agilent Technologies, Palo Alto, USA) using a He carrier gas with a flow of 2 mL min^{-1}. The oven temperature was programmed from 60 °C to 250 °C at 4 °C min^{-1}. The transfer line and ion source temperatures were both set to 300 °C. Using the electron ionisation (EI) mode at 70 eV, mass spectra were acquired across an *m/z* range of 25-400. Compound identification was attained using the NIST Mass Spectrometry library (Stein et al., 2014).

6.3.4. Treatment analysis

The lysed grass treatments' moisture content was determined gravimetrically by oven drying (80 °C, 24 h). Subsequently, grass C:N ratios were determined on the oven-dried and ground samples using a TruSpec® Analyzer. The sheep urine treatment was analysed for dissolved organic C (DOC) and total dissolved N (TDN) using a Multi N/C 2100S Analyzer. River and sea water samples were analysed for pH and EC using standard electrodes in addition to colorimetric determination of NO_3-N, NH_4-N, and P as per the methods described above, TOC and TN were also measured using the Multi N/C 2100S Analyzer. Treatment characteristics are summarised in Appendix 4 Table 1.

6.3.5. Data and statistical analysis

The raw VOC data files were processed using MassHunter Workstation Profinder version B.08.00 (Agilent Technologies, Palo Alto, CA, USA). Feature extraction was achieved using the Batch Feature Extraction algorithm with the subsequent processing parameters: peak filters = 500, ion count threshold = 5, retention time tolerance = 0.3 min, absolute height = 10000 counts, *m/z* range = 25-250, retention time range = 5-35 min. This processing step produced a data output for each independent entity in the form of [intensity × retention time × mass]. These data were then input into MassHunter Mass Profiler Professional version B.14.5 (Agilent Technologies, Palo Alto, CA, USA) and log_{10} transformed and normalised using an external scalar. To moderate the number of features subjected to statistical analysis, data filtering was executed. Briefly, a frequency filter (entities present in >90% of samples in at

least one sample group), fold change filter (14.0) and sample variability filter (coefficient variable < 25%) were applied. This approach identified 18 discriminatory compounds, summarised in Fig. 6.2. Total relative VOC production was also quantified by summing the areas of all peaks considered from each sample's chromatogram, examples of which are shown in Appendix 4 Fig 2.
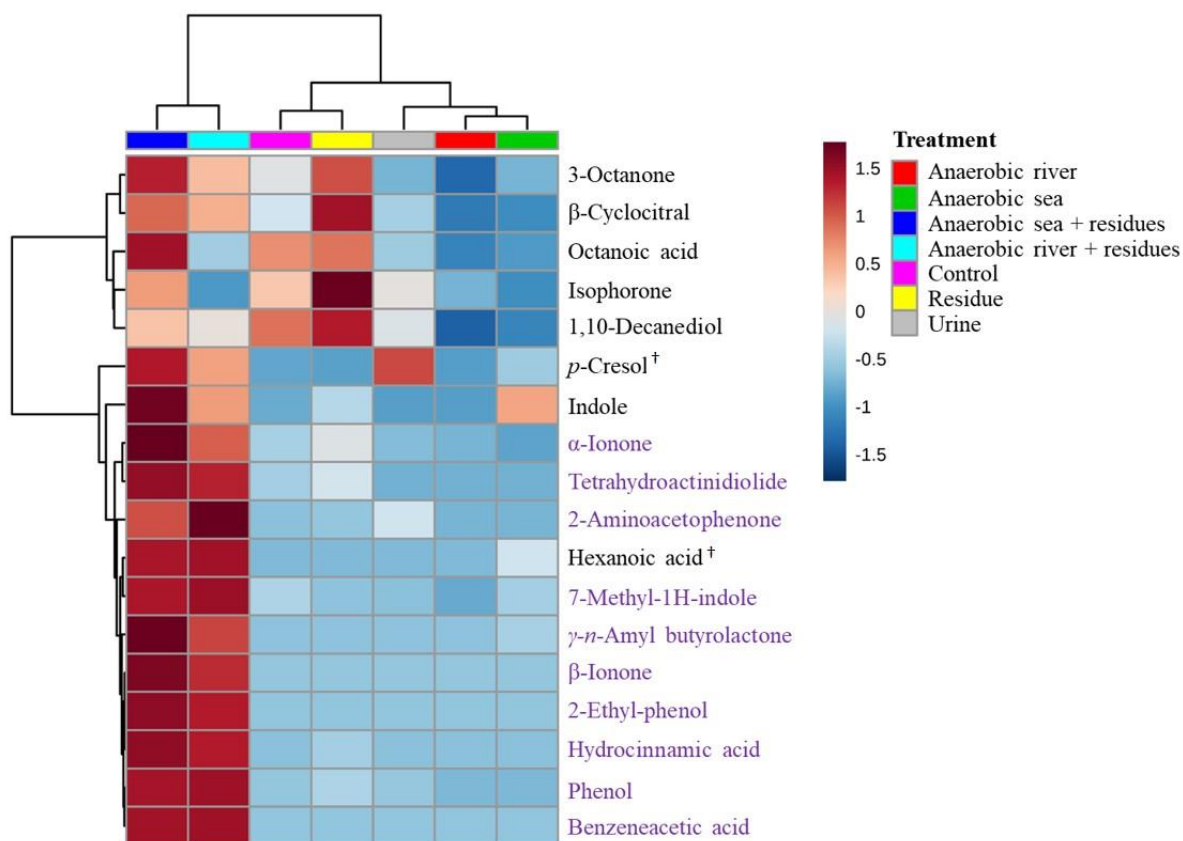
Figure 6.2. *Heatmap of eighteen VOC compounds identified as driving changes between soil treatments. Compounds highlighted in purple are found in significantly higher concentrations in anaerobic + residues treatments. Compounds highlighted with † were not significantly different between treatments ($p > 0.05$). Darker red = large positive relative difference between treatments, darker blue = large negative relative difference between treatments. Samples are ordered using group averages and clustered using a Ward algorithm. Compound structures are shown in Appendix 4 Fig. 1.*

All of the following statistical analysis was performed in R v3.5.3 (R Core Team, 2019). In the analysis of discriminatory VOCs, relative peak heights were used to effectively compare samples based on the chemical diversity of compounds. A non-metric dimensional scaling

(NMDS) approach was used to condense the multivariate VOC data in a comprehensible number of dimensions and visualize the relative degree of similarity among samples using the whole VOC dataset, which was performed using the 'vegan' package. NMDS was also used to analyse PLFA data. All PLFAs detected were used in the analysis, to represent the whole microbial community. Heatmap analysis and an ANOVA with Tukey post-hoc test was performed on $\log_{10}$ transformed and pareto-scaled discriminatory VOC compound data in 'metaboanalyst 4.0' (Chong et al., 2018). This package was also used to perform hierarchical cluster analysis on $\log_{10}$ transformed and pareto-scaled PLFA and VOC data. An ANOVA was also used to test $\log_{10}$ transformed total relative VOC production as well as total number of peaks identified between treatments. Significant differences were further explored using a Tukey HSD post-hoc test. The relationships between total VOCs and number of VOCs to key soil parameters were tested using Spearman correlation analysis, using the 'corrplot' R package (Wei and Simko, 2017). For all analyses the significance threshold was set at $p \leq 0.05$.

6.4. Results

6.4.1. Treatment driven changes in VOCs and PLFAs

NMDS analysis was used to show the clustering for all VOC and PLFA compounds, respectively, produced under the soil treatments. Both, VOC (Fig. 6.3A) and PLFA (Fig. 6.3B) analysis show separation between anaerobic + residues treatments compared to other treatments, implying a different microbial community and production of VOCs under these conditions. Using VOC analysis, anaerobic treatments without residues also grouped closely. Hierarchical clustering analysis (Fig. 6.4A and 6.4B) further illustrated the relationships between treatment levels, with results largely supporting NMDS findings. Overall, VOC analysis was more able to differentiate between anaerobic + residues treatments and aerobic treatments, with anaerobic treatments without residues and urine treatments being more closely related than they appeared using NMDS analysis. PLFA analysis was less able to separate treatments from one another.
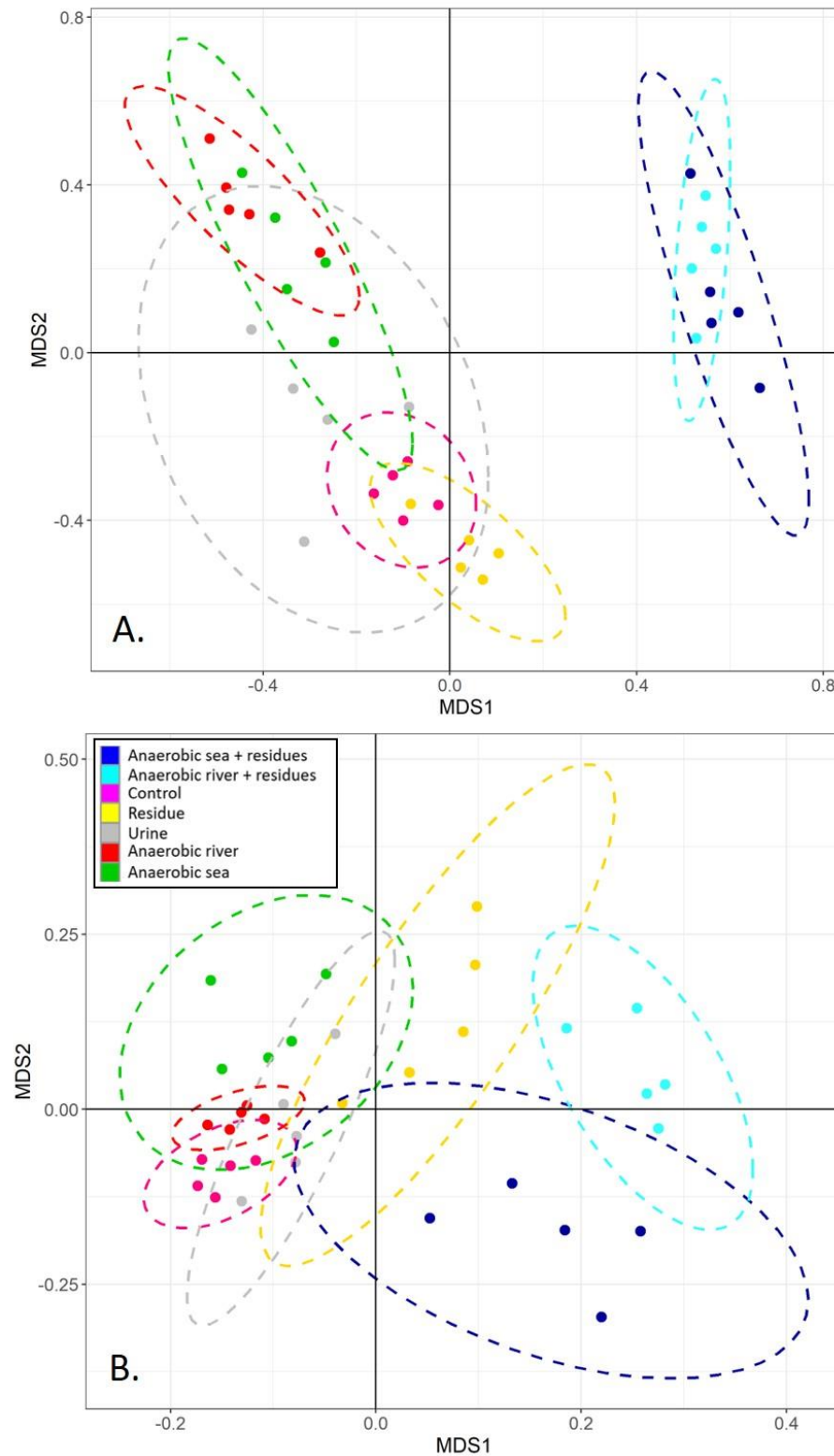
Figure 6.3 *A) NMDS plot of VOC profiles of each soil treatment. B) NMDS plot of PLFA profile for each soil treatment. Ellipses represent 95% confidence intervals for each treatment. The legend is the same for both panels.*
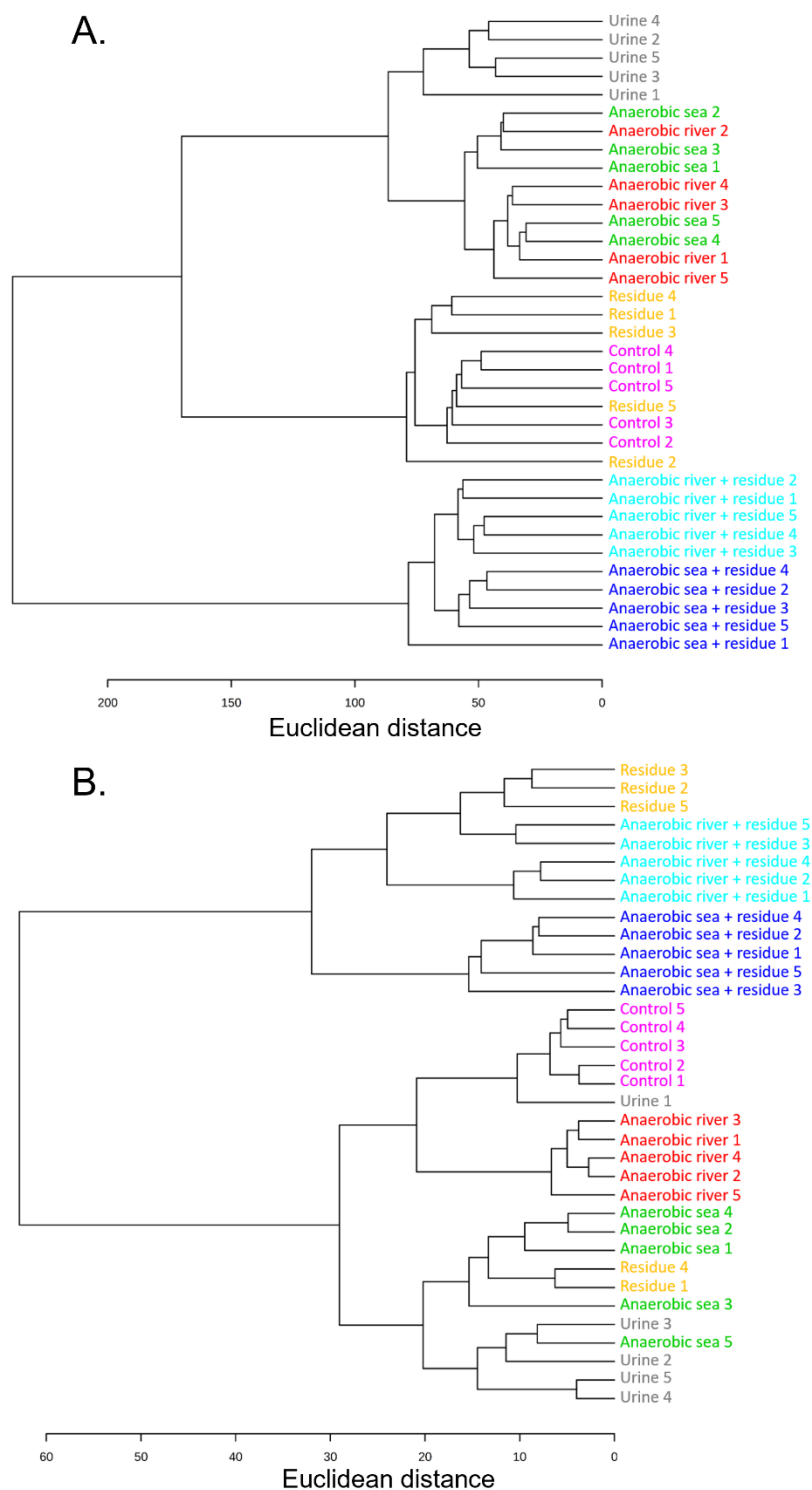
Figure 6.4 *A) Dendrogram, using Euclidean distance measure and a ward clustering algorithm, of VOC profiles of each soil treatment (n = 5). B) Dendrogram, using Euclidean distance measure and a ward clustering algorithm, of PLFA profiles of each soil treatment replicate (n = 5).*

6.4.2. Relative VOC production between treatments

Total relative VOC production was significantly higher from anaerobic + residues treatments ($p < 0.001$) compared to other treatments. Aerobic residue treatments also produced significantly more VOCs than anaerobic without residues and urine treatments ($p < 0.001$). Across all soil treatments 514 unique VOC peaks were detected. The total number of VOC compounds detected was significantly lower for both anaerobic + residues treatments ($p < 0.05$). Control and urine treatments had a significantly higher number of VOC compounds detected ($p < 0.05$).

6.4.3. Soil factors affecting VOC production

Several key soil properties were found to be significant predictors of both, the relative total concentration of VOCs and the number of VOC compounds emitted from soil samples. Particularly, organic matter content, dissolved organic carbon and PLFA microbial biomass were significantly correlated with total VOC concentration and number of VOCs emitted (summarised in Table 6.2). ORP and soil moisture were also significantly correlated to the number of VOCs emitted from soil.

Table 6.2. *Pearson's correlation coefficients for key soil properties in relation to both total amount of VOCs emitted, and the number of VOCs emitted from samples. NS signifies not significant (i.e. p > 0.05).*

	Total VOCs	No. VOCs
pH	*NS*	*NS*
ORP (mV)	*NS*	0.576
Soil moisture (%)	*NS*	-0.64
Organic matter (%)	0.361	-0.425
Total dissolved N (mg kg^{-1})	*NS*	*NS*
Total dissolved organic C (mg kg^{-1})	0.385	-0.391
Microbial biomass (µmol PLFA kg^{-1})	0.388	-0.489

6.4.4. Identities of discriminatory VOCs produced

Across the 7 treatments, software was able to identify 18 VOC compounds that were able to differentiate between the control and treated samples; these compounds are subsequently referred to as discriminatory compounds (Fig. 6.2). Of the 18 compounds, 16 were found to have significant differences between treatments when tested statistically (*p*-cresol and hexanoic acid were not significantly different between treatments). Additionally, 10 compounds were found in significantly higher concentrations in anaerobic + residues treatments compared to the other treatments ($p < 0.001$), as highlighted in Fig. 6.2.

6.5. Discussion

6.5.1. Identification of VOC profile trends

NMDS and hierarchical clustering of VOC data (Fig. 6.3A and 6.4A) illustrate the clustering of anaerobic + residues treatments, suggesting that nutrient-rich anaerobic conditions had the greatest impact upon the VOC profile of the soils relative to the control soils. Anaerobic + residues treatments also had the highest levels of total VOCs, but the lowest number of individual compounds detected. This suggests that addition of plant residues under anaerobic conditions caused a large increase in the concentration, but not number, of VOCs detected. It is generally understood that VOCs are found in greater concentrations under anaerobic conditions, due to the production of metabolic end-products by anaerobic fermentation and extracellular degradation of complex organic metabolites (Insam and

Seewald, 2010; Seewald et al., 2010; Stotzky et al., 1976). However, the results of this study show this to be conditional on the amount of available nutrients and C under anaerobic conditions, as soils under anaerobic conditions but with no plant residues produced significantly less total VOCs than anaerobic soils with plant residues. This is likely due to a lack of easily assimilable C in non-residue amended anaerobic samples, as well as lower levels of microbial activity and thus VOC production. However, although anaerobic + residues treatments resulted in a greater total VOC concentration compared to other treatments, the number of compounds contributing to the signal produced was significantly less. This suggests a limited range of metabolic reactions are responsible for producing the majority of the soil VOC profile, as microbial metabolism is the dominant source of VOC emissions from soil (Leff and Fierer, 2008).

Control and urine amended soils produced a significantly greater number of VOC compounds compared to other treatments. Under the control treatment, it is likely that the soil microbial community will be highly adapted to the prevailing conditions (i.e. low stress) and thus more likely to have a streamlined metabolism and high C use efficiency. Most of the C used for energy production will produce only CO_2 as an end-product rather than respiratory-derived VOCs (e.g. ethanol, volatile organic acids) or those used in secondary metabolism for stress alleviation (Insam and Seewald, 2010). Under these low stress conditions, the large number of different VOC compounds are likely to be characteristic of a diverse soil microbial community (McNeal and Herbert, 2009). Conversely, under the urine treatment, the input of nutrients into the system, particularly K and soluble N (e.g. urea NH_4^+), is likely to induce a rapid change in soil microbial metabolism (Waldrop and Firestone, 2004; Williams et al., 2000). However, it is also likely that the urine itself will contain some unique VOCs (Deev et al., 2020; Mozdiak et al., 2019). The experimental set up used here, however, was unable to distinguish between the direct and indirect effects of urine on the soil VOC profile.

6.5.2. Sources of discriminatory VOC compounds

Following the positive identification of 18 discriminatory VOCs, the abundance of which could be used to determine several soil characteristics key in determining a soils quality (e.g. SOM, levels of oxygen content), prospective sources and functions of the compounds were examined using the existing literature. Notably, of these compounds, 10 were found in

significantly higher relative concentrations in the anaerobic + residues treatment, several of which can be identified as breakdown products of the residues.

For example, the biodegradation of carotenoids is the most likely source of β-ionone and α-ionone. Carotenoids form part of a group of terpenoid organic pigments that play a major role in photosynthesis in addition to the photoprotection of photosystems in plants and photosynthetic bacteria (Lobo et al., 2012). These compounds were only produced under anaerobic + residues treatments suggesting that either under aerobic conditions these molecules were further catabolised or were only produced under nutrient-rich anaerobic conditions.

Benzeneacetic acid, an auxin, was solely identified in anaerobic soils with added grass residues. This suggests that under aerobic conditions all the benzeneacetic acid was mineralised. Alternatively, benzeneacetic acid may only be produced by anaerobic bacteria. For example, it has been reported that *Azoarcus evansii*, an endophytic facultative anaerobic denitrifying bacterium found in several grass species, is a significant producer of benzeneacetic acid (Schulz and Dickschat, 2007; Sun et al., 2019).

Despite many possible sources of the discriminatory compounds, linking a VOC to specific processes, functions or microorganisms is challenging due to the variety of degradation pathways which a VOC can take within the soil, dependant on environmental conditions (i.e. oxygen and nutrient status). Furthermore, the necessity to increase the temperature of the sample during the extraction procedure in order to increase the partition coefficient, generates a degree of ambiguity as to the true source of the compounds in the headspace, as under such conditions there is a possibility of the breakdown of thermolabile compounds within the sample (Kaspar et al., 2008). Accordingly, it is uncertain whether the compounds extracted are a samples' intrinsic VOCs; released by the microorganisms within the soil or as a direct result of an amendment (e.g. urine), or VOCs produced during the extraction procedure due to thermal degradation of the sample. For example, lignin is widely described as stable below temperatures of 100 °C, however, the thermal stability of many soil components is poorly reported within the literature (Brebu and Vasile, 2010).

Additionally, there is potential discrepancy between the production of a volatile compound and its emission from matrix. The emission of VOCs from the soil matrix, is highly dependent on the soil's structure and moisture as well as the rate at which the VOCs are being emitted. These factors ultimately control the rate of VOC production, dispersal and consumption (Aochi and Farmer, 2005). Similarly to greenhouse gases, it is likely that soils act

as sources or sinks for VOCs depending on environmental conditions (Insam and Seewald, 2010; Oertel et al., 2016). For example, VOCs produced further down the soil profile could be consumed or degraded before reaching the soil surface. Particularly in aerobic systems, when in a steady state equilibrium these production-consumption systems may cause emission of very few VOCs, however, on disturbance, emissions may increase. Heating during analysis, to improve the partition co-efficient (the ratio of analyte in matrix-to-analyte in headspace) and drive VOCs into the headspace, is likely to increase the emissions from the soil sample (Turner et al., 2019).

6.5.3. Relationship between soil properties and VOC emission

Overall, levels of soil moisture seemed to be the best predictor of the number of VOCs produced from samples, with higher levels of soil moisture reducing the number of VOCs emitted. Similarly, ORP was positively correlated to the number of VOCs produced. Under anaerobic, reducing conditions it is likely that alcoholic sugar fermentation predominates (Pezeshki and DeLaune, 2012). Previous studies, e.g. Stotzky et al. (1976) and Seewald et al. (2010), have shown that anaerobic conditions increase the diversity and amount of VOCs emitted. However, this study showed that this was only the case if conditions were not nutrient limited. Both anaerobic treatments + residues additions resulted in an increase in total VOCs emitted but in a reduction in the number of VOC compounds emitted. As shown previously, substrate availability and quality are key in the production and emission of VOCs (Wheatley et al., 1996). However, there was no correlation between total dissolved nitrogen and total VOCs or number of VOCs produced, and only a weak correlation between available inorganic N (NO_3 and NH_4) and total VOCs emitted.

6.5.4. Critical analysis of VOCs and PLFAs as soil quality indicators

Whilst both VOCs and PLFAs can be related directly to the soil biological community, each offer very different types of insight. PLFAs focus on the membrane lipid composition, from which links can be made to microbial community composition (Mann et al., 2019). However, use of isotopic substrates can enable PLFA to be used as indicators of soil function, i.e. Bull et al. (2000). VOCs focus on secondary metabolism of the soil biological community,

which relates to community function. Advantages and disadvantages of each method are summarised in Table 6.3.

Sensitivity is a key attribute in biological quality monitoring, as methods must be able to detect subtle changes in soil biochemistry to give an accurate representation of soil quality. Both PLFA and VOC analysis are, in theory, sensitive, as both sets of compounds degrade rapidly under environmental conditions (Li et al., 2019; Zhang et al., 2019). However, this experiment has demonstrated that, in terms of discriminatory power, VOCs can more robustly separate the impact of short-term soil treatments, which correspond to different soil qualities (Fig. 6.3 and 6.4).

Table 6.3. *Summary of the advantages and disadvantages of VOC and PLFA analysis in the analysis of soil quality.*

	VOC analysis	PLFA analysis
Advantages	Functionally relevant Rapid extraction and analysis. Microbial culture not required. Rapid degradation - offers snapshot of current state of biological activity. Possibility of identifying characteristic biomarkers for specific conditions. Non-destructive.	Sensitive and reproducible. Measure of biomass and community structure. Microbial culture not required. Rapid degradation - offers snapshot of current state of biology. Lack of group or species-specific FA's
Disadvantages	Difficult to separate plant and microbial derived VOCs in soil. Lack of fundamental understanding of VOCs in soil. SPME requires destructive sampling. Most relationships have been investigated using laboratory/ pure culture studies – may not reflect diverse soil community response. Medium sample throughput with possibility of automation. High analytical capital costs.	Time consuming and complex extraction required. Little functional relevance. Extraction of entire microbial community – not only the active fraction. Most relationships have been investigated using laboratory/ pure culture studies – may not reflect diverse soil community response. Low sample throughput. High capital and labour costs. Destructive.

Both PLFAs and VOCs relate to the biological function of soil. However, the turnover of VOCs is more rapid and more functionally relevant than PLFAs. The impact of many environmental factors e.g. pH, heat and moisture content are similar between both types of analysis. One advantage of VOC analysis over PLFA analysis is the lack of pre-treatment and multi-stage chemical extraction required, reducing the amount of inherent bias within the method. However, identification of individual VOCs is difficult; while databases exist, they are by no means extensive, and identification may not be absolute without the use of confirmatory standards.

6.5.5. Future research direction

This study highlights several potential future research areas within the soil and environmental sciences. Specifically: Is it possible to increase the rapidity of VOC analysis, by reducing extraction and/ or analysis time, while retaining the resolution and sensitivity to enable compound identification? How do *ex-situ* sampling techniques demonstrated in this study compare to *in-situ*, non-destructive, methods, for example, sorbent tube sampling? To what extent are abiotic VOCs contributing to the overall soil VOC profile compared to biotic VOCs? Under different soil types and cropping regimes, does the core microbiome have similar metabolism and therefore produce similar VOCs, i.e. is analysis of VOCs applicable over large spatial scales? And what is the temporal frequency of analysis required to accurately assess a soils quality? Addressing these questions will further advance sampling and analysis of VOCs in soil and elucidate the role of VOCs as indicators of changes in soil quality. Another key aspect is defining the boundary values for VOC concentrations and profiles for 'healthy' soils. Arguably, the control and aerobic residues treatment in this study have started to provide some data to represent healthy soils.

6.6. Conclusions

This study applied a HSSPME extraction and analysis method for the determination of VOCs in soils. The method was applied to seven soil treatments representing a range of soil qualities and a number of discriminatory compounds were identified. When compared to PLFA analysis, VOC analysis was better able to differentiate between soil treatments. 18

discriminatory compounds were identified, 10 of which were associated with anaerobic treatments with residues. Contrary to previous findings, VOCs under anaerobic treatments with residue additions exhibited a relative increase in concentration of total VOCs emitted but a reduction in the abundance of specific compounds emitted. These results demonstrate the potential of secondary metabolites as an indicator of soil quality and highlights the need for further research into soil VOC analysis to understand nutrient cycling and metabolism as well as the effect of *in-situ* vs. *ex-situ* sampling.

6.7. Acknowledgements

6.8. References

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Chapter 7: Dependence of thermal desorption method for profiling volatile organic compound (VOC) emissions from soil

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RB, JM and DJ conceptually conceived and designed the experiment. RB prepared soil samples and performed soil analysis. JM performed VOC analysis. RB performed data analysis and interpretation and wrote the first draft of the manuscript. All authors contributed to subsequent revisions.

7.1. Abstract

The study of volatile organic compounds (VOCs) in soil and other complex substrates is a rapidly developing field. VOCs in soils originate from a wide variety of biological sources; bacterial, fungal, mesofaunal, and plant. They are vital to inter- and intra-species interaction and soil health, and therefore offer a potential reactive, functional diagnostic tool to determine soil quality. The standard methodology for untargeted VOC profiling in environmental samples has been headspace solid phase microextraction (HS-SPME), avoiding the need for solvent extraction procedures used in many biological soil tests. However, this technique can suffer from a lack of sensitivity due to competition between individual VOCs on the solid phases used for VOC recovery. Other common techniques used to monitor the VOC fingerprints from soils include high capacity sorptive extraction (HCSE). This study presents a novel SPME-trap-enrichment method using an automated, cryogen-free, focussing and pre-concentration trap method to reduce phase competition and increase sensitivity of analysis. This method was evaluated against single-SPME-trap and HCSE methods for sensitivity and number of compounds identified using a range of induced soil qualities (good, medium and poor). Results showed that SPME-trap-enrichment was able to identify 71% and 7% more compounds than single-SPME-trap and HCSE, respectively, using a software-based data processing approach, as well as increased total peak areas. The VOC profile was substantially affected by the extraction method used. The samples within the 'poor' treatment produced a larger number of aromatic, carboxylic acid and sulphur containing compound groups, while the 'good' and 'medium' groups were largely characterised by ketones and aldehydes. The potential ecological significance of the compounds identified were also explored. Of those tested, SPME-trap-enrichment represented the most robust and sensitive technique for VOC analysis, offering the potential to better elucidate the multifaceted interactions of VOCs within plant-microbial-soil systems.

Keywords: Soil quality, Volatilomic profiling, Methods, Secondary metabolites, Complex substrates

7.2. Introduction

The ability of soil to function and sustainably provide an increasing food supply for a rapidly growing global population has become of vital worldwide importance (Gomiero, 2016). Traditionally, soil health; a measure of a soil's ability to function, has been determined on a physicochemical basis with biological characteristics often overlooked (Bünemann et al., 2018). However, microbiology is extremely responsive to environmental and functional change within the soil system (Lehman et al., 2015). One biochemical method proposed as a functional, diagnostic indicator of soil health is profiling or fingerprinting the volatile organic compounds (VOCs) emitted from soil (Brown et al., 2021).

In biochemistry, volatile organic compounds (VOCs) form part of secondary metabolism; small molecule products derived from primary metabolism that are not essential to the survival of an organism (Keller, 2019), with most microbial volatiles formed either as end products of fermentative pathways of primary metabolism or intermediates of detritus decomposition (Korpi et al., 2009; Seewald et al. 2010). These molecules are key to inter- and intra- species signalling and interaction (Schulz-Bohm et al., 2017) and the delivery of a range of soil-based ecosystem services (Choudoir et al., 2019). Further, the type and quantity of VOCs produced in soil can have a considerable effect on below-ground ecology as well as the soil odour profile (Insam and Seewald, 2010; Kesselmeier and Staudt, 1999). Emission and cycling of VOCs are also highly dependent on the health of an organism, its ontogenetic stage and the prevailing environmental conditions (e.g. temperature, availability of water) as well as the amount, composition and bioavailability of organic matter (Insam and Seewald, 2010; Leff and Fierer, 2008). Therefore, the study of volatile compounds emitted from the soil has potential in the determination of biological soil quality. However, the chemical diversity of VOCs emitted from soils and other environmental matrices is often a hindrance to identification (Peñuelas et al., 2014; Stahl and Klug, 1996). As soil is a complex matrix with solid, liquid and biological elements, extraction and analysis, and subsequent data interpretation of VOCs is challenging.

A large variety of sample techniques for VOCs exist. Traditionally, analysis of VOCs was performed using solvent extraction or vapour partitioning methods (Hewitt, 1998). However, the issues of preconcentration of analytes, as well as extraction biases of solvent-based approaches remain challenging (Demeestere et al., 2007; Wardencki et al., 2007) and with a move toward green chemistry, solvent-less methods are now well established (Sheldon, 2018, 2005). The most common solvent free techniques for the extraction of volatiles from soil

is passive head space (HS) solid phase micro-extraction (SPME) (Wypych and Mañko, 2002). However, a drawback of single HS-SPME extraction is the large amount of competition for phase, leading to only the most competitive VOCs being adsorbed to the SPME fibre and consequently analysed, making phase compound choice critical for sensitive and targeted extraction (Pawliszyn, 2000).

Annotation of small molecules using high resolution, untargeted, mass spectrometry based metabolomic and volatilomics analysis remains a major challenge. With the accuracy of analysis techniques ever increasing, the potential to separate and identify a larger number of metabolites is becoming increasingly possible allowing for a more comprehensive view of the metabolome and volatilome. However, the inability to include calibration standards to confirm the wide range of compounds identified in complex samples remains a consistent issue. Software-based identification approaches offer an alternative to conventional preparation of analytical standards (though only to level 2 (putative annotation)); however, they are limited by compound database sizes, the chromatographic resolution of equipment and the ability of the algorithm to identify compounds. It is estimated that $1 - 2$ million of the > 120 million small molecule compounds currently contained with databases are of biological relevance (Aksenov et al., 2017; Milman and Zhurkovich, 2017). However, most metabolites and small molecule compounds elucidated using untargeted analysis remain unassigned, resulting in significant amounts of information about the biochemical system, and environmental interaction, being lost.

Recent developments in automation and pre-concentration technologies have allowed the development of a HS-SPME-trap-enrichment method (Mascrez and Purcaro, 2020a). Traditional approaches to HS-SPME-trap-enrichment or multiple head space (MHS)-SPME offered poor precision as each extraction is analysed by GC-MS separately and an estimated total peak area subsequently calculated from the combined data (Costa et al., 2013; Liang et al., 2019). This requires very stable retention times across runs to accurately match and identify compounds. HS-SPME-trap-enrichment offers the potential to extend VOC extraction by reducing phase competition within the headspace and increasing the sensitivity to compounds with high and low boiling points. It also ensures stability of retention times for accurate compound identification by 'stacking' samples onto a trap and analysing several extractions in one chromatographic run (Górecki et al., 1999; Jeleń et al., 2017).

This study presents a novel method of HS-SPME-trap-enrichment for sampling VOCs from soil, a complex environmental sample, with comparisons to single HS-SPME as well as a high capacity sorptive probe (HS-HCSE). HS-HCSE takes a similar approach to HS-SPME

but offers a larger (100x more) phase volume compared to SPME, thus reducing phase competition equally. Each of these techniques were combined with an innovative cryogen-free focussing and pre-concentration trap, meaning i) all extraction techniques were run on a single platform without the need to change the hardware, and ii) single (SPME-trap) and multiple extractions (SPME-trap-enrichment) were carried out automatically on samples. The aims of the study were to compare the methods for sensitivity and recovery, and to explore the VOC emissions profile associated with each soil treatment.

7.3. Materials and methods

7.3.1. Sample collection and treatment

Independent replicate soil samples ($n = 3$) of a Eutric Cambisol (0 - 10 cm) were collected from a postharvest wheat (*Triticum aestivum* L.) field located at Henfaes Agricultural Research Station, Abergwyngregyn, North Wales (53°14′N; 4°01′W). Further details of the site, management regime and climate can be found in Zang et al. (2020). After collection, soil was sieved through a 2 mm mesh to remove stones and plant material and to ensure sample homogeneity and stored for 2 weeks at 4°C to allow the samples to equilibrate. Mesocosms were created by weighing 300 g of sieved soil into sealable 2 l polypropylene sample containers and treatments applied. Treatments were designed to simulate soils under 'good', 'medium' and 'poor' soil quality. They consisted of the following; (i) 'medium' – no amendment, (ii) 'good' – 5 % soil improver (Verve topsoil conditioner, B&Q, Eastleigh, UK) loading (1:20 w/w soil improver-to-soil ratio), and (iii) 'poor' – 5 % grass residue loading (1:20 w/w lysed grass-to-soil ratio) saturated with river water (to generate anaerobic conditions). Grass residues (green *Lolium perenne* L. shoots) were collected from a nearby un-grazed pasture, lysed by freezing for 1 h at -80°C and then incorporated into the soil. Anaerobic mesocosms were saturated with oligotrophic river water collected from the Afon Rhaeadr-fawr (53°14'N; 4°01'W) on the day the treatments were set up and the meoscosms sealed with a gas-tight lid. Amendment characteristics are summarised in Appendix 5 Table 1. Further details of the river water can be found in Sánchez-Rodríguez et al. (2018). The aerobic mesocosms ('good' and 'medium' treatments) remained unsealed throughout the experiment to allow gas exchange and were maintained in a field moist state throughout the experiment via the addition of deionised water. Mesocosms were subsequently incubated at 25°C for 10 d to stimulate soil microbial

activity and create a new biochemical equilibrium. At the end of the incubation period the mesocosms were destructively sampled as described in Sections 2.2 and 2.3.

7.3.2. Soil analysis

At destructive harvesting, excess water was drained from the 'poor' mesocosms. Subsequently, the soil in all treatments was homogenised by hand for 10 s to ensure a representative sample was obtained for soil chemical and physical analysis. Briefly, soil moisture content was determined gravimetrically by oven drying (105°C, 24 h) and soil organic matter was determined by loss-on-ignition (450°C, 16 h) (Ball, 1964). Soil pH and electrical conductivity (EC) were measured using standard electrodes submerged in 1:5 (w/v) soil-to-deionised water suspensions. The oxidation–reduction potential (ORP) was measured directly in the soil using a SenTix® ORP-T 900 combination electrode (Xylem Analytics, Weilheim, Germany) connected to a mV reader. C:N ratio was determined on oven-dried, ground soil using a TruSpec® Analyzer (Leco Corp., St. Joseph, MI).

Within 12 h of the end of the incubation, 1:5 (w/v) soil-to-0.5 M K_2SO_4, 1:5 (w/v) soil-to-0.5 M AcOH and 1:5 (w/v) soil-to-distilled water (dH_2O) extraction were performed. Total dissolved organic C (DOC) and total dissolved N (TDN) were determined on K_2SO_4 extracts using a Multi N/C 2100S Analyzer (AnalytikJena, Jena, Germany). Nitrate (NO_3-N), ammonium (NH_4-N) and within the K_2SO_4 extracts were measured by the colorimetric methods of Miranda et al. (2001) and Mulvaney (1996), respectively. Phosphate (PO_4-P) was measured on the dH_2O extracts using the colorimetric method of Murphy and Riley (1962). Cations (Na, K and Ca) were measured on the AcOH extracts using a flame photometer (Sherwood Model 410; Sherwood Scientific Ltd, Cambridge, UK). Soil characteristics are summarised in Table 7.1.

Table 7.1. *Characteristics of the good, medium and poor quality Eutric Cambisol soil. Values are expressed on a mean dry soil weight basis $\pm$ SEM (n = 3). Letters denote significant differences between treatments using a Kruskall-Wallis with Dunn Post-hoc test and Bonferroni correction (p < 0.05).*

	Good	Medium	Poor
Texture		Sandy clay loam	
Soil moisture (%)	35.8 ± 0.8^a	30.8 ± 1.6^b	80.0 ± 8.0^c
pH	6.65 ± 0.16	6.02 ± 0.20	6.67 ± 0.10
EC (μS cm^{-1})	109 ± 31^{ab}	65 ± 3^a	393 ± 62^b
ORP (mV)	222 ± 44^a	373 ± 38^b	-37 ± 25^c
Total C (%)	3.87 ± 0.98	2.62 ± 0.15	3.06 ± 0.31
Total N (%)	0.34 ± 0.03	0.31 ± 0.01	0.31 ± 0.00
C:N ratio	10.9 ± 1.6	8.3 ± 0.4	9.8 ± 0.9
Total organic C (mg C kg^{-1})	120 ± 6	114 ± 7	1303 ± 50
Extractable NO$_3^-$ (mg N kg^{-1})	28.3 ± 1.6^a	38.4 ± 3.4^b	2.3 ± 0.6^c
Extractable NH$_4^+$ (mg N kg^{-1})	0.4 ± 0.1	1.1 ± 0.7	270.8 ± 59.7
Extractable P (mg P kg^{-1})	14.0 ± 1.1^a	10.3 ± 1.2^b	24.2 ± 1.1^c
Exchangeable Na (mg Na kg^{-1})	25.8 ± 1.8^a	13.1 ± 1.1^b	18.2 ± 0.5^c
Exchangeable K (mg K kg^{-1})	342.5 ± 33.4^a	223.6 ± 22.3^b	793.3 ± 36.1^c
Exchangeable Ca (mg Ca kg^{-1})	237.3 ± 14.0	418.6 ± 97.2	448.6 ± 57.5
Microbial biomass C (mg C kg^{-1})	1137 ± 114	958 ± 138	1578 ± 806
Microbial biomass N (mg N kg^{-1})	109 ± 17	94 ± 20	165 ± 163

EC, electrical conductivity; ORP, redox potential.

7.3.3. VOC collection and identification

The objective of this study was to examine the resultant, equilibrated, mesocosm soil quality, therefore the treatments (soil improver and residues) were not analysed alone. For single HS-SPME-trap extractions, 2 g of soil sample was placed in a 20 ml glass vial capped with a SPME compatible PTFE septum (Merck, Darmstadt, Germany) and spiked with toluene-d8 (1 ppb) as an internal standard, in order to ensure a reference retention time for data quality assessment. Vials were then placed on to an autosampler (Centri®; Markes International, Bridgend, UK) for extraction. On analysis, the sample was equilibrated for 5 min at 95 °C. A thermally conditioned SPME fibre (85 μm Polyacrylate (PA) ;Restek, USA) was then exposed to the headspace for 15 min. The fibre was then thermally desorbed (5 min, 260 °C, 50 ml min⁻

^{1}) to a cryogen-free focusing trap (U-T12ME-2S; Markes International). The trap was cooled to 20 °C during fibre desorption. Finally, a 1 min purge of the trap was performed at 50 ml min$^-$^{1} before desorption (5 min, 280 °C, 5:1 split) directly into the GC.

HS-SPME-trap-enrichment extraction analysis was performed using the sample parameters as described above; however, a 1 min enrichment delay was used before the following extraction. Enrichment was achieved by repeating the exposure and desorption to a cryogen-free focusing trap for a total of three cycles from each vial. The trap was then purged and desorbed directly into the GC, as described above.

For HS-HCSE extraction, 4 g of soil sample was placed in a 20 ml glass vial capped with a HCSE compatible PTFE septum (Merck, Darmstadt, Germany) and spiked with toluene-d8 (1 ppb) as an internal standard, in order to ensure a reference retention time for data quality assessment.. Vials were then placed on to a Centri$^®$ autosampler for extraction. On analysis, the sample was equilibrated for 5 min at 85 °C. A thermally conditioned polydimethylsiloxane (PMDS) probe (HiSorb™ PDMS; Markes International) was subsequently exposed to the headspace for 90 min. The probe was then thermally desorbed (5 min, 260 °C, 50 ml min^{-1}) to a cryogen-free focusing trap (Centri$^®$ with a U-T12ME-2S; Markes International, Bridgend, UK). The trap was cooled to 20 °C during probe desorption. Finally, a 1 min purge of the trap was performed at 50 ml min^{-1} before desorption (5 min, 280 °C, 5:1 split) directly into the GC. For all methods described above, extraction efficiencies for a range of standard and soil related compound were tested, from matrix-less vials prior to the main study (Appendix 5 Fig. 1 and 2), to ensure methods were applicable.

Injections (280 °C trap desorption, 32 min, split mode) were performed using an autosampler (Centri$^®$; Markes International) integrated to a single quadrupole GC-MS (ISQ 1300; Thermo Fisher Scientific, Waltham, MA, USA). Chromatographic separation was obtained on a mid-polarity column (DB-624 60 m $\times$ 0.25 mm $\times$ 1.4 µm; Agilent Technologies, Palo Alto, USA) using helium flowing at 2 mL min^{-1} as the carrier gas. The GC oven program was: 35 °C for 3 min; then temperature was increased to 100 °C at 10 °C min^{-1}, then increased to 220 °C at 20 °C min^{-1}, holding for 5 min and then increased to 260 °C at 20 °C min^{-1} holding for 10 min. The transfer line and ion source temperatures were both set to 230°C. Using the electron impact mode at 70 eV, mass spectra were acquired across an m/z range of 35-350 with an acquisition frequency of 5 Hz.

7.3.4. Data processing and statistics

Extraction methods were assessed by their sensitivity and the range of volatiles extracted. The total peak area of the chromatogram was used as a proxy of the sensitivity of extraction, defined as the intensity of signal recorded for a fixed concentration of sample (Brown et al., 2021; Tavares et al., 2019). Range of volatiles was assessed by the number of compounds successfully/putatively identified using the untargeted software-based approach, described subsequently. The number and breadth of volatiles extracted is incredibly relevant to the end user, as in untargeted analysis (using software-based identification approaches) it is important to have a high conversion ratio of potential compounds (peaks) to (high confidence) punitively identified compounds (Blaženović et al., 2018).

To assess total peak areas of samples, all chromatogram features were manually aligned and extracted using Xcalibur™ v2.2 (Thermo Fisher Scientific). Features with a peak area or peak height less than 0.01% of the total chromatogram were removed to decrease chromatographic noise. Total relative VOC production was then quantified by summing the areas of all remaining peaks from each sample's chromatogram. Total number of compounds detected was also calculated by summing the number of peaks detected on each chromatogram.

Untargeted analysis was undertaken in order to putatively identify as many compounds as possible in the samples. Analysis of VOC chromatograms was performed in Chromspace (v1.0, Sepsolve Analytical, Peterborough, UK). Background subtraction, deconvolution and compound identification was performed prior to compound identification, which was achieved through comparison with the NIST mass spectral library. Only compounds with a match factor (MF; a software processing method combining forward and backward library comparison and probability weighting) greater than 700 were accepted as putatively positively identified. A MF value of 700 was assessed to be the best value to balance the number of potential matches while ensuring matches were of a high quality (Viana et al., 2018). As a further quality control (QC) step, all compounds were manually examined; only compounds with consistent retention times, that were present in three or more samples were taken forward for analysis. Duplicated compound identities were removed as well as the Toluene D8 internal standard peak. Siloxane peaks were also removed as they have been shown to be associated with the breakdown of the silicone polymers in the sorbents (Jiang et al., 2006). This produced a total of 214 compounds positively identified across all samples and extraction methods. Peak areas were used as a qualitative measure of compound concentration.

All analysis was performed in MetaboAnalyst 5.0 unless otherwise stated (Chong et al., 2018; Pang et al., 2020). Heatmapping was performed; during data processing features with constant or single values across samples were removed and data was pareto scaled and glog transformed. '*ggplot2*' (Wickham, 2016) was used to produce bar charts of the number of compounds identified by each method, by presence or absence across all treatments, using R v 3.6.0 (R Core Team, 2021). R was also used to perform Kruskall-Wallis tests to determine significant differences between treatment properties. Significant differences were further explored using Dunn-Sidàk posthoc tests (Table 7.1).

The method giving the largest number of positive identifications and highest sensitivity, was determined to be SPME-trap-enrichment. Data from this method was therefore subsequently used to further examine treatment differences. Scaled and normalised data were used to graphically examine the relationship between extraction methods using principal component analysis (PCA) using the '*vegan*' and '*ggplot2*' packages (Oksanen et al., 2020; Wickham, 2016). The multiplicative beta (β) diversity of the VOCs detected in each sample was also calculated (Whittaker, 1972). One-way ANOVAs were used to test the differences between concentrations of VOCs across treatments groups. In cases of significant differences, these were further explored using a Tukey HSD. Kendall correlation analysis was used to examine relationships between sample characteristics and VOC emission using the '*stats*' package in R. In all analyses the significance level was defined as $p < 0.05$.

7.4. Results

7.4.1. Influence of method on HS sample extraction efficacy

As discussed in section 7.2.4., extraction methods were assessed for their sensitivity and the range of volatiles extracted. In terms of sensitivity, SPME-trap-enrichment produced a higher total peak area yield, 455% greater than single SPME and 46% greater than HCSE across all treatments (Fig. 7.1A). The total number of chromatographic features detected was highest using the single SPME method, 27% greater than SPME-trap-enrichment and 14% greater than HCSE across all treatments (Fig. 7.1B). The SPME-trap-enrichment was also able to successfully identify the greatest number of compounds using the software-based approach, 55% greater than single SPME and 4% greater than HCSE across all treatments (Fig. 7.1C). Overall, the conversion rate of total peaks detected (making the assumption that every peak was potentially a unique compound) to successfully identified compounds was greatest using

the SPME-trap-enrichment (39%) and smallest using single SPME (18%), while HCSE had a conversion rate of 32%.
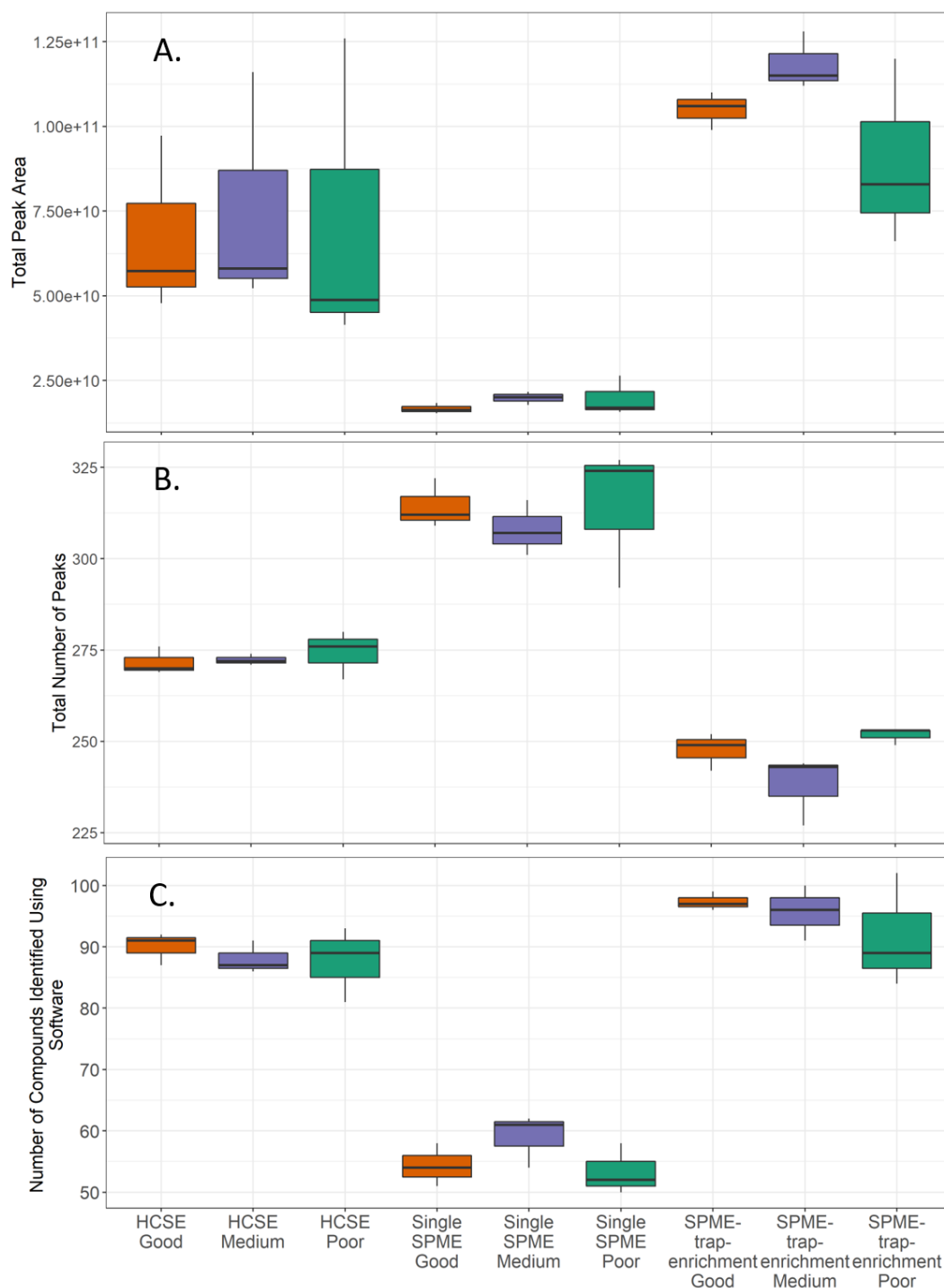
Figure 7.1. *The influence of extraction method (SPME-trap, SPME-trap-enrichment, HCSE) on the recovery of VOCs from a 'good', 'medium' and 'poor' quality soil. A) total peak area, B) total peaks (features) identified and C) total number of compounds putatively identified using software (MF > 700).*

7.4.2. Putative identification of individual VOCs

In total, 212 unique VOCs were positively identified (MF > 700), using the software-based approach, across all samples and extraction methods. SPME allowed the identification of 96 unique compounds, HCSE; 137 and SPME-trap-enrichment; 156. Across all methods, several compounds were found consistently under all soil treatments ($n = 11$). We note that compounds deemed to be high quality matches (with an MF score > 700 and QC), may still only be considered to be putatively identified, as calibration standards were not used to quantify compounds, with a large number of peaks (compounds) remaining unidentified from each sample.

7.4.2. Variability of VOCs detected between methods

The VOC profile was substantially affected by the extraction method used (Fig. 7.2). Each method allowed the identification of compounds that remained undetected using other methods e.g. furfural using SPME-trap-enrichment or 2-methyl-butanal, using HCSE. HCSE and SPME-trap-enrichment methods were both able to detect an equal, or greater number of compound groups, consistently across treatment, compared to Single SPME (Fig. 7.3).
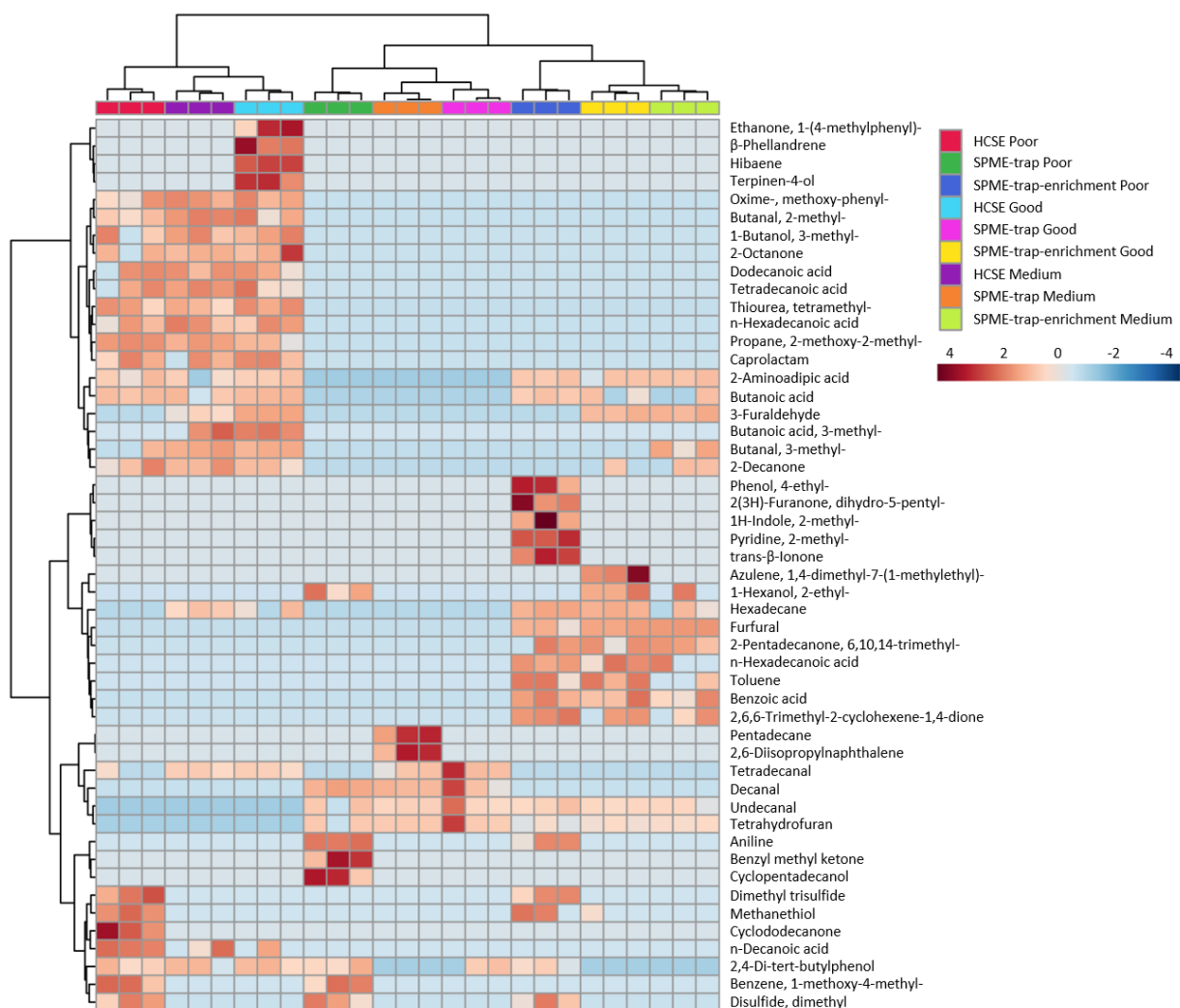
Figure 7.2. *Hierarchically clustered heatmap showing the influence of extraction method (SPME-trap, SPME-trap-enrichment, HCSE) on the recovery of VOCs from a 'good', 'medium' and 'poor' quality soil. Showing the top 50 VOC compounds tentatively identified, by significant difference as determined by ANOVA ($p < 0.05$). VOCs are clustered using Euclidean distance and Ward linkage. Data was normalised using log10 transformation and Pareto scaling. The colour of samples range from red to blue, indicating metabolite concentration z-score; numbers 4 to -4 on the scale bar indicate the number of standard deviations from the mean.*
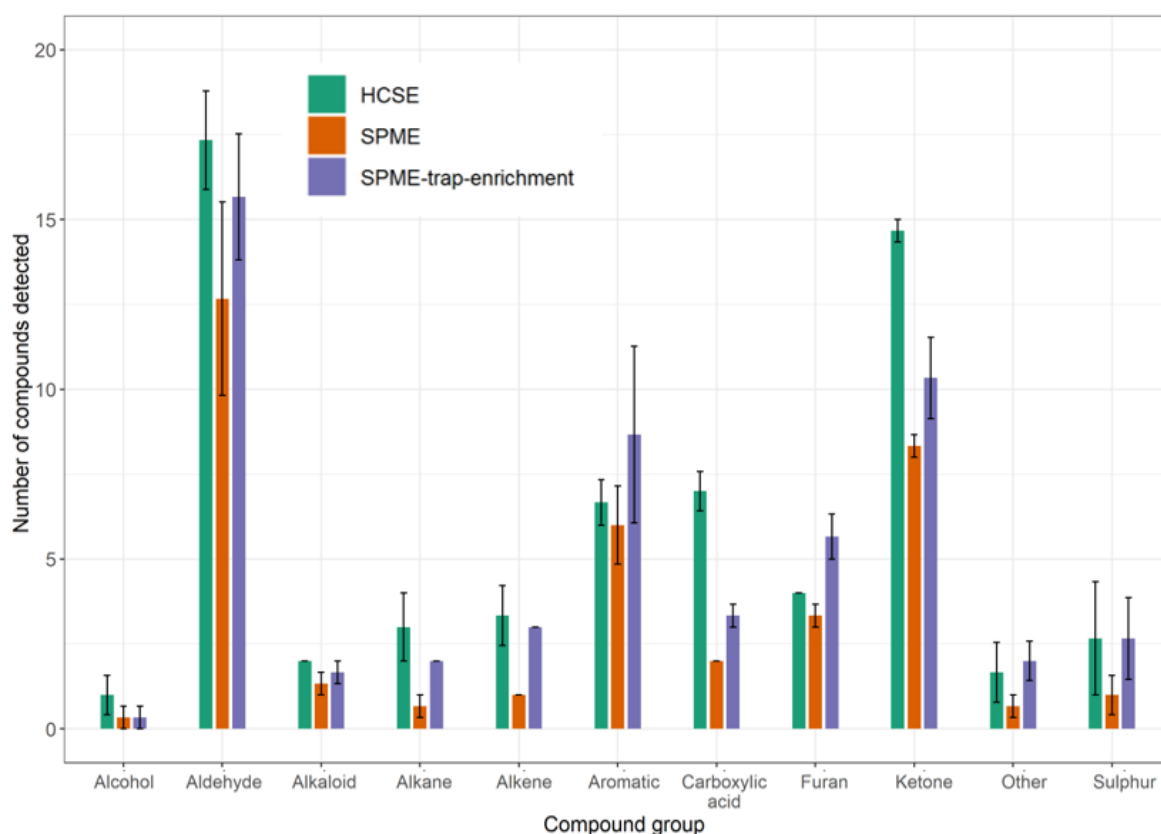
Figure 7.3. *Average number of compounds detected consistently in all replicates (n = 3), in each broad VOC compound category identified using three different extraction methods (SPME-trap, SPME-trap-enrichment, HCSE) across all soil samples, independent of treatment. Error bars indicate the SEM.*

7.4.3. Variability of VOCs detected between treatments using SPME-trap-enrichment data

From the results presented in section 7.3.1. SPME-trap-enrichment was determined to be the best method of those evaluated in terms of sensitivity and the number of compounds identified. From these data, the 'poor' soil quality treatment was the most diverse (β-diversity = 0.40), followed by the 'good' treatment (β-diversity = 0.36) then the 'medium' treatment (β-diversity = 0.34). Compounds detected in all replicates of each treatment are summarised in Table 7.2. Twenty-four compounds were detected across all treatments. A single compound was emitted in all replicates of each of the 'good' and 'medium' treatments, respectively. Ten compounds were emitted only by the 'poor' treatment. Finally, 3 compounds were emitted only by the 'good' and 'medium' treatments. Generally, compounds emitted under the 'poor' treatment were malodourous, whereas compounds from 'good' and 'medium' treatments were characterised by more pleasant odour compounds. PCA analysis visually confirmed that the

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'good' and 'medium' treatments were grouped more similarly, with some overlap compared to the 'poor' treatment (Fig. 7.4).
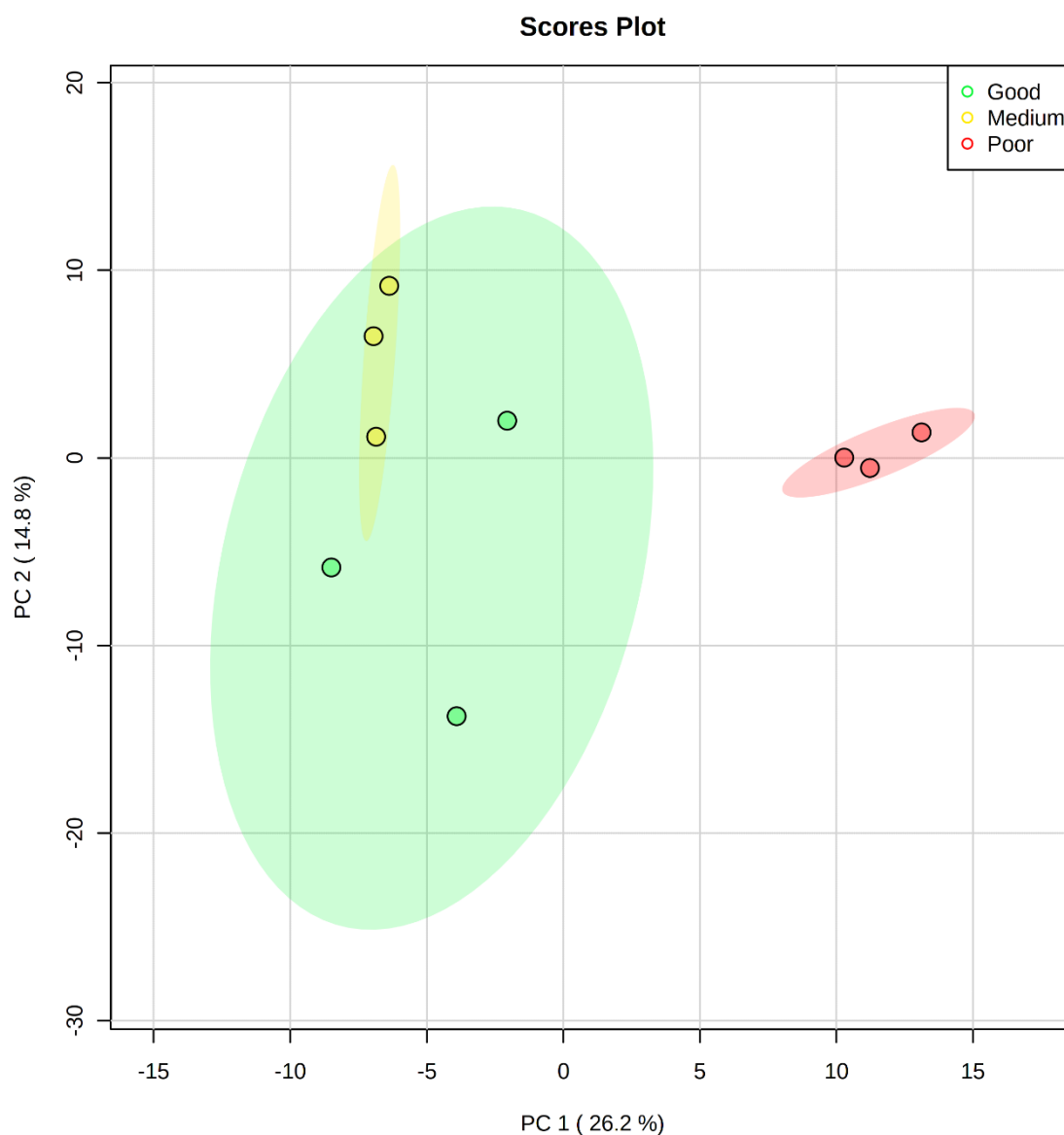
Figure 7.4. *Influence of soil quality (good, medium or poor) on the VOC profile determined by SPME-trap-enrichment. 2D principal component analysis (PCA) of VOC compounds. Data was normalised using log10 transformation and Pareto scaling. Principal component 1 (PC1) explains 26.2 % of the total variance, while principal component 2 (PC2) explains 14.8 % of the total variance. Ellipses represent 95% confidence levels for each treatment group.*

Table 7.2. Summary of tentatively identified compounds found in all replicates of each treatment using the SPME-trap-enrichment extraction method, and possible metabolic functions. Compound marked with an * indicates compounds that were statistically significant between treatments.

	Compound	Potential functional role in soil
Compound(s) emitted under all treatments	2-Aminoadipic acid	Key metabolite in penicillin (antibiotic) formation in fungi (Nigam and Singh, 2014; de Valmaseda et al., 2005)
	2-Butanone	Ubiquitous in the natural environment (Schühle and Heider, 2012). Bacterial VOC (non-toxic) (Audrain et al., 2015).
	2-Butenal, 3-methyl-	Endogenously formed during lipid peroxidation or after oxidative stress, derivative of acrolein. Found in plants as a regulator of cytokinin (hormone involved in cell division etc.) degradation (Kieber and Schaller, 2014).
	2-Heptanone	Natural component of some foods and plants. Bacterial metabolite shown to attract nematodes (Man et al., 2019). Can have inhibitory effect on the survival of bacteria (Melkina et al., 2017).
	2-Heptanone, 6-methyl-	VOC associated with a number of plant, fungal and bacterial species (Owens et al., 1997; Goeminne et al., 2012; Cramer et al., 2005) Methylation product of 2-hepanone, metabolism shown in *B. subtilis* (Man et al., 2019).
	2-Hexenal	Common plant metabolite showing antibacterial and antifungal properties (De Lucca et al., 2013; 2011).
	Acetone	Ubiquitous soil VOC (Insam and Seewald, 2010). Key in ketone metabolism (alternative metabolic fuel source) (Puchalska and Crawford, 2017)
	Acetophenone	Secondary metabolite in some species (*Evodia merrillii* (Chou et al., 1992), *Melicope semecarpifolia* (Chen et al., 2008)). May be metabolised by some bacteria (Arthrobacter) (Cripps, 1975) and involved in the anaerobic degradation of ethylbenzene (Kanehisa et al., 2017). Antifungal (Ma et al., 2013).
	Benzene	Naturally present in low concentrations in the environment. Metabolised by some plants into organic acids (Ugrekhelidze et al., 1997). Benzene present in soil

		can be enzymatically degraded by aerobic bacteria (Kuykendall, 2008). Potentially toxic to soil microbes, reduces microbial diversity (Fahy et al., 2005).
	Benzeneacetaldehyde	Prokaryotic and eukaryotic metabolite derived from phenylalanine (essential amino acid) (*Saccharomyces cerevisiae* (Manzoni et al., 1993), *Escherichia coli* (Parrott et al., 1987), tomato plants (Tieman et al., 2006)), also breakdown product of styrene in humans. Important VOC in flower scent (Kaminaga et al., 2006).
	Benzoic acid	Growth stimulant for bacteria and fungi but may have inhibitory effects for specific species (Liu et al., 2017).
	Benzonitrile	Precursor to some herbicides and fungicides (Veselá et al., 2010). Biodegradation by some bacteria (Mukram et al., 2015).
	Butanal, 3-methyl-	Plant and bacterial metabolite (Tait et al., 2014). Involved in leucine (amino acid) degradation and glycine betaine (osmolyte) biosynthesis from betaine aldehyde.
	Creatinine	Potentially antibacterial (McDonald et al., 2012).
	Furan, 2-ethyl-	Derived from furan, bacterial and plant metabolite (Farag et al., 2006).
	Furan, 2-pentyl-	Emitted from *Bacillus megaterium* with possible growth promoting effects on *Arabidopsis thaliana* (Zou et al., 2010).
	Furfural *	Shown to inhibit enzymatic hydrolysis of lignocellulosic material (Sun et al., 2016). Highly toxic to most microorganisms (Wierckx et al., 2011)
	Heptanal	Common plant volatile. An endogenous aldehyde coming from membrane lipid oxidation.
	Hexenal	Produced through degradation of unsaturated fatty acids during microbial growth (Korpi et al., 1998). Anti-fungal VOC (Gardini et al., 1997).
	Nonanal	Eukaryotic metabolite found in numerous plants (Pophof et al., 2005; Wildt et al., 2003; Maganha et al., 2010). Potentially antifungal/ antimicrobial (Abanda-Nkpwatt et al., 2006; Kobaisy et al., 2001; Sharifi et al., 2018).
	Nonanoic acid	Eukaryotic metabolite (common in pelargonium plants) (Pohanish, 2015). Herbicidal, antimicrobial and antifungal properties (Ciriminna et al., 2019; Sahin et al., 2006; Jang and Jung, 2012).

	Octanal	Plant secondary metabolite, growth inhibitory volatile (Mishyna et al., 2015). Bacterial and fungal metabolite, involved in fatty acid decomposition (oleic acid) (Hamilton-Kemp et al., 2005; Beck et al., 2011).
	Tetrahydrofuran	Environmental pollutant, few bacterial and fungal species are able to metabolise it (He et al., 2014).
	Undecanal	Role as an antimycobacterial and nematocide, a volatile oil component and fatty acid derived plant metabolite (Esquivel-Ferriño et al., 2012; Kim et al., 2008; Pickett et al., 2016)).
Compound(s) emitted under good treatment only	Azulene, 1,4-dimethyl-7-(1-methylethyl)- *	Sesquiterpenoid, shown to possess antimicrobial properties (Li et al., 2012).
Compound(s) emitted under medium treatment only	Butanal, 2-ethyl-3-methyl- *	No evidence supporting function found.
Compound(s) emitted under good and medium treatment only	2-Heptenal, (Z)- *	Associated with lipid autoxidation (Lorenzo et al., 2013).
	3-Furaldehyde *	More commonly present as 2-furanaldehyde (furfural). Likely a component of ligno/hemicellulose (Jönsson et al., 2013).
	Formic acid, octyl ester	Plant and animal metabolite present in the cell membrane and used in energy storage.
Compound(s) emitted under poor treatment only	1H-Indole, 2-methyl-	Highly toxic to microorganisms, few bacteria or fungi can metabolise it (Ochiai et al., 1986; Arora et al., 2015).
	2(3H)-Furanone, dihydro-5-pentyl- *	Microbial quorum-quenching activity (Cirou et al., 2012).
	2,4-Di-tert-butylphenol *	Common secondary metabolite produced by various groups of organism with potential insecticidal, nematocidal, anti-bacteria, -viral and -fungal activities (Zhao et al., 2020)
	3-Octanone *	Secondary metabolite produced in fungi (Takeuchi et al., 2012; Kanchiswamy et al., 2015). Molluscicide (Khoja et al., 2019).
	Aniline	Used in the synthesis of herbicides. Bacterial degradation has been seen in a number of species (Arora, 2015). Antifungal activity (Zhang et al., 2020).
	Dimethyl trisulfide *	Terminal electron acceptor during anaerobic respiration (Hedderich et al., 1998). Antimicrobial (Tyc et al., 2015).

	Disulfide, dimethyl	Terminal electron acceptor during anaerobic respiration (Hedderich et al., 1998). Nematocidal (Yan et al., 2019). Quorum-sensing-inhibiting compound (Chernin et al., 2011). Stimulate bacterial growth and inhibit fungal growth (Garbeva et al., 2014).
	Phenol, 4-ethyl- *	Can act as the sole carbon source for some fungi (Jones et al., 1994). Associated with the biological decomposition of plant material (Akdeniz et al., 2007).
	Pyridine, 2-methyl- *	Bacterial and fungal secondary metabolite, fungicidal activities (Seifert and King, 1982; Asari et al., 2016).
	trans-β-Ionone *	Associated with carotenoid degradation (Reese et al., 2019). Antifungal and antibacterial properties (Borras-Hidalgo, 2010; Lamikanra and Richard, 2002).

Significant differences between treatments, as determined using ANOVA with a Tukey post-hoc test, were found for a number of compounds (Table 7.2). Furfural and butanal, 2-ethyl-3-methyl- were found in higher concentrations under the medium treatment. Azulene, 1,4-dimethyl-7-1-methylethyl- was detected in greater concentrations under the 'good' treatment. Pentanal, 3-furaldehyde and (Z)-2-heptenal were all detected in significantly higher concentrations under the 'good' and 'medium' treatments compared to the 'poor' soil quality treatment. While 2-methyl-1H-indole,; trans-β-ionone; 2(3H)-furanone, dihydro-5-pentyl-; 2,4-di-tert-butylphenol; 3-octanone; 2-methyl-pyridine,; dimethyl trisulfide and 4-ethyl-phenol, were all found in significantly higher concentrations under the 'poor' treatment. In general, the samples within the 'poor' treatment had a larger number of aromatic, carboxylic acid and sulphur containing compound groups, while the 'good' and 'medium' groups were characterised by ketones and aldehydes. The treatments that were enriched with organic matter (good and poor) produce a larger number of aromatic compounds, as summarised in Fig 7.5.
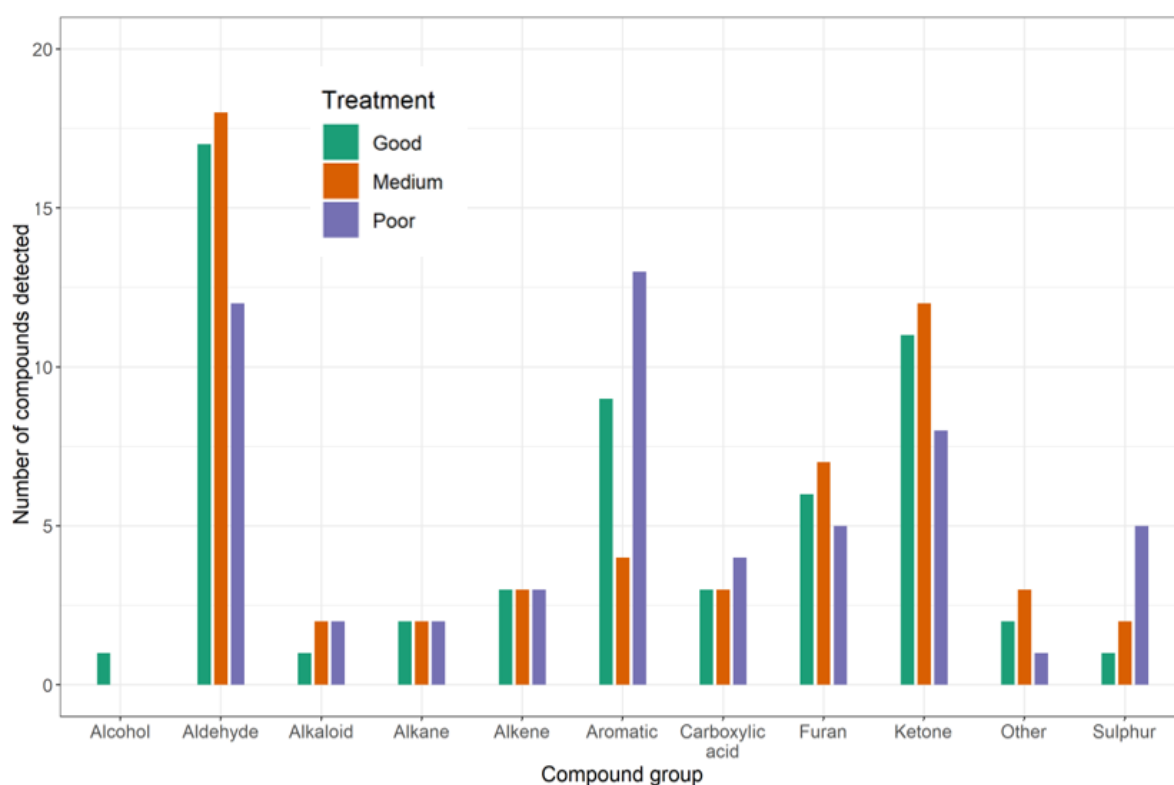
Figure 7.5. *Number of compounds detected consistently in all replicates (n = 3) in each broad VOC compound category identified using SPME-trap-enrichment, by treatment.*

7.5. Discussion

7.5.1. Evaluation of different soil VOC extraction techniques

Single extraction SPME methods represent a common technique for the environmental sampling of VOCs. However, this approach can also suffer from issues associated with competition for phase adsorption on the fibre itself (Górecki et al., 1999). This study compared standard SPME methods against alternative and novel sample methods (HCSE and SPME-trap-enrichment) to assess their performance on complex sample matrices. In general, HCSE and SPME-trap-enrichment both improved the sensitivity and analyte range of extractions compared to single SPME extraction, across all treatments (Fig. 7.4). This was likely a result of decreasing the phase competition, particularly for compounds with low and high boiling points, respectively. While single SPME yielded the highest number of chromatographic features, this did not translate into greater compound identification, likely due to the smaller peak areas, making compounds difficult to distinguish from chromatographic noise. Both SPME-trap enrichment and HCSE had larger total peak areas for a reduced number of peaks, allowing more successful putative software-based compound identification.

To reduce the generation of pressure, a lower extraction temperature was used for the HCSE extractions compared to the other HS sampling methods tested in this study, resulting in a comparatively higher partition co-efficient (k) value than the other HS extraction methods. A key aspect of this analysis was to keep sample preparation to an absolute minimum, allowing this approach to be easily scalable to a greater number of samples. To achieve this, while not disturbing the sample, the original moisture content was not altered in any way. At higher temperatures, close to the boiling point of water, we reached the physical limitations of the probe holder. For instrumental continuity, a lower temperature was used to compensate for the limitations of the higher pressure. This had the potential to lower the number of compounds emitted to the headspace in the HCSE extractions compared to the other methods. However, the larger sorbent volume of the HCSE (65 µl, as opposed to 0.6 µl and 1.8 µl, for SPME-trap and SPME-trap-enrichment, respectively) was likely to have played a role in good VOC recovery rates, as the rate of adsorption is directly proportional to the surface area (Ouyang et al., 2007). The molecular weight of an analyte has a large effect on its retention, longer extraction times allow heavier analytes to reach equilibrium increasing the number and intensity of peaks (Giri et al., 2010; Yamaguchi et al., 2018).

It must also be noted that sampling sorbents differed between extraction methods; SPME fibres were made of PA (commonly used for recovery of polar semi-volatiles; 80 – 300

molecular weight (MW)) while HCSE uses PDMS (widely responsive to volatiles; 60 – 275 MW), both of which extract analytes via absorption and greatly reduce competition for phase. As fibre coating section is a key determinant of compound recovery, this is likely to have influenced the types of compounds recovered using each method (Fig. 7.2). PDMS has been the most commonly used fibre for soil VOC sampling, particularly with regard to pollution sampling and monitoring (James and Stack, 1996; Zhao and Zhai, 2010). However, for more subtle changes in the VOCs associated with soil ecology, a combined phase approach (e.g. polydimethylsiloxane/divinylbenzene (PDMS/DVB), Carboxen/polydimethylsiloxane (CAR/PDMS) or Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS)) is often utilised in order to maximise the range of compound recovery from the headspace (Brown et al., 2021). However, mixed coatings extract analytes via adsorption, physically interacting with the analytes and thus creating competition for phase (Gherghel et al., 2018). Longer sampling times will increase competition for the finite number of adsorption sites on the surface of a mixed coating fibre, possibly biasing analysis towards compounds with the greatest affinity for the fibre (dependent on a number of factors including sampling material and analyte polarity) (Kallenbach et al., 2014; Mascrez and Purcaro, 2020b).

The SPME-trap-enrichment technique warrants further investigation using multi-phase fibres; allowing more analyte to be sampled from the headspace, preconcentrated and subsequently analysed. Once optimised, this has the potential to increase the ability to monitor subtle changes in the ecological VOC profile of complex environmental samples at increased sensitivity and wider range of analyte molecular weight and polarity, ultimately reducing bias in the extraction method.

7.5.2. Chemical diversity of compound detection using SPME-trap-enrichment

The soil VOC emission profile is generally a product of the composition of the microbial community and the prevailing environmental conditions (Abis et al., 2020; Insam and Seewald, 2010; Misztal et al., 2018). The three contrasting treatments used here were diverse in their VOC profiles with the aerobic 'good' and 'medium' soil quality treatments being more closely related in their VOC emission profiles relative to the 'poor' treatment (Fig. 7.4). The SPME-trap-enrichment method detected the most compounds across the 11 groups summarised in Fig. 7.5. The 'poor' treatment produced the greatest diversity of VOCs. We ascribe this to the anaerobic conditions where a greater proportion of C is partitioned into metabolic by-products from respiration and secondary stress-related metabolites, which often

results in an increase in the diversity of VOCs emitted (Seewald et al., 2010). Equally, the addition of organic material (lysed grass in poor treatments and soil improver in good treatments, respectively) can increase the substrate quality, providing a readily accessible source of nutrients, driving the production of VOCs. Though, organic additions are likely to have different substrate qualities; soil improver is more stable with more recalcitrant (cellulose and lignin) C and N than the lysed grass.

In the cases of the 'good' and 'poor' treatments, it is likely that changes in the soil system (increased availability of nutrients) generated conditions more favourable for the net emission of VOCs (i.e. production $\geq$ consumption). However, when a biochemical steady state equilibrium has been reached (i.e. no significant change in biotic or abiotic conditions), soil may be a VOC sink rather than a source (Asensio et al., 2007). Accordingly, it is possible that, with no significant inputs or changes to the 'medium' treatment, it was in an equilibrium state (i.e. consumption $\geq$ production), and it was only the heating of the sample on analysis that VOC emission occurred. All the major VOC chemical groups that have been previously reported being emitted from soil were identified in this study (Insam and Seewald, 2010). We also acknowledge that, as treatment VOCs signatures were not directly measured, some of the VOCs identified may be due to direct amendment and not subsequent microbial metabolism. However, as the existing literature shows, soil microbial metabolism has the potential to rapidly degrade (particularly labile) compounds and we therefore expect the majority of compounds to be microbially synthesised (Bore et al., 2017; Gougoulias et al., 2014; Ray et al., 2020).

7.5.3. Ecological significance of VOC compounds

The diffusive potential and mobility of VOCs allows microorganisms to increase their sphere of influence ('volatisphere') within the soil. While the role of VOCs in soil is diverse, generally these can be categorised into the following: quorum sensing, biological control, or inter-/ intra-organismal interactions (Evans, 2009; Keller, 2019; Yergeau et al., 2017).

In this study, using data from the most sensitive method (SPME-trap-enrichment), a number of compounds were emitted ubiquitously from all treatments, a summary and possible ecological role within the system are explored in Table 7.2. These were low molecular weight ketones (e.g. acetone and 2-butanone), aldehydes (e.g. octantal and hexanal) and furans (furfural and furan, 2-ethyl), which have been shown to be commonly emitted from a range of soils, as by-products of microbial anabolism or catabolism (Huber et al., 2010; McBride et al., 2019; Peñuelas et al., 2014). Other compounds were treatment specific, likely produced as

result of, or in response to, the perturbed microbial community. Generally, the compounds summarised in Table 7.2, have been shown to have a possible function or benefit for the emitting organism, e.g. bacterial or fungal inhibition. However, while many of the compounds identified have well defined roles within microbial metabolism, there are still knowledge gaps regarding the applicability of VOCs measured in a laboratory setting under favourable conditions, to an applied or field setting.

7.6. Conclusions

SPME-trap-enrichment with some partition coefficient modification and cryogen free focusing technology, allows increased sensitivity, particularly of compounds with low and high boiling points, and subsequently allows more compounds to be positively identified (MF > 700). This in turn allows for a much more comprehensive VOC profiling and the potential to determine the functions of specific VOC produced by the microbial community. Ultimately, these novel methods of VOC extraction provide the key to elucidating environmental biochemical interactions on a micro-scale.

7.7. Acknowledgements

7.8. References

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Chapter 8: The fact of the (organic) matter - soil metabolomics: current challenges and future direction

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8.1 Abstract

Soil is an extremely complex and dynamic matrix, in part, due to the wide diversity of organisms living within it. Soil organic matter (SOM) is the fundamental substrate on which the provision of ecosystem services depends, providing the metabolic fuel to drive soil function. As such, the study of metabolomics (diversity and concentration of low molecular weight metabolites) holds the potential to greatly expand our understanding of the behaviour, fate, interaction and functional significance of small organic molecules in soil. Encompassing a wide range of chemical classes (including amino acids, peptides, lipids and carbohydrates) and a large number for individual molecules (estimated 2×10^5 to 1×10^6) the metabolome is a product of several layers of a biological hierarchy, namely the genome, transcriptome and proteome. Therefore, it may also provide support and validation for metatranscriptomic and metagenomic datasets. We present the case for the increased use of metabolomics in soil biochemistry, particularly for furthering our fundamental understanding of SOM composition and cycling, as well as the scale of the challenge of in terms of extraction and interpretation in a complex system. Further, we identify several key areas for future research focus, including the importance of untargeted analysis, the emission and fate of volatile organic compounds in and from soil and monitoring ecological interaction in the rhizosphere, which will aid the fundamental comprehension of biochemical interactions and transformations between the soil substrate and soil-dwelling biology.

Keywords: Carbon, Pools and fluxes, Biochemistry, Soil organic matter, Nutrient cycling.

8.2. Introduction

Soil is key to the survival of life on Earth. Its resources not only provide food, fuel and fibre for a growing human population, but the large range of additional ecosystem services, for example, nutrient cycling, carbon (C) sequestration, climate regulation and flood prevention (Pereira et al., 2018). However, soil research faces major challenges into the future, with increasing agricultural intensification (Kopittke et al., 2019), salinisation (Clarke et al., 2018), desertification and urbanisation (Li et al., 2018), in addition to increasingly unpredictable weather patterns (Borrelli et al., 2020). Consequently, a deeper understanding and improved management of soil processes will become key in maintaining soil health (Baveye, 2015). Soil biology is now recognised as the key driver of soil functioning; however, it is generally underrepresented in soil quality assessments, likely due to its immense complexity, both in terms of multispecies interaction and interpretation, compared to more traditional chemical and physical measures (Bünemann et al., 2018). A combinatorial suite of approaches is therefore needed to fully integrate biological indicators into routine soil monitoring. While major advances have been made in evaluating the diversity of organisms which live in soil (e.g., via metagenomics and metabarcoding), directly linking this to changes in the amount and chemical properties of soil organic matter (SOM) and soil functioning has proven difficult.

SOM is the universal substrate on which the majority of life on Earth relies upon to some extent. The amount and quality of SOM underpins most of the ecosystem services provided by soil. Biology, being the driving force behind nutrient cycling in soil, facilitates the decomposition and transformation of organic residues to organic matter (Jiao et al., 2019; Roth et al., 2019). The soil's cycling of these nutrients, while occurring on a microscale, has global implications in terms of C cycling (net emission or sequestration), as well as other key chemical cycles (e.g. nitrogen, phosphorus and sulphur cycling) (Gougoulias et al., 2014). Generally, there is understanding of the higher-level process governing the cycling of nutrients (i.e. how environmental conditions affect aerobic and anaerobic soil respiration, carbon use efficiency, or nitrification/denitrification), although potential rates must be interpreted with caution (Hazard et al., 2021). Yet, there is little understanding of the small molecule interactions underpinning these processes. To date, most characterisation of SOM has been associated with techniques that provide a broad view of its chemical composition (e.g. NMR, FTIR). In addition, the turnover rate of SOM is normally quantified by measuring total soil respiration or heat production (calorimetry). While this provides a total integration of biological activity occurring in soil, it provides no detail on the processes involved. The study of environmental

metabolomics is in an excellent position to explore this as the output of the metabolome is a product of several layers of a biological hierarchy, namely the genome, transcriptome and proteome, as summarised in Fig. 8.1.
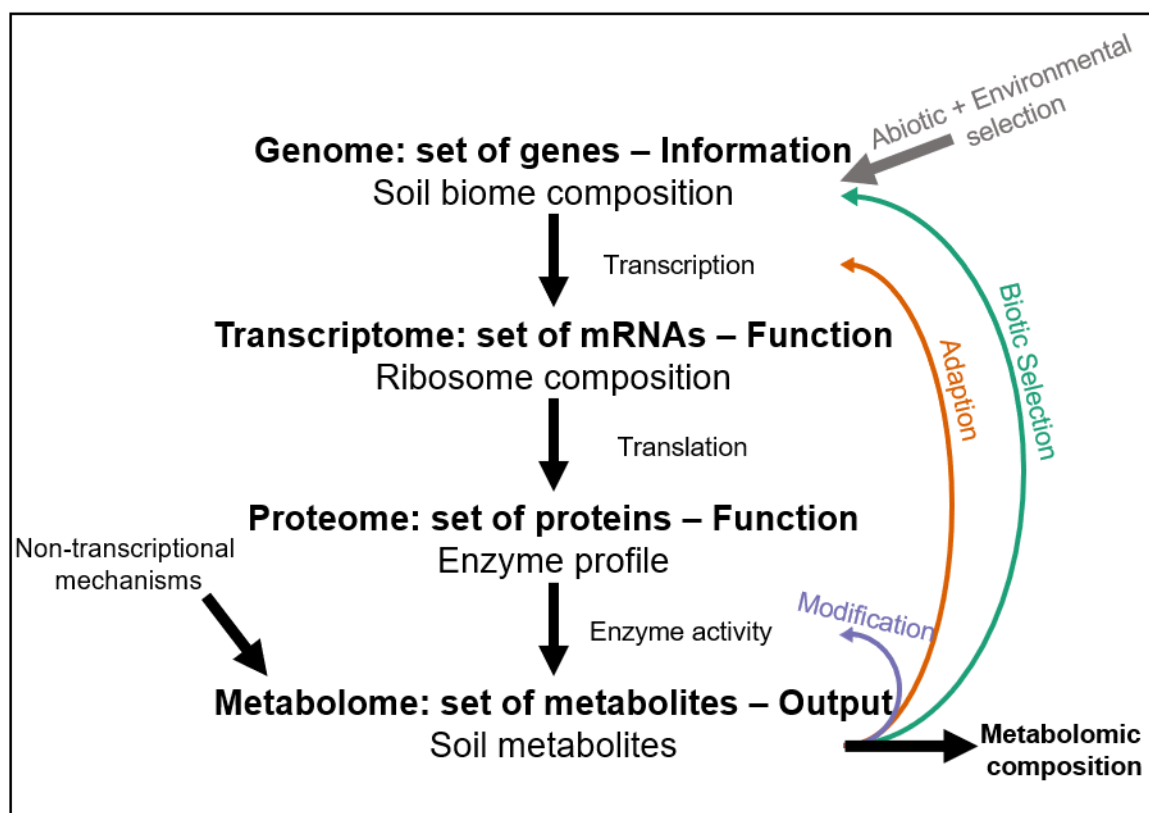
Figure 8.1. *A conceptual representation of the biological hierarchy from genome to metabolome, illustrating the metabolome's sensitivity to environmental and organismal change. Adapted from Takahashi et al., (2012).*

Metabolomics, the study of small molecules (typically 100 – 1500 Da; i.e. metabolites, chemical substances produced as a result of organismal metabolism), has been widely applied in a number of fields. A brief summary of the methods discussed in this chapter are contained within Table 8.1. The application of metabolomic analysis to a single cell, organism or species is widely practiced, e.g., to understand virus-host interactions (Kumar et al., 2020), environmental stresses on shellfish (Viant et al., 2003), or understanding nutritional requirements of single species (Creek et al., 2013). However, in recent years the field of environmental metabolomics has evolved, allowing the ability to characterise the interactions between a community of organisms and their environment (Bundy et al., 2009). The application of metabolomics to soil science is a nascent field, with large potential.

Table 1 8.1. *A brief description of the subtypes of metabolomics discussed in this chapter.*

Sub-types of metabolomic analysis	Brief description	References
Targeted	Analysis of defined groups of chemically characterized and biochemically annotated metabolites.	Roberts et al., (2012)
Untargeted	Comprehensive analysis of all the measurable analytes including chemical unknowns, limited by analysis and extraction technique	Want et al., (2018)
Primary metabolites	Compounds involved directly in the normal growth, development and reproduction of an organism or community. Generally, endogenic, often produced in the log phase of growth.	Crueger and Crueger, (1990) Sanchez et al., (2008)
Secondary metabolites	Compounds that are not required for the growth or reproduction of an organism or community but are usually produced in order to gain a selective advantage. Usually, exogenic, often produced in the stationary phase of growth.	Karlovsky, (2008) Chomel et al., (2016) Erb and Kliebenstein, (2020) Ruiz et al., (2010) Isah, (2019)
Lipidomics	Lipid compounds generally transcend the categories of primary and secondary metabolites. For example, some groups are crucial for cell function e.g. PLFAs as membrane lipids and TAGs as storage lipids whereas others are used as signalling compounds.	Zhao et al., (2018) Soto et al., (2019) Mahfouz et al., (2020) Frostegard et al., (2015)
Volatilomics	A subset of secondary metabolomics concerning all volatile metabolites, often referred to as volatile organic compounds (VOCs).	Leff and Fierer, (2008) Insam and Seewald, (2010) Kesselmeirer and Staudt, (1999) Brown et al., (2021) Brilli et al., (2019)

Metabolomics encompasses a broad range of analytical techniques and metabolic subdivisions, therefore the ability to tailor analysis to answer specific hypothesis and research questions is a strength. However, the complexity of the metabolome is often a hindrance to interpretation (Fig. 8.2), with many laboratory-based studies performed under controlled conditions. Often these are not subsequently applied to field scenarios, where many variables are uncontrollable.
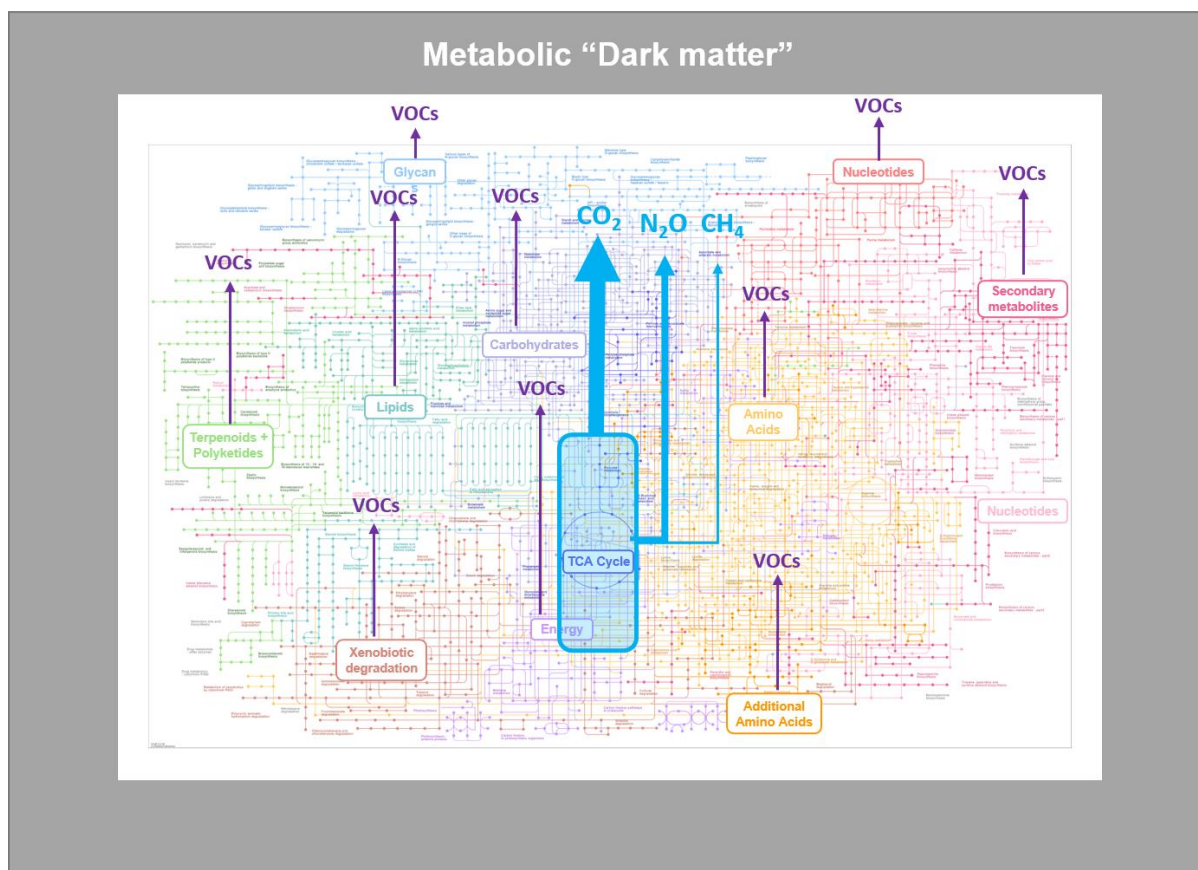
Figure 8.2. *The Kyoto Encyclopedia of Genes and Genomes (KEGG) schematic representation of known metabolomic pathways (Kanehisa et al., 2016), however the database is not extensive, with the potential for many other as yet identified metabolic pathways, or 'metabolic dark matter'. Equally, while the main focus of most studies to date is the emission of the three most prolific greenhouse gases from soil (CO_2, CH_4 and N_2O), VOCs may potentially be emitted as metabolic intermediates from the anabolism and catabolism of various other primary and secondary metabolites (Insam and Seewald, 2010), potentially representing a significant flux of carbon from the micro- and phyto-biome.*

8.3. The importance of metabolomics in the study of soil biochemistry

Metabolic reactions underpin soil biological life and function within the environment. They are the microscale interactions that determine the soil's ability to provide ecosystem services for example, C storage, nutrient cycling and buffering of pollutants (Brown et al., 2021b; Chomel et al., 2016; Jones et al., 2014). Particular focus has been on understanding SOM quality (segregated into water extractable (i.e. accessible compounds), and solvent

extractable, (i.e. intracellular compounds, and more polar (less biologically accessible) extracellular compounds)) (Swenson et al., 2015; Swenson and Northen, 2019). However, still little is known about the small molecule composition of soils, and how these molecules are cycled. Where this work has been undertaken, it has tended to focus on a specific group of compounds (e.g. organic acids, amino acids, allelochemicals) providing only a small piece of the metabolic jigsaw puzzle. However, an untargeted approach, examining the whole metabolome, may provide a greater understanding of C cycling, with potentially significant effects for C budgets and fluxes within and from soils (Overy et al., 2021). When linked to stable isotope labelling of SOM (e.g. $^{12}C/^{13}C$, $^{14}N/^{15}N$, $^{32}S/^{34}S$, $^{16}O/^{18}O$) it is also possible to simultaneously trace the flux of individual metabolites in soil. This isotope pool dilution approach has been used extensively for phospholipids (Maxfield and Evershed et al., 2014; Watzinger et al., 2019) and amino acids (Dippold and Kuzyakov, 2013; Dippold et al., 2014), however, it has not yet been widely applied to other compound groups. This approach also has the potential to track the source and fate of metabolites derived from plants by $^{13}CO_2$ labelling the shoots (Chen et al., 2021).

Equally, ecological metabolomics (ecometabolomics), the exploration of the ecophysiological and ecological function of organisms and ecosystems, is also a field of growing importance (Sardans et al., 2011). In this sense its application is not only limited to soil microbial communities, the metabolic health of soil dwelling macrofauna (e.g. earthworms (Rochfort et al., (2008); Tang et al., (2020); Zhu et al., (2020)) may also be explored. As the sensitivity of analytical equipment increases, the resolution at which inter- and intra-species interactions may be observed also increases. This is particularly the case with extracellular volatile metabolites, which potentially may be profiled in real-time, non-invasively (Seewald et al., 2010).

While only beginning to be elucidated, small molecule interactions are likely to play a central role in the fundamental understanding of environmental interactions and ultimately environmental quality as a whole. Examples of its use include pollution monitoring (Jones et al., 2014), soil quality assessment (Brown et al., 2021a; Withers et al., 2020) and quantifying soil stress (Lankadurai et al., 2013) as well as the discovery of new compounds for pesticide and drug development (Aliferis et al., 2010; Atanasov et al., 2021; Gupta et al., 2018). While soil biological function is currently, generally measured on the output of processes (i.e. GHG emission or crop yields), metabolomics may be the key to elucidating functional change on a much finer scale. With the potential to increase our understanding of functional metabolomics

to that of functional genomics (Danczak et al., 2020; Ungerer et al., 2007; Yan and Xu, 2018), ascribing particular metabolites to be indicative of specific processes and further elucidating metabolic pathways adding to the Kyoto Encyclopaedia of Genes and Genomes (KEGG; and equivalent databases) (Kanehisa et al., 2017; Kanehisa and Goto, 2000). Known examples of this include the role of organic acids in regulating P availability in soil (Menezes-Blackburn et al., 2016; Zhu et al., 2021), amino acids in drought stress tolerance (Kushal et al., 2015; You et al., 2019) and specific peptides in disease regulation (Datta et al., 2015; Khademi et al., 2020).

8.4. Current limitations

Soil is, by definition, an extremely complex analytical substrate with biological, chemical and physical factors all interacting dynamically, with small perturbations in extrinsic or intrinsic factors leading to changes in the equilibrium. Soil is also extremely spatially heterogeneous, reflecting soil quality, land use and management (Gravuer et al., 2020; Mao et al., 2014), down to the fine-scale variations in exudate composition along a root (Dong et al., 2019). Potentially rapid temporal changes add to soil's complexity, with changes in metabolic profiles occurring within minutes of perturbation (Gunina et al., 2017), as well as diurnally based on plant rhizospheric excretion fluxes (Hubbard et al., 2017). Metabolomic extraction methods are often optimised for specific soil types (Jenkins et al., 2017; Swenson et al., 2015), or environmental conditions, for example saline soils are likely to hinder the analysis of metabolites (Annesley, 2003; Xu et al., 2021), or potentially are not optimised for soil at all (Fiehn, 2016; Withers et al., 2020). This combined with a range of available extraction solvents (Table 8.2), targeting different fractions of organic matter (OM) (water extractable OM (i.e. directly biologically available) vs. solvent extractable OM (examining intracellular, available and soil adsorbed/absorbed depending on choice of solvent), leads to difficulty of direct comparison between datasets during meta-analysis.

Table 8.2. *A summary of common metabolite extraction techniques and analysis platforms.*

Extraction techniques	Brief description	References
Fumigation-extraction	Fumigation of a soil sample with chloroform has been shown to lyse cells and release intracellular metabolites prior to extraction. This may be combined with an H_2O or solvent based extraction.	Vance et al. (1987) Swenson et al. (2015)
H_2O	For the extraction of water extractable organic matter (WEOM) or soil exometabolites. Effective at extracting a broad range of polar compounds. However, may significantly underrepresent fatty acids and sterol compounds. Low concentrations of salt may be included to reduce the effect of cell lysis and osmotic shock, however salt must be removed before analysis.	Swenson et al. (2015) Gregorich et al. (2000)
Solvent based	Solvents may be used either individually or in combination, common extraction solutions include, methanol as well as combinations of isopropanol, methanol and water or isopropanol, acetonitrile and water. Generally, combining solvents allows for the extraction of a broader range of metabolite compound classes including a greater number of non-polar molecules compared to H_2O only.	Swenson et al. (2015) Ser et al. (2015) Capriel et al. (1986) Withers et al. (2020) Roberts et al. (2012)
Analysis platforms		
GC-MS	Flexible, high resolution and sensitivity to a broad range of compounds, including volatile organic compounds, lipids and derivatizable molecules, allowing for targeted or untargeted analysis. Often identifying > 100 compounds per sample. The technology is easily combined with mass spectrometers and accurate mass measurements. This is complemented by large supporting spectral libraries and standardised methodologies.	Schauer and Fernie (2006) Fiehn et al. (2016)
NMR spectroscopy	Highly repeatable, easy metabolite identification and non-destructive analysis. However, generally low resolution and sensitivity hinders the determination of metabolites to ≤ 50 per sample.	Emwas et al. (2019)
LC-MC	Flexible, high resolution and sensitivity to a broad range of compounds; capable of identifying > 400 compounds per sample. Often provides increased resolution for semi-polar metabolites.	Zeki et al. (2020)
Other	PTR-MS – high sensitivity, real-time measurement of VOCs, requiring no sample pre-preparation. However, a limited range of molecules detectable (only molecules with a proton affinity higher than water) and limited total concentration. FT-ICR-MS – the most advanced mass analysers in terms of high accuracy and resolving power and sensitivity, with sub-parts-per-million mass accuracy. Potentially greater information about heteroatom-rich samples	Mancuso et al. (2015) Hewitt et al. (2003) Han et al. (2008) Kirwan et al. (2014) Ghaste et al. (2016) Simon et al. (2018)

Traditionally, untargeted analysis has been associated with hypothesis forming, whilst targeted analysis is associated with hypothesis testing and is often viewed as more powerful. The data output from targeted analysis is generally quantitative, absolute quantification and annotation, whereas untargeted results are often qualitative or, in some cases where a range of standards have been included, semi-quantitative. Equally, the diversity of analysis techniques (LC/GC-MS, NMR, FT-ICR-MS, Table 8.2) creates further issues for direct comparisons of datasets.

The sheer scale and size of the metabolome is an additional hinderance to analysis, particularly considering the significant chemical diversity of metabolites and their dynamic range (D'Auria and Gershenzon, 2005; Fernie et al., 2004). For example, from the 4×10^5 vascular plant species on the planet (Willis, 2017) it is estimated there are between 2×10^5 and 1×10^6 individual metabolites (Rai et al., 2017; Fang et al., 2019), with any single species containing $\geq 5 \times 10^4$ compounds (Fernie et al., 2004). Furthermore, while primary metabolism is generally conserved, the synthesis of secondary (specialised) metabolites, which can account for a large proportion of their metabolome, can be hugely diverse between species (Alseekh and Fernie, 2018). This diversity of metabolism is replicated across the fungal kingdom, and while bacteria are generally considered to be less metabolically diverse, they still exhibit significant variation (depending on their energy requirements and metabolic mechanisms (e.g. phototrophs and chemotrophs) (Gomez, 2011).

Linking metabolite analysis to the underlying genomic, transcriptomic, proteomic and enzymatic (although this may only indicate potential rates (Greenfield et al., 2020)) analysis remains the gold standard in environmental 'omic research, potentially allowing insight into microbial function in unprecedented detail. Whilst it is possible to correlate the presence of metabolites to specific members of the biological community (Li et al., 2020; Taylor et al., 2018), metabolites have also been linked to specific genes (Finn et al., 2020; Hooft et al., 2020; Kjærbølling et al., 2018). This translation of 'omics metrics into a functional understanding of ecosystem processing and ecosystem function and service provision, will be key to cementing their relevance in the study environmental biology into the future (Bahamonde et al., 2016; Biswas and Sarkar, 2018). However, this requires the systematic construction of high-resolution metabolome libraries, including the continual characterisation of as yet unidentified metabolites (or 'metabolic dark matter', Fig. 8.2). Diverse pathway maps will also be required to reflect the variety of metabolism (particularly of secondary metabolism) within the taxonomic kingdoms, as this is often not reflected in current, generic metabolic maps.

Ultimately, this should aid effective and efficient integration with the other 'omics techniques currently utilised in soil research.

8.5. Perspectives on the study of soil metabolomics

8.5.1. The importance of untargeted analysis

As alluded to in section 8.3, targeted metabolomic analysis is often viewed as more powerful compared to untargeted analysis, due to its quantitative, authenticated nature. However, untargeted analysis should not be overlooked. A large number of metabolic compounds are yet to be characterised, this so called 'metabolic dark matter' (Fig. 8.1) represents an exciting and, as yet, untapped source of new analogues of clinically used antibiotics theoretically allowing the discovery of metabolites capable of circumventing clinically important resistance mechanisms (Peek et al., 2018; Sharrar et al., 2020). In addition, this will aid in the understanding of the chemical ecology of soil (Kellogg and Kang, 2020) and fundamental fluxes of small organic molecules through soil and the inter- and intra-species interactions between soil organisms. It is only by performing untargeted, 'discovery' analysis that the elucidation of molecular biomarkers, for example typical osmolytes associated with drought e.g. trehalose, mannitol and glycine betaine, may be associated with the conditions (Nawaz and Wang, 2020; Warren, 2020). We have only scratched the surface in terms of our comprehension the high-resolution C cycling in soils, analytical advances in resolution, annotation and quantification are likely to enhance this into the future.

8.5.2. The emission and fate of VOCs

The emission of C from soil is also an area of interest for future research, current models focus overwhelmingly on the three most potent greenhouse gases from soil, carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O). While the effect of these gases are well known in terms of climate change, the emission of VOC from soil has been little explored. For example, biogenic secondary metabolite emission from soil (e.g., isoprene and monoterpenes) may also contribute to tropospheric ozone and secondary organic aerosol formation (Ahlberg et al., 2017; Fitzky et al., 2019; Sindelarova et al., 2014). With soil VOC emissions having shown to be up to $5.0\% \pm 2.0$ of CO_2 emissions (molar C equivalent) on rewetting (Rossabi et

al., 2018), this may be a significant, previously unaccounted for, loss of C from soil. While, VOCs have been incorporated into atmospheric models to some extent, they do not usually focus on total VOC emission, rather, a small number of the most abundant VOCs, and often not from soil itself (Navarro et al., 2014).

Nevertheless, modelling has estimated the annual global emission of VOCs from vegetation is 6.34×10^8 t C yr^{-1} (Henrot et al., 2017). Plants are also major emitters of VOCs to soil through their roots (Dudareva et al., 2007), of which many are related to abiotic or biotic stressors. Therefore, monitoring their emission and diffusion in a non-invasive and non-destructive way may provide further insight into plant health. Equally, VOCs provide a labile substrate and are capable of being consumed in soil (Owen et al., 2007). Therefore, methods of measurement throughout the whole soil profile, not just at the surface, will be key to understanding the mechanisms of anabolism and catabolism throughout the soil. Future work is required to quantify the loss of C from soil. Further, it is not only the emission of these compounds, but the interaction effects with the environment and inhabiting biology, as well as the degradation pathways and the ultimate fate of this C that is largely unknown and warrants further investigation.

8.5.3. The rhizosphere – a key hotspot for biochemical and organismal interaction

The rhizosphere was long been recognised as a 'hotspot' for nutrient transformation, biochemical and organismal interaction (Kuzyakov and Blagodatskaya, 2015). Plants exude significant amounts of their photosynthetically fixed C and N through their roots, releasing up to 20% and 15%, respectively (Mohanram and Kumar, 2019; Venturi and Keel, 2016). Yet, the complete reasoning behind this large investment remains elusive. Rhizosphere exudate composition is largely defined by the genotype of the plant (Mönchgesang et al., 2016), its developmental stage (Chaparro et al., 2013) and level of abiotic stress experienced (Carvalhais et al., 2013), all of which have been shown to influence and self-select the plants rhizosphere microbiome (Sasse et al., 2018) and subsequent inter-kingdom (Durán et al., 2018) and inter-species interactions (Badri et al., 2009; Foster and Bell, 2012). This interaction is of significance to plants, aiding in their resilience to disease and tolerance to stress (Mohanram and Kumar, 2019). There is also great interest in manipulating the plant microbiome to improve agricultural productivity and reduce potential N pollutants (N_2O and NO_3 leaching) through

selecting for plant genes that promote nitrification inhibition (Arif et al., 2020; Subbarao et al, 2021).

However, further research is required to understand the fundamental relationship between the soil metabolite profile and the microbial community, as well as the relationship to the regulation of plant rhizo-processes and subsequent nutrient transformations and processing. Particularly, investigation into the rhizosphere metabolome and microbiome under different plant growth stage and abiotic stresses, including those that are likely to become more prevalent with climate change (e.g. drought, flood, salinisation (Jansson and Hofmockel, 2019)), anthropogenic perturbation (e.g. plastic and microplastic loading and other pollution events (de Souza Machado et al., 2019; Rillig et al., 2017)), and agriculture (e.g. nutrient loading and multi-species planting (Overy et al., 2021)). The increased knowledge gained from such studies will further guide our ability to engineer the rhizosphere to promote resistance, resilience, productivity and sustainability. This is likely to be a challenge, as extracting compounds from the rhizosphere without disturbing the root itself (which may lead to a stress response) is extremely difficult (Oburger and Jones, 2018). Therefore, the task of disentangling the complex relationship between plant-microbe interactions in the rhizosphere should not be underestimated.

8.5.4. Understanding temporal metabolite fluxes

The temporal flux of metabolites through the soil system, through the processing of SOM is a key component of soil function. The relative cost of primary metabolomic extraction and analysis remains relatively high and is often a hinderance to large-scale experiments with measurement over time. Equally, there is currently little analytical scope for real-time monitoring, with a focus on the study of metabolic pools (Warren, 2013). Equally, stable- and radioisotope analysis is a little explored method which has the potential to be a powerful tool for enhancing the understanding of metabolic pathways and networks, as well as overall metabolome change within the soil environment (Bore et al., 2017; Dijkstra et al., 2011; Nakabayashi and Saito, 2020; Tian et al., 2018; Watzinger, 2015). Ultimately, understanding the dynamics of the soil system is key to understanding the rates of C and nutrient cycling and the functional implications (Canarini et al., 2019).

While the gaseous nature of VOCs is better suited to real-time measurement, for example, using proton-transfer-reaction mass spectrometry (PTR-MS), compound detection is not extensive, and compound identification is putative and qualitative, unless combined with other analytical techniques (e.g. TOF-MS) (Mancuso et al., 2015). However, significant advances in calibrated sensor technology (e.g. Photoionization detectors, PIDs) may allow the non-selective measurement of total VOC fluxes from the soil (Bocos-Bintintan et al., 2019), potentially quantitatively aiding the understanding of VOC emission and secondary metabolic changes (see section 8.4.2).

8.5.5. Towards functional environmental and ecological 'omics

Functional metabolomics is a concept originating from the biomedical sciences with the aim of overcoming the descriptive nature of interpretation, largely limited to speculating on metabolite function based on previous literature (Yan and Xu, 2018). An integrative approach is required to truly understand function, drawing on genomic, transcriptomic and proteomic approaches. The function of the majority of genes are yet to be elucidated, particularly in the case of the gene-protein-metabolite regulation network. This integration of multi-omics data can be utilised to tentatively reconstruct the multi-layer regulation network, potentially providing a more comprehensive and informative understanding of the regulation of genes to transcripts, transcripts to proteins and proteins to metabolites.

What is more, different level 'omics data can be used to validate each other. While likely to be resource intensive work and require significant collaboration between disciplines (microbiologists, biochemists, chemical ecologists, environmental scientists, bioinformaticians and modellers), future research towards functional environmental 'omics must aim to integrate and draw on multiple levels of 'omics data. This integration also requires minimum reporting standards for metabolomic data and metadata and publicly accessible data repositories, similar to that of genomic sequencing data, to allow future comparison in meta-analyses and modelling to maximise use and compatibility of datasets. Previously minimum reporting standards have been suggested e.g. Sumner et al., (2007), Viant et al., (2019) and Fiehn et al., (2007), however not widely adopted, particularly in the environmental sciences.

8.6. Conclusions

Soil metabolomics provides greater characterisation and elucidation of soil biochemistry and chemical ecology at high resolution, aiding the understanding of the complex small molecule interactions taking place within soils. Due to its relative infancy there any many further possibilities for its application. The use of untargeted 'discovery' metabolomics should not be undervalued, particularly in such a complex system such as soil. Equally, both the rhizosphere and volatile products of secondary metabolism are relatively unexplored research areas using metabolomic and volatilomic techniques. Looking to the future, the ultimate goal should be the integration of metabolomics with other 'omics platforms, with an emphasis on providing a functional understanding to key soil processes e.g., C and nutrient cycling, which are essential to (agro-)ecosystem service provision, as well as higher resolution insights into ecological interaction.

8.7. Acknowledgements

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Appendix 1

Supplementary material from Chapter 3

Brown, R.W, Chadwick, D.R., Zang, H., Jones, D.L., 2021. Use of metabolomics to quantify changes in soil microbial function in response to fertiliser nitrogen supply and extreme drought. Soil Biology and Biochemistry, 160, 108351

Figure 1. *Aerial photograph of experimental layout at Henfaes Agricultural Research Station taken May 2017.*
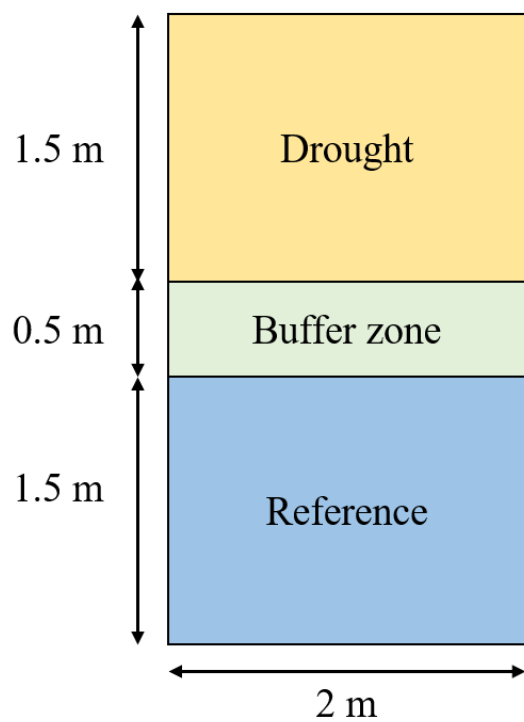
Figure 2. *Split plot experimental sampling design.*

| Aber Echo 300 kg N ha^{-1} | Aber Echo 0 kg N ha^{-1} | Aber Niche 300 kg N ha^{-1} | Aber Niche 0 kg N ha^{-1} |

Reference — Reference — Reference — Reference

Drought — Drought — Drought — Drought
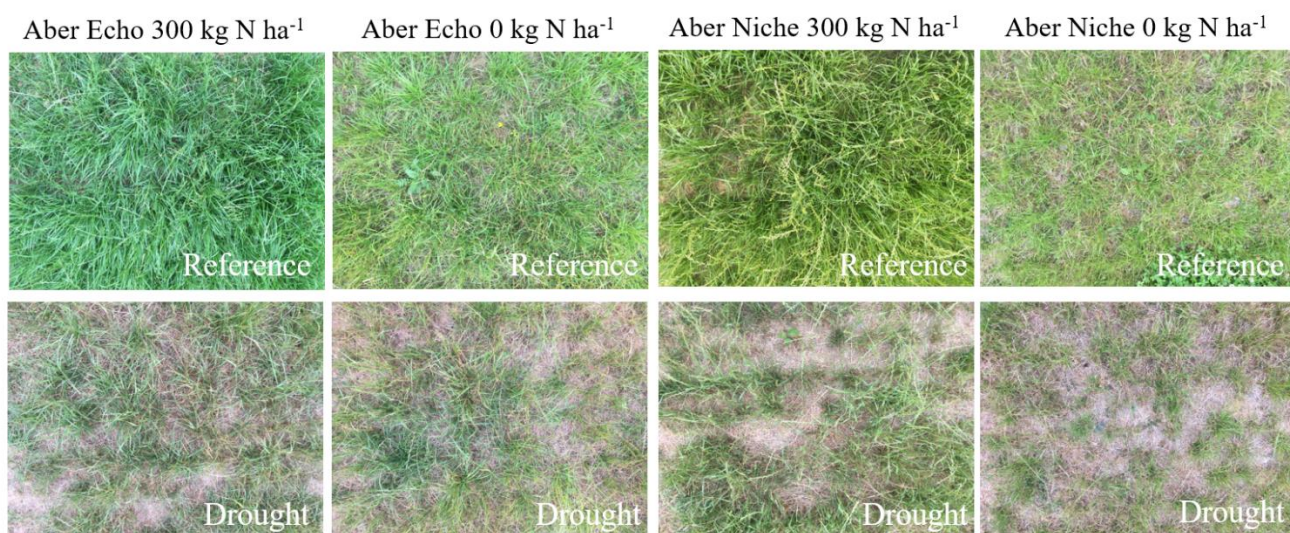
Figure 3. Photos of drought and Reference plots during the drought, illustrating the difference in plant health.

Reference 0 kg N ha^{-1}
Reference 300 kg N ha^{-1}
Drought 0 kg N ha^{-1}
Drought 300 kg N ha^{-1}
Recovery Reference 0 kg N ha^{-1}
Recovery Reference 300 kg N ha^{-1}
Recovery Drought 0 kg N ha^{-1}
Recovery Drought 300 kg N ha^{-1}
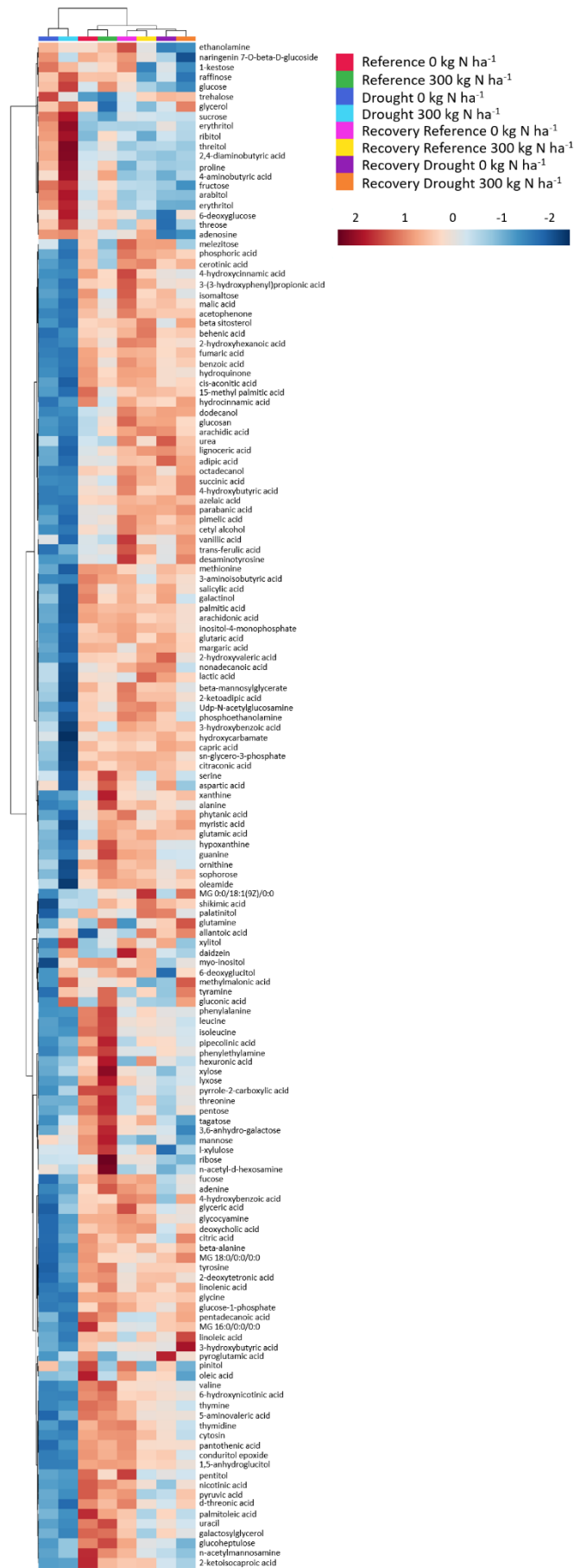
Figure 4. Influence of drought, N fertilisation and grass species on the metabolic profile of soil. Heatmap showing expression profiles of soil treatment groups (n = 8). Metabolites were subsequently clustered using Euclidean distance and Ward linkage. Data were normalised using a log transformation and Pareto scaling. The colour of samples ranges from red to blue, indicating metabolite concentration z-score; numbers 2 to -2 on the scale bar indicate the number of standard deviations from the mean.

Table 1. *Characteristics of bulked water used for irrigation of control plots. Results are expressed on a mean basis ± SEM (n = 4).*

Irrigation water properties	
pH	6.78 ± 0.1
EC ($\mu S\ cm^{-1}$)	72 ± 1
Dissolved organic C ($mg\ C\ l^{-1}$)	2.81 ± 0.8
NO_3^- ($mg\ N\ l^{-1}$)	0.05 ± 0.0
NH_4^+ ($mg\ N\ l^{-1}$)	0.01 ± 0.0
PO_4^- ($mg\ P\ l^{-1}$)	0.03 ± 0.0

Table 2. *UPLC data acquisition parameters.*

Parameter	
Column	Waters Acquity UPLC CSH C18 (100 mm length x 2.1 mm internal diameter; 1.7 µm particles)
Positive mode	Mobile phase A: 60:40 v/v acetonitrile:water + 10 mM ammonium formate + 0.1% formic acid. Mobile phase B: 90:10 v/v isopropanol:acetonitrile + 10 mM ammonium formate + 0.1% formic acid
Column temperature	65°C Flow-rate: 0.6 mL/min
Injection volume	1.67 µL for ESI(+) and 5 µL for ESI(–)
Injection temperature	4°C
Gradient	0 min 15% (B), 0–2 min 30% (B), 2–2.5 min 48% (B), 2.5–11 min 82% (B), 11–11.5 min 99% (B), 11.5– 12 min 99% (B), 12–12.1 min 15% (B), 12.1–15 min 15% (B)
ESI capillary voltage	ESI(+): +3.5 kV; ESI(–): –3.5 kV
Precursor/product isolation width	4 Da
Collision energy	25 eV for ESI(+); 25 eV for ESI(–)
Scan range positive mode	m/z 120 – 1200 Da
Scan range negative mode	m/z 60–1200 Da
Spectral acquisition speed	2 spectra/s Mass resolution: 10,000 for ESI(+) on an Agilent 6530 QTOF MS; 20,000 for ESI(–) on an Agilent 6550 QTOF MS

Table 3. *A summary of PLFA biomarkers summed to represent microbial groups.*

Microbial group category	Peaks			
AM Fungi	16:1 w5c			
Saprophytic Fungi	18:2 w6c			
Gram Negative	10:0 2OH	10:0 3OH	12:1 w8c	12:1 w5c
	13:1 w5c	13:1 w4c	13:1 w3c	12:0 2OH
	14:1 w9c	14:1 w8c	14:1 w7c	14:1 w5c
	15:1 w9c	15:1 w8c	15:1 w7c	15:1 w6c
	15:1 w5c	14:0 2OH	16:1 w9c	16:1 w7c
	16:1 w6c	16:1 w4c	16:1 w3c	17:1 w9c
	17:1 w8c	17:1 w7c	17:1 w6c	17:1 w5c
	17:1 w4c	17:1 w3c	16:0 2OH	17:0 cyclo w7c
	18:1 w8c	18:1 w7c	18:1 w6c	18:0 cyclo w6c
	18:1 w3c	19:1 w9c	19:1 w8c	18:1 w5c
	19:1 w6c	19:0 cyclo w9c	19:0 cyclo w7c	19:1 w17c
	20:1 w9c	20:1 w8c	20:1 w6c	19:0 cyclo w6c
	20:1 w4c	20:0 cyclo w6c	21:1 w9c	21:1 w8c
	21:1 w6c	21:1 w5c	21:1 w4c	21:1 w3c
	22:1 w9c	22:1 w8c	22:1 w6c	22:1 w5c
	22:1 w3c	22:0 cyclo w6c	24:1 w9c	24:1 w7c
	11:0 iso 3OH	14:0 iso 3OH		
Methanotroph	16:1 w8c			
Eukaryote	15:4 w3c	15:3 w3c	16:4 w3c	16:3 w6c
	18:4 w3c	18:3 w6c	19:4 w6c	19:3 w6c
	19:3 w3c	20:4 w6c	20:5 w3c	20:3 w6c
	20:2 w6c	21:3 w6c	21:3 w3c	22:5 w6c
	22:6 w3c	22:4 w6c	22:5 w3c	22:2 w6c
	23:4 w6c	23:3 w6c	23:3 w3c	23:1 w5c
	23:1 w4c	24:4 w6c	24:3 w6c	24:3 w3c
	24:1 w3c			
Gram Positive	11:0 so	11:0 anteiso	12:0 iso	12:0 anteiso
	3:0 iso	13:0 anteiso	14:1 iso w7c	14:0 iso
	14:0 anteiso	15:1 iso w9c	15:1 iso w6c	15:1 anteiso w9c
	15:0 iso	15:0 anteiso	16:0 iso	16:0 anteiso
	17:1 iso w9c	17:0 iso	17:0 anteiso	18:0 iso
	17:1 anteiso w9c	17:1 iso w10c	17:1 anteiso w7c	18:1 w9c
	19:0 iso	19:0 anteiso	20:0 iso	22:0 iso
	16:0 10-methyl	17:1 w7c 10-methyl	17:0 10-methyl	18:1 w7c 10-methyl
	18:0 10-methyl	19:1 w7c 10-methyl	20:0 10-methyl	22:0 10-methyl

Appendix 2

Supplementary material from Chapter 4

Brown, R.W., Chadwick D.R., Thornton H., Marshall M.R., Bei, S., Distaso, M.A., Bargiela, R., Marsden, K.A., Clode, P.L., Murphy, D.V., Pagella, S., Jones, D.L., (In submission). Field application of pure polyethylene microplastic has no significant effect on soil biological quality and function. Soil Biology and Biochemistry.
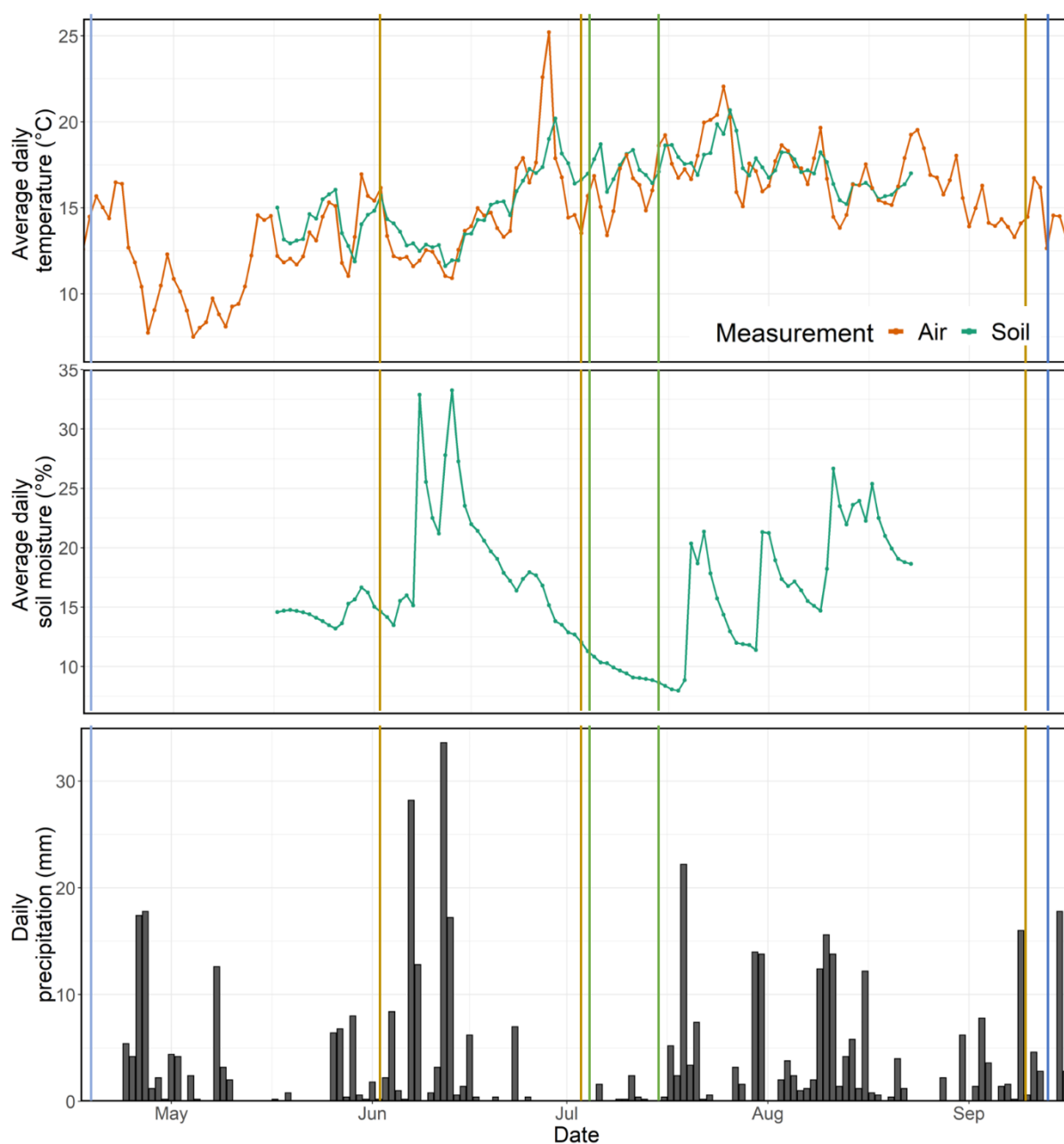
Figure 1. *Weather data from sampling period. Air temperature and precipitation were collected from an adjacent weather station, soil moisture and soil temperature were collected*

using Acclima sensors in control plots (depth = 10 cm, n = 4). Vertical lines correspond to the following sampling points; Light blue - Wheat sown and MP applied; Yellow 1 - pH, EC, NO₃, NH₄; Yellow 2 - pH, EC, NO₃, NH₄, BD, PLFA; Green 1 – N cycling genes NO₃ and NH₄; Green 2 – N cycling genes, NO₃ and NH₄; Yellow 3 - pH, EC, NO₃, NH₄, BD, PLFA, BA, 16S sequencing; Blue – Wheat harvest; Purple – Earthworms.
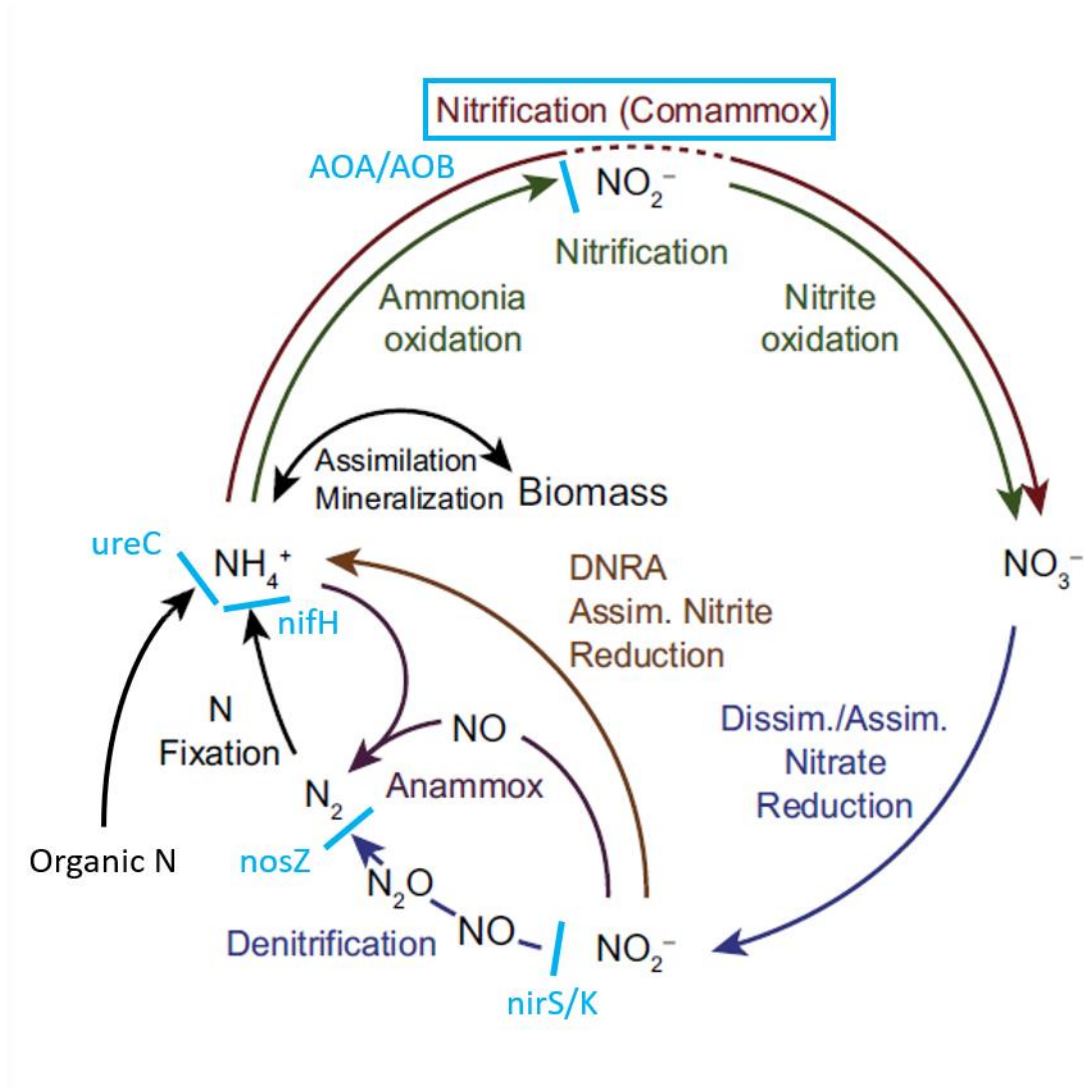
Figure 2. *Soil nitrogen cycling genes were measured in this experiment (blue) in relation to the N cycle.*
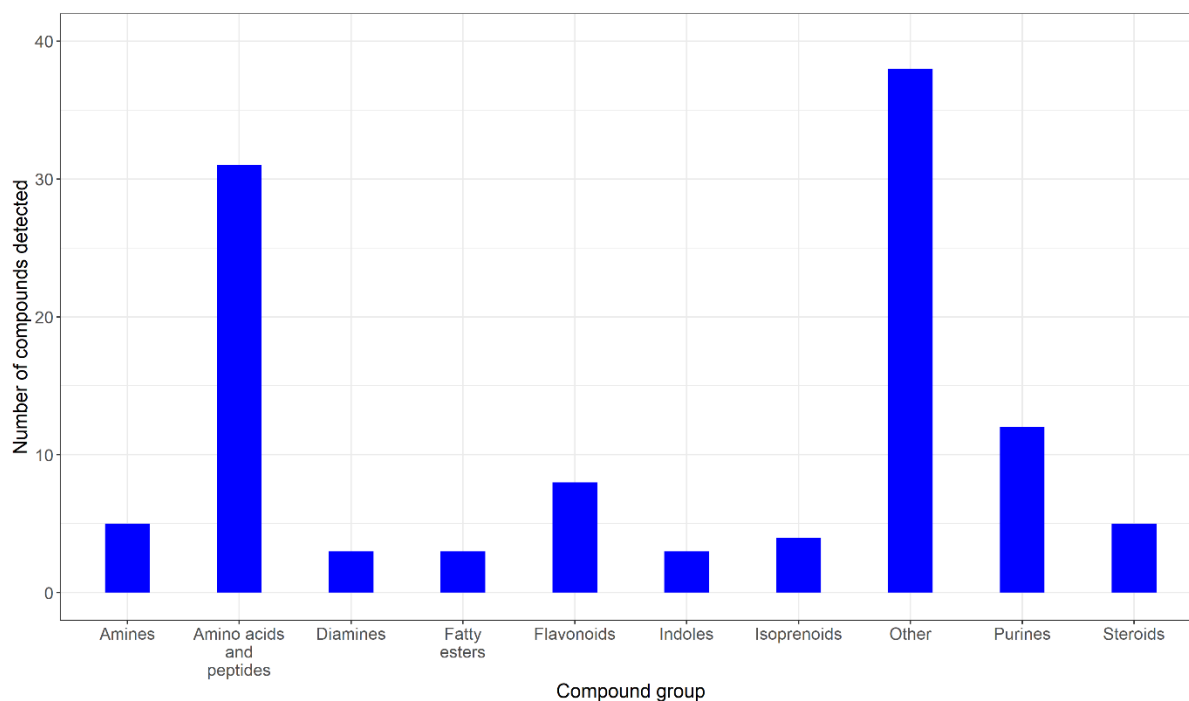
Figure 3. *Dominant biogenic amine compound classes within the soil samples. All compounds identified were found in all samples. 'Other' includes; alkaloids, benzoic acids, fatty amines, pyridines and quinolines. A full compound list can be found in the additional supplementary information.*
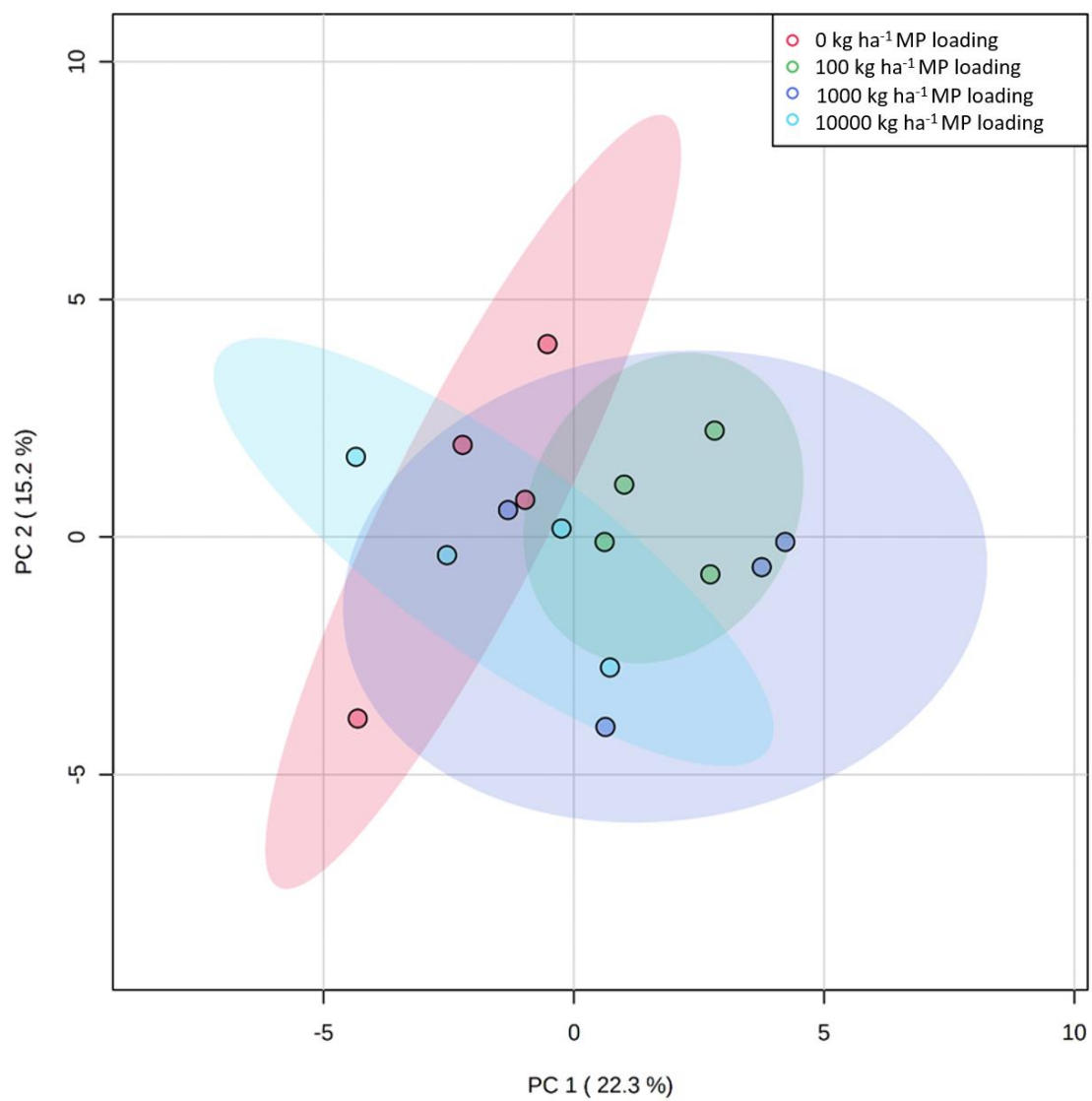
Figure 4. *Principal component analysis (PCA) ordination plot of biogenic amines (BAs) by*

MP loading treatment. Data were normalised using a log transformation and Pareto scaling.

1 ***Table 1.*** *Summary of N cycling gene primers and thermocycling conditions.*

2

Function	Targeted phyla and genes	Primers	Sequence (5′–3′)	Thermocycling conditions	References
Nitrogen metabolism	*ureC gene*	ureC1F ureC2R	ACCTAATGTACAGGAGGAT GGGCTATCTTCCAAAAT	94°C for 10 min; 30 cycles of 94°C for 1 min; 55°C for 1 min, and 72°C for 2 min; and a final elongation at 72°C for 10 min.	Ouyang et al., 2018; Koper et al., 2004
Nitrification	archaeal *amoA*	Arch-amoAF Arch-amoAR	STAATGGTCTGGCTTAGACG GCGGCCATCCATCTGTATGT	95°C for 5 min; 40 cycles consisting of 94°C for 45 s, 53°C for 1min, and 72°C for 1 min; and final elongation at 72°C for 15 min.	Francis et al., 2005
	bacterial *amoA*	amoA-1F amoA-2R	GGGGTTTCTACTGGTGGT CCCCTCKGSAAAGCCTTCTTC	94°C for 30s; then 40 cycles consisting of 15 s at 94°C, 20 s at 55°C, 40 s at 72 °C, and a final elongation at 72 °C for 3 min.	Rotthauwe et al., 1997
	comammox Nitrospira	Ntsp-amoA 162F Ntsp-amoA 359R	GGATTTCTGGNTSGATTGGA WAGTTNGACCACCASTACCA	94 °C for 5 min, 40 cycles of 94°C for 30 s,48 °C for 30 s and 72 °C for 60 s and a final elongation at 72°C for 10 min.	Fowler et al., 2018
Denitrification	*nirK*	nirK1F nirK3R	GG(A/C)ATGGT(G/T)CC(C/G)TGGCA GAACTTGCCGGT(A/C/G)G(C/T)CCAG AC	See Braker et al., 1998.	Throbäck et al., 2004; Braker et al. 1998
	nirS	nirS1F nirS4R	CCTA(C/T)TGGCCGCC(A/G)CA(A/G)T TTCGG(G/A)TG(C/G)GTCTTGA(T/C)G AA	As above.	Throbäck et al., 2004; Braker et al. 1998
	nosZ	nosZ2 nosZ2R	FCGCRACGGCAASAAGGTSMSSGT CAKRTGCAKSGCRTGGCAGAA	95 °C for 3 min, 40 cycles of 95°C for 30 s,62 °C for 30 s and 72 °C for 60 s.	Henry et al., 2006
Free nitrogen fixation	*nifH*	nifH-F nifH-R	AAAGGYGGWATCGGYAARTCCACC AC TTGTTSGCSGCRTACATSGCCATCAT	95 °C for 3 min, with 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s, and final elongation at 72 °C for 10 min.	Rösch et al., 2002; Feng et al., 2018

3

Table 2. *A summary of PLFA biomarkers summed to represent microbial groups.*

Microbial group category	Peaks			
AM Fungi	16:1 w5c			
Saprophytic Fungi	18:2 w6c			
Gram Negative	10:0 2OH	10:0 3OH	12:1 w8c	12:1 w5c
	13:1 w5c	13:1 w4c	13:1 w3c	12:0 2OH
	14:1 w9c	14:1 w8c	14:1 w7c	14:1 w5c
	15:1 w9c	15:1 w8c	15:1 w7c	15:1 w6c
	15:1 w5c	14:0 2OH	16:1 w9c	16:1 w7c
	16:1 w6c	16:1 w4c	16:1 w3c	17:1 w9c
	17:1 w8c	17:1 w7c	17:1 w6c	17:1 w5c
	17:1 w4c	17:1 w3c	16:0 2OH	17:0 cyclo w7c
	18:1 w8c	18:1 w7c	18:1 w6c	18:0 cyclo w6c
	18:1 w3c	19:1 w9c	19:1 w8c	18:1 w5c
	19:1 w6c	19:0 cyclo w9c	19:0 cyclo w7c	19:1 w17c
	20:1 w9c	20:1 w8c	20:1 w6c	19:0 cyclo w6c
	20:1 w4c	20:0 cyclo w6c	21:1 w9c	21:1 w8c
	21:1 w6c	21:1 w5c	21:1 w4c	21:1 w3c
	22:1 w9c	22:1 w8c	22:1 w6c	22:1 w5c
	22:1 w3c	22:0 cyclo w6c	24:1 w9c	24:1 w7c
	11:0 iso 3OH	14:0 iso 3OH		
Methanotroph	16:1 w8c			
Eukaryote	15:4 w3c	15:3 w3c	16:4 w3c	16:3 w6c
	18:4 w3c	18:3 w6c	19:4 w6c	19:3 w6c
	19:3 w3c	20:4 w6c	20:5 w3c	20:3 w6c
	20:2 w6c	21:3 w6c	21:3 w3c	22:5 w6c
	22:6 w3c	22:4 w6c	22:5 w3c	22:2 w6c
	23:4 w6c	23:3 w6c	23:3 w3c	23:1 w5c
	23:1 w4c	24:4 w6c	24:3 w6c	24:3 w3c
	24:1 w3c			
Gram Positive	11:0 so	11:0 anteiso	12:0 iso	12:0 anteiso
	3:0 iso	13:0 anteiso	14:1 iso w7c	14:0 iso
	14:0 anteiso	15:1 iso w9c	15:1 iso w6c	15:1 anteiso w9c
	15:0 iso	15:0 anteiso	16:0 iso	16:0 anteiso
	17:1 iso w9c	17:0 iso	17:0 anteiso	18:0 iso
	17:1 anteiso w9c	17:1 iso w10c	17:1 anteiso w7c	18:1 w9c
	19:0 iso	19:0 anteiso	20:0 iso	22:0 iso
	16:0 10-methyl	17:1 w7c 10-methyl	17:0 10-methyl	18:1 w7c 10-methyl
	18:0 10-methyl	19:1 w7c 10-methyl	20:0 10-methyl	22:0 10-methyl

Table 3. *N cycling gene linear mixed model outputs.*

Predictors	log(UreC) Estimates	CI	p	log(AOA) Estimates	CI	p	log(AOB) Estimates	CI	p	log(Comammox) Estimates	CI	p	log(nirK) Estimates	CI	p	log(nirS) Estimates	CI	p	log(nosZ) Estimates	CI	p	log(nifH) Estimates	CI	p
(Intercept)	15.04	14.88 – 15.20	**<0.001**	13.02	12.93 – 13.11	**<0.001**	11.44	11.28 – 11.60	**<0.001**	12.81	12.64 – 12.98	**<0.001**	10.63	10.48 – 10.78	**<0.001**	13.13	12.96 – 13.31	**<0.001**	12.60	12.42 – 12.77	**<0.001**	14.04	13.93 – 14.15	**<0.001**
MP.loading [100]	-0.10	-0.32 – 0.12	0.383	-0.13	-0.26 – -0.00	**0.045**	-0.09	-0.31 – 0.14	0.458	-0.03	-0.28 – 0.21	0.785	0.10	-0.11 – 0.31	0.351	0.13	-0.12 – 0.38	0.296	0.02	-0.22 – 0.27	0.858	0.09	-0.07 – 0.24	0.265
MP.loading [1000]	-0.01	-0.24 – 0.21	0.907	-0.08	-0.21 – 0.04	0.196	-0.31	-0.54 – -0.09	**0.007**	-0.02	-0.27 – 0.22	0.858	-0.28	-0.49 – -0.07	**0.009**	0.03	-0.22 – 0.27	0.829	-0.33	-0.57 – -0.08	**0.009**	-0.05	-0.20 – 0.10	0.529
MP.loading [10000]	0.09	-0.13 – 0.32	0.428	-0.02	-0.15 – 0.10	0.714	-0.15	-0.37 – 0.08	0.203	0.05	-0.20 – 0.29	0.700	-0.03	-0.24 – 0.17	0.744	-0.01	-0.26 – 0.23	0.906	-0.07	-0.32 – 0.17	0.558	-0.06	-0.22 – 0.09	0.405
c.Date	-0.26	-0.57 – 0.06	0.107	-0.05	-0.23 – 0.13	0.607	-0.48	-0.80 – -0.16	**0.003**	-0.03	-0.37 – 0.31	0.857	-0.27	-0.56 – 0.02	0.069	-0.16	-0.51 – 0.19	0.368	-0.18	-0.53 – 0.17	0.324	0.08	-0.11 – 0.27	0.385
MP.loading [100] * c.Date	0.46	0.02 – 0.90	**0.042**	0.20	-0.05 – 0.46	0.123	0.38	-0.07 – 0.83	0.099	0.21	-0.27 – 0.69	0.391	0.17	-0.24 – 0.58	0.419	0.24	-0.26 – 0.73	0.351	0.18	-0.32 – 0.67	0.481	0.07	-0.20 – 0.33	0.627
MP.loading [1000] * c.Date	0.13	-0.32 – 0.57	0.577	0.22	-0.03 – 0.48	0.084	0.65	0.20 – 1.11	**0.005**	0.10	-0.37 – 0.58	0.669	0.48	0.07 – 0.89	**0.021**	0.26	-0.23 – 0.75	0.304	0.59	0.09 – 1.08	**0.019**	0.12	-0.15 – 0.39	0.370
MP.loading [10000] * c.Date	0.24	-0.21 – 0.68	0.296	0.11	-0.14 – 0.37	0.392	0.06	-0.39 – 0.51	0.804	0.11	-0.37 – 0.59	0.651	0.40	-0.01 – 0.81	0.055	0.38	-0.11 – 0.88	0.129	0.11	-0.38 – 0.61	0.658	-0.12	-0.39 – 0.15	0.370

Random Effects

	log(UreC)			log(AOA)			log(AOB)			log(Comammox)			log(nirK)			log(nirS)			log(nosZ)			log(nifH)		
σ^2	0.05			0.02			0.05			0.06			0.04			0.06			0.06			0.02		
τ_{00}	0.00 Paired			0.00 Paired			0.00 Paired			0.00 Paired			0.00 Paired			0.00 Paired			0.00 Paired			0.00 Paired		
ICC	0.02									0.02			0.02									0.13		
N	16 Paired			16 Paired			16 Paired			16 Paired			16 Paired			16 Paired			16 Paired			16 Paired		
Observations	32			32			32			32			32			32			32			32		
Marginal R² / Conditional R²	0.198 / 0.211			0.282 / NA			0.445 / NA			0.060 / 0.076			0.399 / 0.413			0.127 / NA			0.343 / NA			0.281 / 0.371		

Table 4. *Cumulative N$_2$O fluxes by treatment and event. Letters denote the lack of statistical significance between treatments upon each event.*

Event	MP loading rate (kg ha^{-1})	Cumulative flux (µg N$_2$O-N m^{-2})
Initial MP application	0	-0.25[a] ± 6.3
	100	0.13[a] ± 2.8
	1000	-1.25[a] ± 7.2
	10000	2.41[a] ± 2.7
N fertiliser application 1	0	66.70[b] ± 16.8
	100	79.02[b] ± 21.1
	1000	51.17[b] ± 1.7
	10000	87.57[b] ± 6.3
N fertiliser application 2	0	16.17[c] ± 11.6
	100	27.88[c] ± 27.0
	1000	45.44[c] ± 19.5
	10000	67.71[c] ± 12.6

Supplementary information references

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Appendix 3

Supplementary material from Chapter 5

Brown, R.W., Chadwick, D.R., Jones, D.L., in prep. Labile nutrient enrichment of soil induces significant change in its metabolite profile and carbon usage. Soil Biology and Biochemistry.
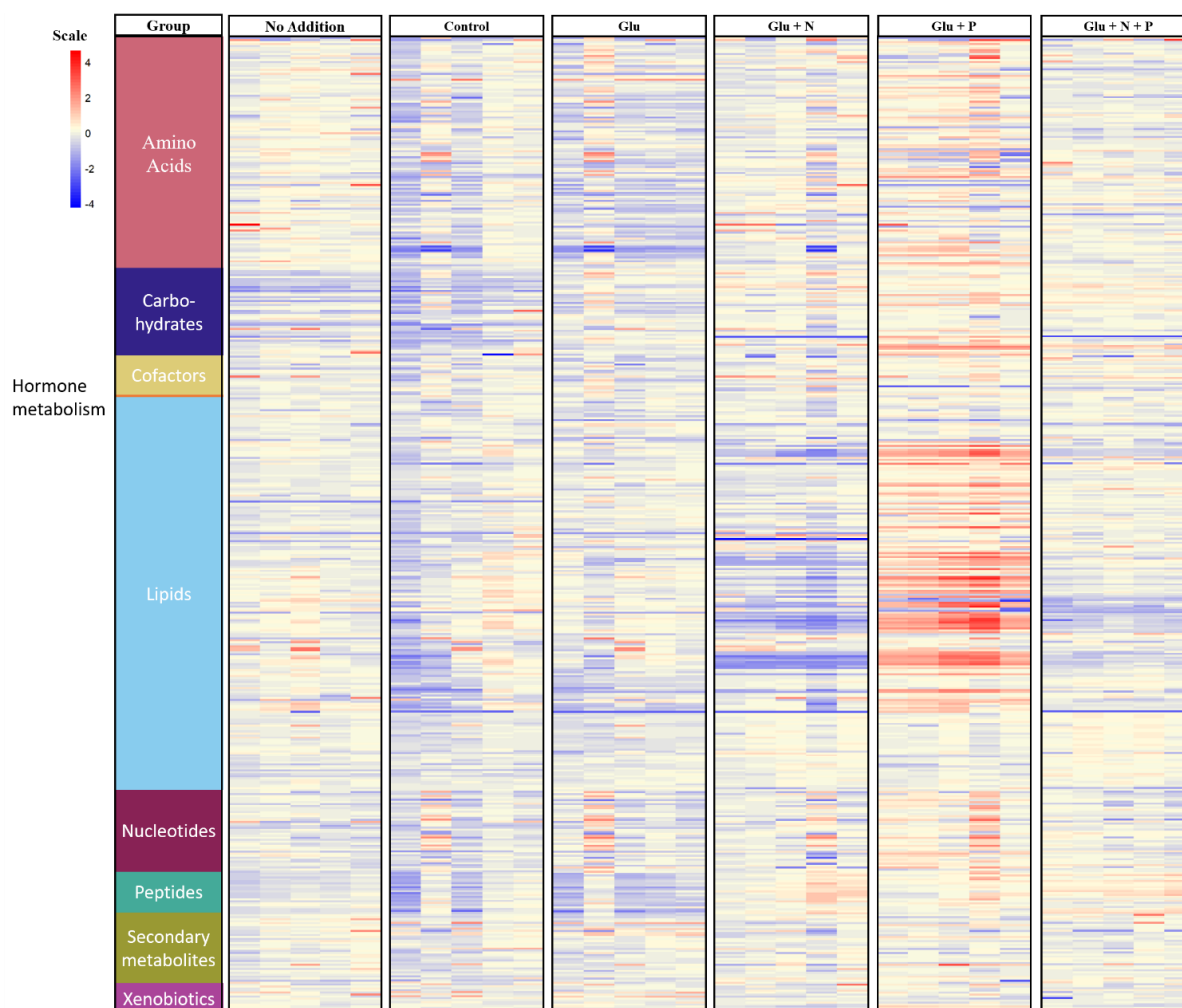
Figure S1. Influence of nutrient addition on the primary metabolite profile of soil. Heatmap showing expression profiles of soil treatment groups ($n = 5$). Compounds are grouped into their superpathways and treatment groups. Plotting was performed on natural log (ln) transformed median scaled imputed data. The colour of samples ranges from red to blue, indicating metabolite concentration z-score; numbers 4 to −4 on the scale bar indicate the number of standard deviations from the mean. Red illustrates a relative increase in the compound concentration, blue indicates a relative decrease in compound concentration.
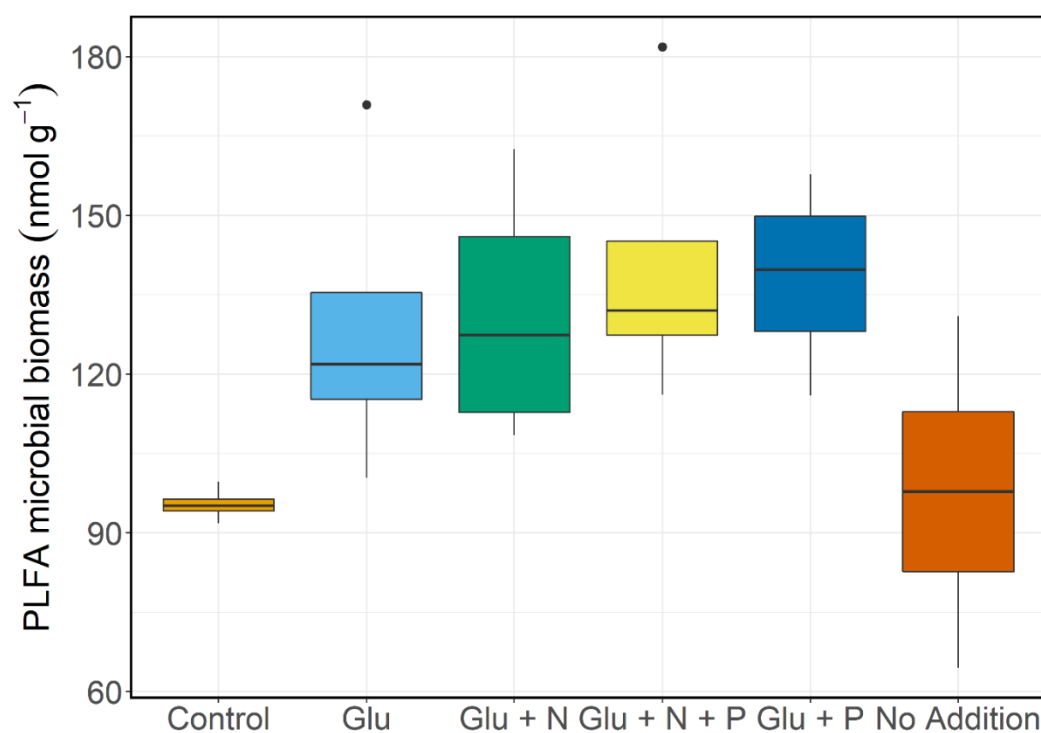
Figure S2. Influence of nutrient addition on the PLFA derived microbial biomass. There was no significant difference between treatments by Tukey HSD.

Table S1. Summary of PLFA biomarkers summed to represent microbial groups.

Microbial group category	Peaks			
AM Fungi	16:1 w5c			
Saprophytic Fungi	18:2 w6c			
Gram Negative	10:0 2OH	10:0 3OH	12:1 w8c	12:1 w5c
	13:1 w5c	13:1 w4c	13:1 w3c	12:0 2OH
	14:1 w9c	14:1 w8c	14:1 w7c	14:1 w5c
	15:1 w9c	15:1 w8c	15:1 w7c	15:1 w6c
	15:1 w5c	14:0 2OH	16:1 w9c	16:1 w7c
	16:1 w6c	16:1 w4c	16:1 w3c	17:1 w9c
	17:1 w8c	17:1 w7c	17:1 w6c	17:1 w5c
	17:1 w4c	17:1 w3c	16:0 2OH	17:0 cyclo w7c
	18:1 w8c	18:1 w7c	18:1 w6c	18:0 cyclo w6c
	18:1 w3c	19:1 w9c	19:1 w8c	18:1 w5c
	19:1 w6c	19:0 cyclo w9c	19:0 cyclo w7c	19:1 w17c
	20:1 w9c	20:1 w8c	20:1 w6c	19:0 cyclo w6c
	20:1 w4c	20:0 cyclo w6c	21:1 w9c	21:1 w8c
	21:1 w6c	21:1 w5c	21:1 w4c	21:1 w3c
	22:1 w9c	22:1 w8c	22:1 w6c	22:1 w5c
	22:1 w3c	22:0 cyclo w6c	24:1 w9c	24:1 w7c
	11:0 iso 3OH	14:0 iso 3OH		
Methanotroph	16:1 w8c			
Eukaryote	15:4 w3c	15:3 w3c	16:4 w3c	16:3 w6c
	18:4 w3c	18:3 w6c	19:4 w6c	19:3 w6c
	19:3 w3c	20:4 w6c	20:5 w3c	20:3 w6c
	20:2 w6c	21:3 w6c	21:3 w3c	22:5 w6c
	22:6 w3c	22:4 w6c	22:5 w3c	22:2 w6c
	23:4 w6c	23:3 w6c	23:3 w3c	23:1 w5c
	23:1 w4c	24:4 w6c	24:3 w6c	24:3 w3c
	24:1 w3c			
Gram Positive	11:0 so	11:0 anteiso	12:0 iso	12:0 anteiso
	3:0 iso	13:0 anteiso	14:1 iso w7c	14:0 iso
	14:0 anteiso	15:1 iso w9c	15:1 iso w6c	15:1 anteiso w9c
	15:0 iso	15:0 anteiso	16:0 iso	16:0 anteiso
	17:1 iso w9c	17:0 iso	17:0 anteiso	18:0 iso
	17:1 anteiso w9c	17:1 iso w10c	17:1 anteiso w7c	18:1 w9c
	19:0 iso	19:0 anteiso	20:0 iso	22:0 iso
	16:0 10-methyl	17:1 w7c 10-methyl	17:0 10-methyl	18:1 w7c 10-methyl
	18:0 10-methyl	19:1 w7c 10-methyl	20:0 10-methyl	22:0 10-methyl

Section S1. Comparison of metabolite extraction methods

S1.1. Soil metabolite extraction

Four independent replicates of soil were collected from the Henfaes Agricultural Research Station, Abergwyngregyn, North Wales (53°14′N, 4°01′W). On collection, field-moist soil was sieved through a 2 mm mesh to remove stones and plant material and ensure sample homogeneity. Subsequently, samples were chloroform fumigated (24 h), then frozen (-80°C) and lyophilised, and mechanically ground using the methods described in section 2.3.

All glassware used for extraction was cleaned and sterilised as described in section 2.3. Soils were then extracted using either HPLC-plus grade H_2O or acetonitrile (MeCN)/isopropyl alcohol (IPA)/HPLC-plus grade water (H2O) (3:3:2 v/v/v). For extraction, 6 g of soil was weighed into a glass centrifuge tube and the respective extraction solvent added (pre-cooled to either 4°C (water) or -20°C (3:3:2)). Samples were then horizontally shaken on ice (4°C) at a frequency of 200 min^{-1} for 1 h then centrifuged at 3320 × g for 15 min (Swenson et al., 2015). Supernatants were then pipetted, using glass pipettes, into 20 ml glass vials and stored at -20°C or 4°C (to ensure metabolic activity was quenched but the supernatant was not frozen). Samples were left unfiltered due to the potential of contamination from the filter paper and plastic housing. The supernatant was lyophilised in 2 ml glass vials using a Modulyo Freeze Dryer with RV pump (Edwards Ltd., Crawley, UK) attached to a SpeedVac vacuum concentrator (Savant; ThermoFisher, Waltham, MA, USA). The vials were periodically topped up with the supernatant, taking note of the quantity added (~15 ml total) and lyophilised to complete dryness. Samples were then shipped on dry ice (-78.5 °C) to Metabolon Inc. (Morrisville, North Carolina, USA) for untargeted LC/MS metabolomic analysis. Upon analysis, samples were dissolved in methanol:water (4:1 v/v) and subjected to the standard Metabolon sample preparation procedure. LC/MS analysis parameters, bioinformatics, compound ID and data curation are summarised in supplementary information (section S2).

S1.2. Soil metabolite extraction comparison results

The present dataset comprises a total of 443 compounds of known identity (named biochemicals). The number of compounds detected in the individual soil samples (Table S2) showed some variation, with slightly higher numbers (+6 %) being detected in the samples extracted with 3:3:2 as compared to H_2O. In the case of both extraction methods, the number

of detected metabolites were much greater in the soil samples than the matrix blanks. Metabolites detected commonly within soil extraction samples and matrix blanks were also typically found at much higher levels in the soil extracts. We therefore concluded that the methods were comparable in terms of number of compounds detected, although 3:3:2 generally was more sensitive to non-polar compounds (i.e. lipids).

Table S2. Comparison of the number of biochemical detected using 3:3:2 vs. H_2O extraction methods.

Extraction Method	Client Sample ID	Sample Description	Biochemicals Detected
lyophilized water	1	Soil extraction	354
	2	Soil extraction	360
	3	Soil extraction	358
	4	Soil extraction	357
	5	Matrix Blank	121
lyophilized acetonitrile, isopropanol, water (3:3:2)	6	Soil extraction	392
	7	Soil extraction	399
	8	Soil extraction	395
	9	Soil extraction	384
	10	Matrix Blank	102

Section S2. Metabolite analysis by ultrahigh Performance Liquid Chromatography-Tandem Mass Spectroscopy (UPLC-MS/MS)

S2.1. Sample Accessioning

Following receipt, samples were inventoried and immediately stored at -80ºC. Each sample received was accessioned into the Metabolon LIMS system and was assigned by the LIMS a unique identifier that was associated with the original source identifier only. This identifier was used to track all sample handling, tasks, results, etc. The samples (and all derived aliquots) were tracked by the LIMS system. All portions of any sample were automatically assigned their own unique identifiers by the LIMS when a new task was created; the relationship of these samples was also tracked. All samples were maintained at -80ºC until processed.

S2.2. Sample preparation

Samples were prepared using the automated MicroLab STAR® system from Hamilton Company. Several recovery standards were added prior to the first step in the extraction process for QC purposes. To remove protein, dissociate small molecules bound to protein or trapped in the precipitated protein matrix, and to recover chemically diverse metabolites, proteins were precipitated with methanol under vigorous shaking for 2 min (Glen Mills GenoGrinder 2000) followed by centrifugation. The resulting extract was divided into five fractions: two for analysis by two separate reverse phase (RP)/UPLC-MS/MS methods with positive ion mode electrospray ionization (ESI), one for analysis by RP/UPLC-MS/MS with negative ion mode ESI, one for analysis by HILIC/UPLC-MS/MS with negative ion mode ESI, and one sample was reserved for backup. Samples were placed briefly on a TurboVap® (Zymark) to remove the organic solvent. The sample extracts were stored overnight under nitrogen before preparation for analysis.

S2.3. Quality control

Several types of controls were analyzed in concert with the experimental samples: a pooled matrix sample generated by taking a small volume of each experimental sample (or alternatively, use of a pool of well-characterized human plasma) served as a technical replicate throughout the data set; extracted water samples served as process blanks; and a cocktail of QC standards that were carefully chosen not to interfere with the measurement of endogenous

compounds were spiked into every analyzed sample, allowed instrument performance monitoring and aided chromatographic alignment. Tables S2 and S3 describe these QC samples and standards. Instrument variability was determined by calculating the median relative standard deviation (RSD) for the standards that were added to each sample prior to injection into the mass spectrometers. Overall process variability was determined by calculating the median RSD for all endogenous metabolites (i.e., non-instrument standards) present in 100% of the pooled matrix samples. Experimental samples were randomized across the platform run with QC samples spaced evenly among the injections, as outlined in Figure S2.

Table S3. Description of Metabolon QC samples

Type	Description	Purpose
MTRX	Large pool of human plasma maintained by Metabolon that has been characterized extensively.	Assure that all aspects of the Metabolon process are operating within specifications.
CMTRX	Pool created by taking a small aliquot from every customer sample.	Assess the effect of a non-plasma matrix on the Metabolon process and distinguish biological variability from process variability.
PRCS	Aliquot of ultra-pure water	Process Blank used to assess the contribution to compound signals from the process.
SOLV	Aliquot of solvents used in extraction.	Solvent Blank used to segregate contamination sources in the extraction.

Table S4. Metabolon QC Standards

Type	Description	Purpose
RS	Recovery Standard	Assess variability and verify performance of extraction and instrumentation.
IS	Internal Standard	Assess variability and performance of instrument.
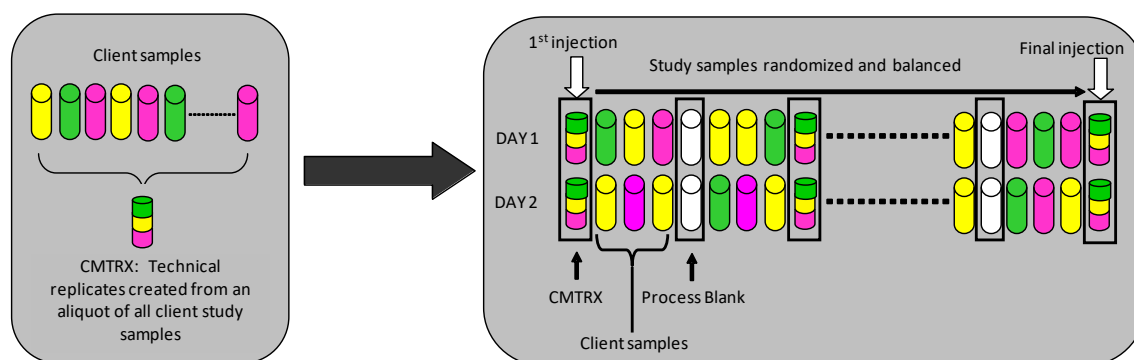
Figure S2. Preparation of technical replicates. A small aliquot of each sample (colored cylinders) is pooled to create a CMTRX technical replicate sample (multi-colored cylinder), which is then injected periodically throughout the platform run. Variability among consistently

detected biochemicals can be used to calculate an estimate of overall process and platform variability.

S2.4. Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectroscopy (UPLC-MS/MS)

All methods utilized a Waters ACQUITY ultra-performance liquid chromatography (UPLC) and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution. The sample extract was dried then reconstituted in solvents compatible to each of the four methods. Each reconstitution solvent contained a series of standards at fixed concentrations to ensure injection and chromatographic consistency. One aliquot was analyzed using acidic positive ion conditions, chromatographically optimized for more hydrophilic compounds. In this method, the extract was gradient eluted from a C18 column (Waters UPLC BEH C18-2.1x100 mm, 1.7 µm) using water and methanol, containing 0.05% perfluoropentanoic acid (PFPA) and 0.1% formic acid (FA). Another aliquot was also analyzed using acidic positive ion conditions; however, it was chromatographically optimized for more hydrophobic compounds. In this method, the extract was gradient eluted from the same aforementioned C18 column using methanol, acetonitrile, water, 0.05% PFPA and 0.01% FA and was operated at an overall higher organic content. Another aliquot was analyzed using basic negative ion optimized conditions using a separate dedicated C18 column. The basic extracts were gradient eluted from the column using methanol and water, however with 6.5 mM ammonium bicarbonate (pH 8). The fourth aliquot was analyzed via negative ionization following elution from a HILIC column (Waters UPLC BEH Amide 2.1x150 mm, 1.7 µm) using a gradient consisting of water and acetonitrile with 10mM ammonium formate, pH 10.8. The MS analysis alternated between MS and data-dependent MSn scans using dynamic exclusion. The scan range varied slighted between methods but covered 70-1000 m/z. Raw data files are archived and extracted as described below.

S2.5. Bioinformatics

The informatics system consisted of four major components, the Laboratory Information Management System (LIMS), the data extraction and peak-identification software, data

processing tools for QC and compound identification, and a collection of information interpretation and visualization tools for use by data analysts. The hardware and software foundations for these informatics components were the LAN backbone, and a database server running Oracle 10.2.0.1 Enterprise Edition.

S2.6. LIMS

The Metabolon LIMS system provided a fully auditable laboratory automation through a secure and highly specialized data tracking system. The scope of the Metabolon LIMS system encompasses sample accessioning, sample preparation and instrumental analysis and reporting and advanced data analysis. All of the subsequent software systems are grounded in the LIMS data structures. It has been modified to leverage and interface with the in-house information extraction and data visualization systems, as well as third party instrumentation and data analysis software.

S2.7. Data extraction and compound identification

Raw data was extracted, peak-identified and QC processed using Metabolon's hardware and software. These systems are built on a web-service platform utilizing Microsoft's .NET technologies, which run on high-performance application servers and fiber-channel storage arrays in clusters to provide active failover and load-balancing. Compounds were identified by comparison to library entries of purified standards or recurrent unknown entities. Metabolon maintains a library based on authenticated standards that contains the retention time/index (RI), mass to charge ratio (*m/z*), and chromatographic data (including MS/MS spectral data) on all molecules present in the library. Furthermore, biochemical identifications are based on three criteria: retention index within a narrow RI window of the proposed identification, accurate mass match to the library +/- 10 ppm, and the MS/MS forward and reverse scores between the experimental data and authentic standards. The MS/MS scores are based on a comparison of the ions present in the experimental spectrum to the ions present in the library spectrum. While there may be similarities between these molecules based on one of these factors, the use of all three data points can be utilized to distinguish and differentiate biochemicals. More than 3300 commercially available purified standard compounds have been acquired and registered into LIMS for analysis on all platforms for determination of their analytical characteristics.

Additional mass spectral entries have been created for structurally unnamed biochemicals, which have been identified by virtue of their recurrent nature (both chromatographic and mass spectral). These compounds have the potential to be identified by future acquisition of a matching purified standard or by classical structural analysis.

S2.8. Curation

A variety of curation procedures were carried out to ensure that a high quality data set was made available for statistical analysis and data interpretation. The QC and curation processes were designed to ensure accurate and consistent identification of true chemical entities, and to remove those representing system artifacts, mis-assignments, and background noise. Metabolon data analysts use proprietary visualization and interpretation software to confirm the consistency of peak identification among the various samples. Library matches for each compound were checked for each sample and corrected if necessary.

S2.9. Metabolite quantification and data normalization

Peaks were quantified using area-under-the-curve.

Appendix 4

Supplementary material from Chapter 6

Brown, R.W, Bull, I.D, Journeaux, T., Chadwick, D.R., Jones, D.L., 2021. Volatile organic compounds (VOCs) allow sensitive differentiation of biological soil quality. Soil Biology and Biochemistry, 156, 108187.
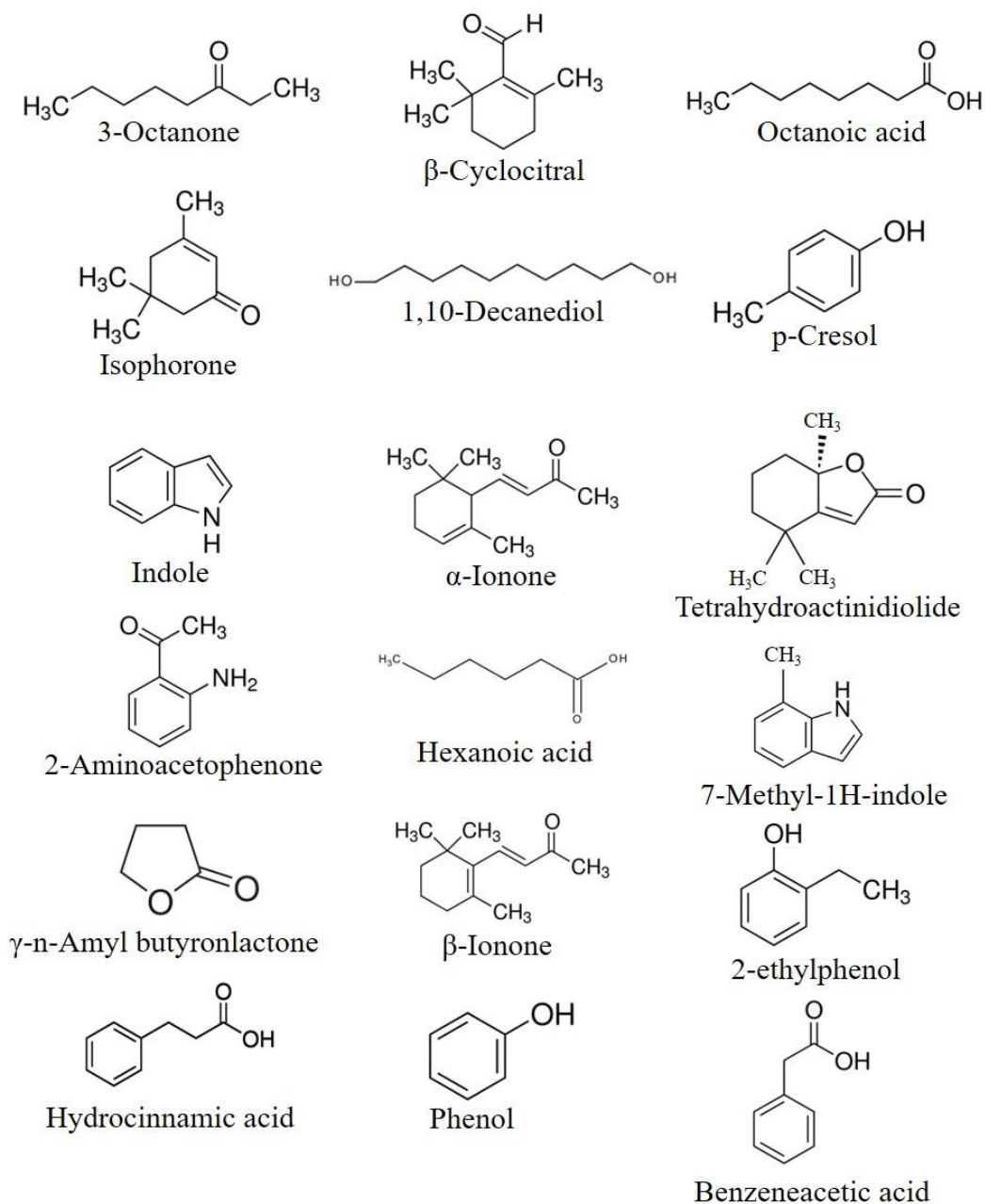
Figure 1. Structures of discriminatory compounds highlighted in Fig 5.2.
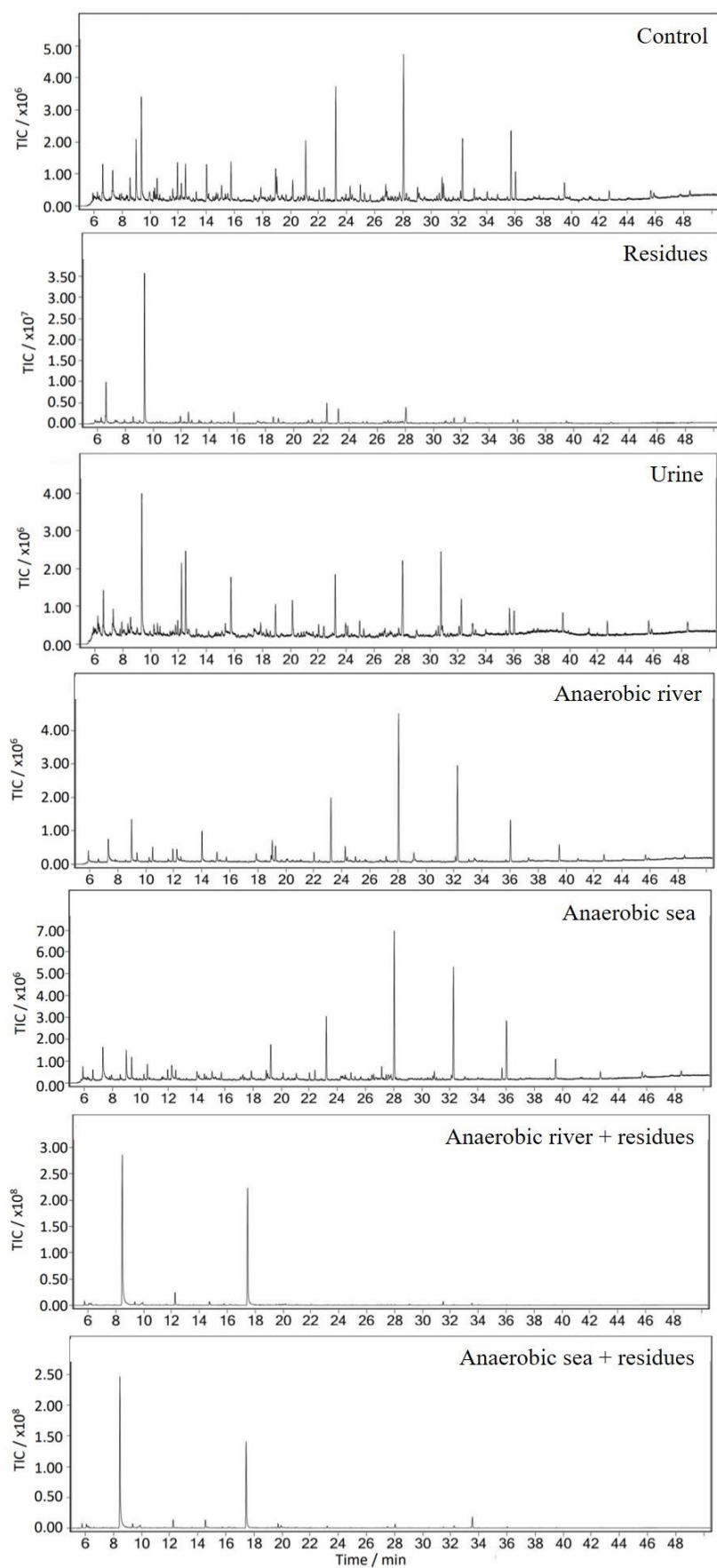
Figure 2. *Raw data chromatogram examples of samples representative of each of the soil treatments.*

Table 1. Characteristics of treatments applied to soils. Values represent mean (n = 5) ± SEM. Urine characteristics were taken from Marsden et al., (2018), as the same urine was used.

	River water	Sea water	Sheep urine	Lysed grass
pH	7.70 ± 0.11	7.94 ± 0.08	8.48	-
EC (mS cm^{-1})	0.01 ± 0.00	7.92 ± 0.02	13.80	-
Total organic C (mg C kg^{-1})	1.71 ± 0.05	4.34 ± 0.28	10.1 ± 0.40	-
Total N (mg N kg^{-1})	0.35 ± 0.01	0.71 ± 0.04	6.35 ± 0.22	-
Extractable NO$_3^-$ (mg N kg^{-1})	0.28 ± 0.01	0.32 ± 0.01	0.61 ± 0.04	-
Extractable NH$_4^+$ (mg N kg^{-1})	0.01 ± 0.01	0.39 ± 0.08	3.75 ± 0.06	-
Extractable PO$_4^+$ (mg P kg^{-1})	0.13 ± 0.00	0.27 ± 0.00	-	-
Moisture (%)	-	-	-	83.6 ± 0.3
Total C (%)	-	-	-	47.0 ± 0.1
Total N (%)	-	-	-	5.14 ± 0.08
C:N ratio	-	-	-	9.17 ± 0.14

Appendix 5

Supplementary material from Chapter 7

Brown, R.W., Mayser, J.P., Widdowson, C., Chadwick, D.R., Jones, D.L., 2021. Dependence of thermal desorption method for profiling volatile organic compound (VOC) emissions from soil. Soil Biology and Biochemistry, 160, 108313.

Table 1. *Summary of amendment characteristics applied to soils. Values represent mean (n = 4) ± SEM. River water collected from the Afon Rhaeadr-fawr (53°14'N 4°01'W), Beds and bordered topsoil (Verve, Chandlers Ford, UK) was used as the soil improver addition, and grass residues consisted of green Lolium perenne L. shoots, lysed by freezing for 1 h at -80°C. Soil improver and lysed grass values are expressed on a dry weight basis.*

	River water	Soil improver	Lysed grass
pH	6.82 ± 0.04	8.54 ± 0.13	-
EC (μS cm^{-1})	69.5 ± 0.75	274 ± 12	-
Total organic C (mg C l^{-1} or mg C kg^{-1})	2.19 ± 0.04	66.8 ± 9.4	-
Total N (mg N l^{-1} or mg N kg^{-1})	0.23 ± 0.01	6.15 ± 1.9	-
Extractable NO_3^- (mg N l^{-1} or mg N kg^{-1})	0.45 ± 0.01	0.99 ± 0.11	-
Extractable NH_4^+ (mg N l^{-1} or mg N kg^{-1})	0.00 ± 0.00	0.53 ± 0.04	-
Extractable PO_4^+ (mg P l^{-1})	0.03 ± 0.03	-	-
Organic matter (%)	-	23.6 ± 0.7	-
Moisture (%)	-	81.1 ± 1	15.7 ± 0.6
Total C (%)	-	-	43.1 ± 0.9
Total N (%)	-	-	3.72 ± 0.23
C:N ratio	-	-	11.6 ± 0.5
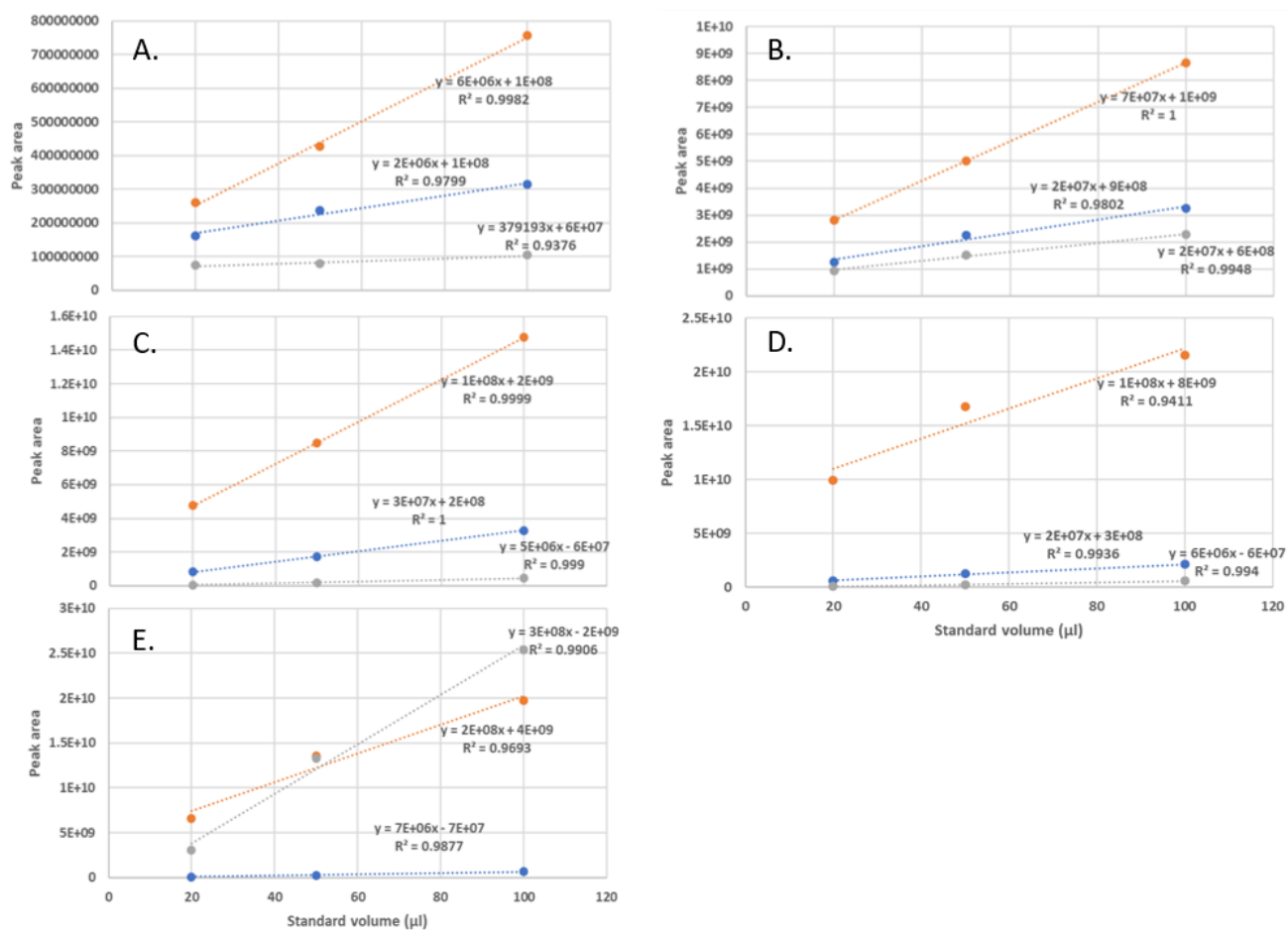
Figure 1. *Extraction efficiencies for soil-related VOC compounds, using matrix-less (no soil) vials. A) Phenol, B) Isophorone, C) p-Cresol, D) Indole, E) β-ionone.*
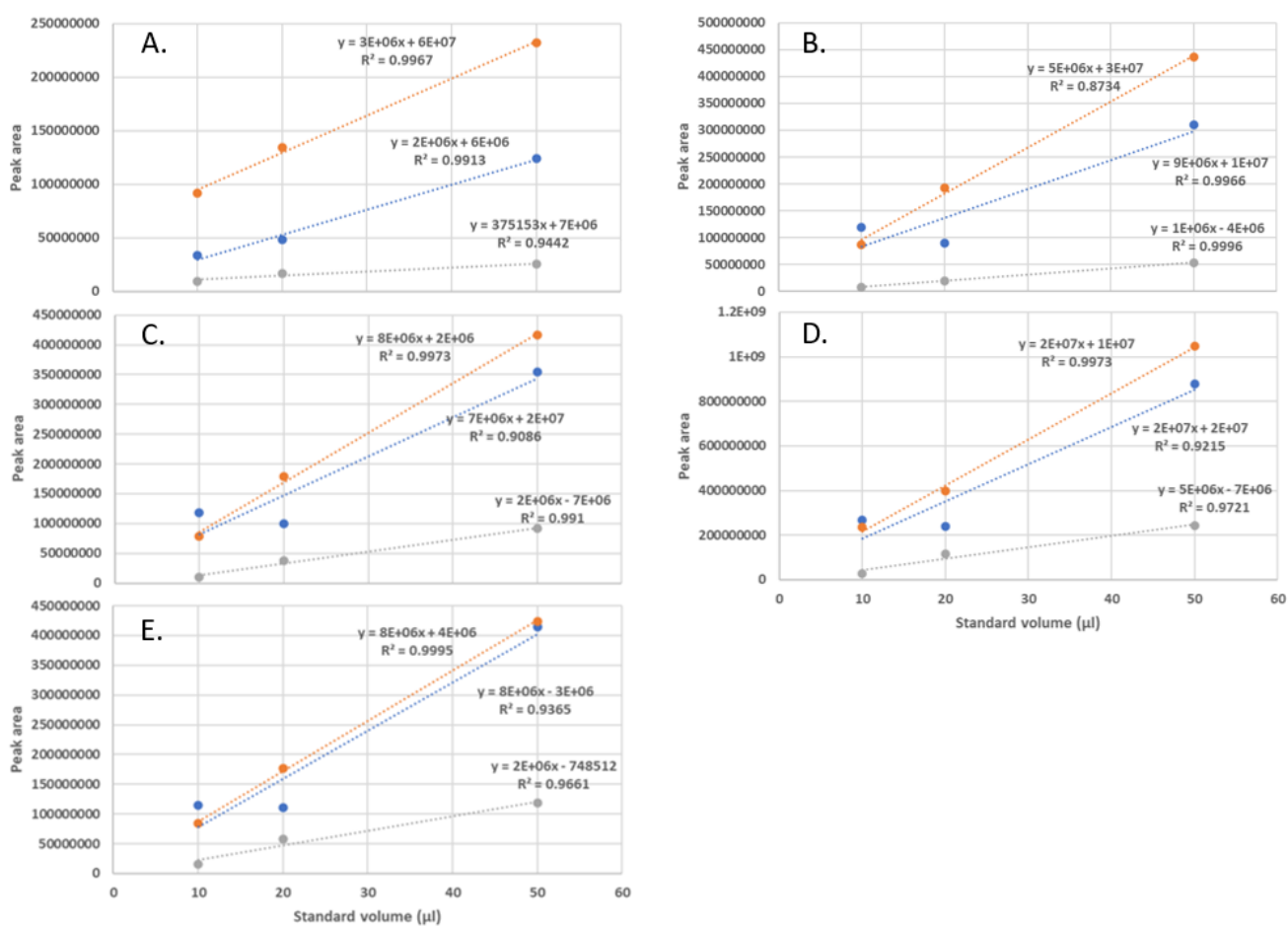
Figure 2. Extraction efficiencies for standard VOC compounds, using matrix-less (no soil) vials. A) Benzene, B) Toluene, C) Ethyl-benzene, D) p-Xylene, E) m-Xylene.

Appendix 6

Saltwater intrusion induces shifts in soil microbial diversity and carbon use efficiency in a coastal grassland ecosystem

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This experiment was designed, performed and the resultant manuscript written during this PhD project; however, it was felt that it does not fit within the overarching themes of this thesis and therefore has only been included as an appendix.

This manuscript is in preparation for submission to *Soil Biology and Biochemistry*.

All authors contributed to the conception and design of the experiment. DJ performed soil sampling. RB performed soil analysis. JR performed data analysis. Data interpretation was performed by all authors. RB and JR wrote the first draft of the manuscript. All authors contributed to subsequent revisions.

ABSTRACT

Salt accumulation and salinisation of coastal soils is a global issue. Further, climate change is likely to increase the amount of land affected through increasing the frequency and severity of coastal flooding and brackish water ingress. The impact of this on the ability of soils to deliver ecosystem services, particularly carbon (C) storage, however, remains unclear. We hypothesized that coastal inundation would negatively affect C storage by reducing plant C inputs and by placing greater osmotic stress on the microbial community leading to a reduced C use efficiency (CUE). Here, we use a coastal grassland ecosystem, which is becoming increasingly subject to sea and brackish water flooding, to explore the relationship between plant/microbial growth and CUE along a natural salinity gradient. To reflect steady state conditions, we traced the turnover and partitioning of a low (ambient) dose of ^{14}C-labelled glucose into microbial anabolic and catabolic pools, from which microbial CUE was calculated. In addition, we added a high dose of ^{14}C-labelled glucose to soil to stimulate microbial growth, and again calculated CUE. This was supported by measurements of the diversity of the bacterial community across the salinity gradient using 16S metabarcoding. Our results showed that coastal flooding aboveground biomass, increased soil C content and induced a small increase in microbial CUE under low glucose-C conditions. Conversely, no difference in CUE or microbial growth was apparent when a high glucose-C dose was used. We ascribe the differences in CUE under the different substrate-C availabilities to microbial community life strategy (i.e. *r*- vs. *K*-strategists) and the ability of the microbial community to readily adapt to life under high salt concentrations. Soil bacterial community alpha (α) diversity increased with soil salinity while beta (β) diversity also shifted in response to the higher saline conditions. Our analysis suggests that the biggest impact of coastal flooding on soil C cycling was the inability of the plant community to adapt, leading to higher plant residue inputs and also a decline in soil structure. Conversely, the microbial community had adapted to the increased salinity, resulting in only small changes in the uptake and metabolic partitioning of C.

1. Introduction

It is estimated that 33% of irrigated land and up to 20% of total cultivated land globally is suffering from salinisation; the excess accumulation of salt in soils (Otlewska et al., 2020). This phenomenon is likely to be further exacerbated through climate change induced sea level rise and an increased frequency and severity of extreme weather events leading to vulnerability to coastal flooding in many regions of the world (Chen and Zong, 1999; Vitousek et al., 2017; Zemp et al., 2019). For example, in the UK, coastal flood risk is expected to increase over the next century and beyond, under all Intergovernmental Panel on Climate Change (IPCC) representative concentration pathway (RCP) climate change scenarios, mainly due to changes in time-mean sea level (predicted to be up to 0.5 m under RCP2.6 to 4.3 m under RCP8.5) (Met Office, 2018a). Consequently, mitigating coastal flooding is recognised as one of the top priorities for many nations worldwide (Kirezci et al., 2020). Globally, a large proportion of productive agricultural land is situated in low lying and reclaimed coastal regions (Gould et al., 2020), threatening food security and sustainable development into the future (Karim and Mimura, 2008; Kirezci et al., 2020).

The progressive accumulation of salt in soil is known to directly impact soil quality, defined here as the capacity of the soil to function (Karlen et al., 1997). For example, the saline intolerance of plant roots and soil macrofauna is likely to lead to a reduction in bioturbation and aeration, eventually leading to a decline in soil physical structure (Otlewska et al., 2020). Additionally, salt-induced dispersion of soil particles can lead to a clogging of soil pores and a reduction in hydraulic conductivity (Qadir and Schubert, 2002) which alters the solubility and accessibility of soil organic matter (SOM) (Mavi et al., 2012; Wong et al., 2010, 2009). Consequently, a negative feedback mechanism may occur leading to a further downward trend in physical, chemical and biological soil quality, changing the nature of ecosystem service provision.

Salinity-induced changes to the size, structure, activity and functioning of the soil microbial community (i.e. ion toxicity, osmotic and oxidative stress) are known to have significant consequences on terrestrial carbon (C) cycling (Yan et al., 2015). In some circumstances, the controlled ingress of seawater onto former agricultural land (i.e. managed sea realignment) can lead to a major increase in soil C storage (Andrews et al., 2008), however, conversely it may also lead to a depletion of soil C stocks (Sjøgaard et al., 2017; Yang et al.,

2019). Our understanding of this is limited and further research is needed to understand the complex feedbacks that may occur in the plant-soil C cycle upon coastal flooding.

Carbon use efficiency (CUE), the efficacy with which microorganisms metabolise available organic substrates into stable biosynthetic products, is critical to ecosystem C cycling and C storage (Geyer et al., 2016). The amount of additional biomass C produced per unit substrate C metabolised ultimately determines the rate of C accumulation (via SOM) or loss (via CO_2/CH_4 efflux) from a soil (Poeplau et al., 2019). Ultimately, CUE is a critical control on the capacity of soil and wider ecosystem to store C (Bradford and Crowther, 2013; Manzoni et al., 2012; Wang et al., 2021). A limited amount of work has been performed to evaluate the changes in microbial CUE in saline stressed soils. However, environmental drivers have been shown to potentially uncouple growth and respiration, changing CUE (Sinsabaugh et al., 2013).

Microbial community stability and resilience determines how a soil responds to and recovers from environmental stresses (Bardgett and Caruso, 2020). Previous work on community stability has shown mixed effects, depending on the nature of the disturbance (e.g. heavy metal vs. salt loading) (Tobor-Kapłon et al., 2006, 2005). It has been suggested that in recently or currently stressed systems, organisms have less energy to cope with further disturbance, as in the first instance energy will be allocated to detoxification and repair, rather than immediate growth (Griffiths and Philippot, 2013). However, there is evidence to show that communities have the potential to adapt and/or develop tolerance to environmental conditions resulting in a more stable community, with a high proportion of energy available for growth (i.e. higher CUE) (Kallenbach et al., 2019). Previous studies have shown that a shift in the composition of the soil bacterial community and reduction in phylogenetic diversity occurs with increasing salinity in naturally saline environments (Canfora et al., 2014; Hollister et al., 2010; Rath et al., 2019a, 2019b; van Horn et al., 2014). However, the response of soils to periodic coastal inundation and salt stress remains poorly understood.

This study aimed to assess the impact of salinisation on the soil microbial community and its resultant CUE across a gradient of salinity in a coastal soil becoming increasing susceptible to periodic saltwater flooding from sea level rise and storm surges. We hypothesised that; i) CUE would decrease under higher salt conditions, due to increased levels of environmental stress, and ii) the soil microbial community under higher salt-stress would exhibit change and increased tolerance compared to the unstressed community.

2. Materials and methods

2.1. Study site

The study site was located at the Henfaes Agricultural Research Station, Abergwyngregyn, North Wales (53°14' 30"N, 4°01'22"W). It comprises a sheep-grazed grassland agricultural field located next to the Menai Strait, which forms part of the Irish Sea (Fig. S1) and is adjacent to the Afon Rhaeadr-fawr river. The site has experienced an increased frequency of tidal storm surges and associated brackish riverine flooding over the last decade, leading to the progressive ingress of salt water onto productive agricultural land (Ganguli and Merz, 2019; Hendry et al., 2019; Met Office, 2018b). This area has also been identified by Welsh Government as an area of very high risk from coastal flooding (NRW, 2014, 2020) due to its low-lying nature (< 2 m a.s.l) and lack of coastal protection. The vegetation is dominated by *Lolium perenne* L. while the soil is classified as a sandy loam textured Eutric Cambisol (Typic Hapludalf) developed on a mixture of glacial till and windblown sand. The site has a temperate-oceanic climate regime with mean annual rainfall of 1060 mm and mean annual temperature of 10°C (10 y average).

Three separate areas with clear salt accumulation at the soil surface were chosen for this study. Within each of these individual field areas, three individual linear transects were demarcated at least 3 m from each other (i.e. nine transects in total). Each transect had a visually clear gradient in salt accumulation and vegetation cover (Fig. 1, Fig. S2). Samples of soil were collected from the Ah horizon (0-10 cm depth) at four locations along this gradient, representing different levels of visual vegetation damage and surface salt accumulation (Fig. 1). The percentage vegetation cover along the transect was assessed using gridded 60 × 60 cm quadrats with 400 individual measurement squares. The relative amount of vegetated and bare soil squares was measured for each quadrat alongside a photographic record of the transects.
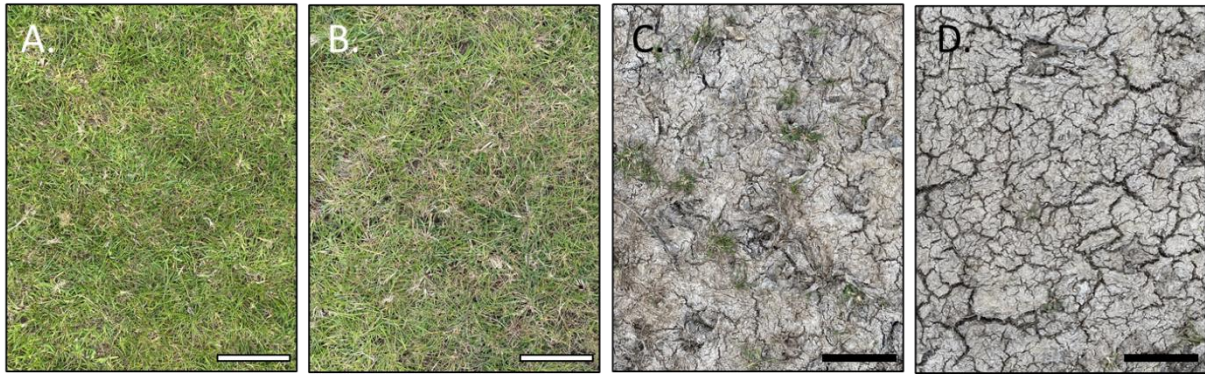
Figure 1. *Visual representation of the salinity gradient sampled. A) Control, B) Control Edge, C) Salt Edge and D) Salt patch, scale bars represent 10 cm.*

2.2. Soil characterisation

Bulk density cores (0 – 5 cm, 100 cm^3) were oven dried (105°C, 24 h) before being sieved, to 2 mm, to remove stones, and weighed. After collection, fresh soil was homogenised and sieved to pass 8 mm to remove stones, mesofauna and roots. This sieve size was chosen to minimise changes in microbial activity (Jones and Willett, 2006). Additionally, a subsample of fresh sieved soil was further sieved to 2 mm, to further homogenise and remove stones and vegetation, before being stored at -80°C to await bacterial sequencing. Soil pH and electrical conductivity (EC) were measured on 1:5 (w/v) soil-to-ultrapure water (UPW) suspensions by submerging standard electrodes. Soil salinity across the patch gradients is visualised in Fig. 2. Subsequently, 1:5 (w/v) soil-to-0.5 M acetic acid (AcOH) and 1:5 (w/v) soil-to-UPW extracts were performed to measure nutrient availability (MISR/SAC, 1985). Extractable nitrate (NO_3-N) and ammonium (NH_4-N) concentrations within the UPW extracts were determined by the colorimetric methods of Miranda et al. (2001) and Mulvaney (1996), respectively. Bioavailable phosphate (PO_4-P) concentrations within the AcOH extracts were determined using the molybdate blue colorimetric method of Murphy and Riley (1962). Exchangeable soil cations (Na, K and Ca) were measured on the AcOH extracts using a Sherwood Model 410 Flame Photometer (Sherwood Scientific Ltd, Cambridge, UK). Dissolved organic C (DOC) and total dissolved N (TDN) were determined on UPW extracts using a Multi N/C 2100S Analyzer (AnalytikJena, Jena, Germany). Soil moisture content was determined gravimetrically on the sieved soils by oven drying (105°C, 24 h). The C and N content of the soil was determined on oven-dried soil using a TruSpec CN analyzer (Leco Corp, St Joseph, MI). All chemical and

physical analysis described subsequently was performed within 48 h of soil collection, during which soil samples were stored at 4°C.
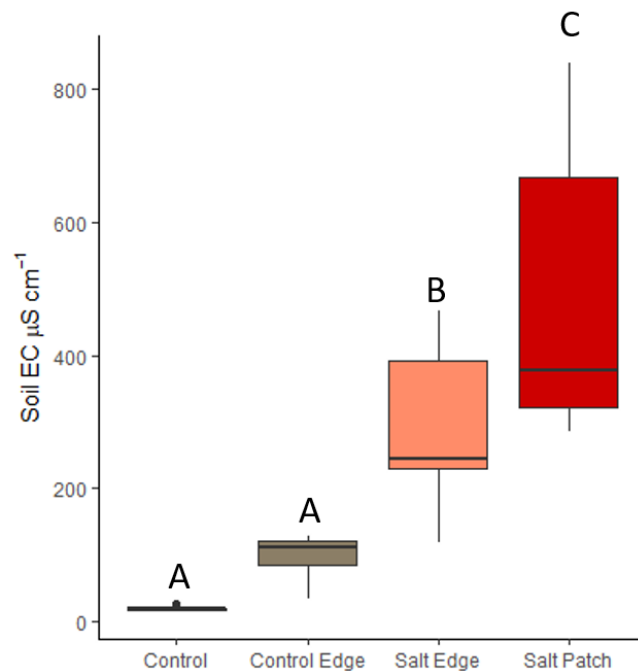
Figure 2. *Variation in electrical conductivity (EC) between location treatments along a natural salinity gradient caused by coastal flooding (n = 9). Horizontal lines show the median, boxes the 25th to 75th percentiles, whiskers the 5th to 95th percentile range. Letters denote significant differences between location treatments.*

2.3. Soil microbial activity and carbon use efficiency

To determine microbial activity and CUE, we measured the mineralization of ^{14}C-labelled glucose in each soil sample. Briefly, 5 g of each soil was placed in individual sterile 50 cm^3 polypropylene tubes. Subsequently, 0.5 ml of uniformly ^{14}C-labelled glucose solution (10 kBq ml^{-1}) with either a low (100 µM) or high concentration (100 mM) was added to the soil surface. After addition of the ^{14}C-labelled glucose, a 1 M NaOH trap (1 ml) was suspended above the soil to catch any respired ^{14}CO$_2$. The tubes were then hermetically sealed and incubated at room temperature (20 ± 1°C). The NaOH traps were replaced periodically (after 1, 3, 6, 9, 24, 34, 48, 58, 72 h and subsequently every 24 h) for one week after glucose application. The efficiency of the NaOH traps was > 98% (as determined by collecting ^{14}CO$_2$

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generated from adding excess 0.1 M HCl to 0.001 M NaH^{14}CO$_3$). The amount of ^{14}C in the NaOH traps was measured using Optiphase HiSafe 3 liquid scintillation cocktail (PerkinElmer Inc., Waltham, MA, USA) and a Wallac 1404 scintillation counter (Wallac EG&G, Milton Keynes, UK) with automated quench correction. At the end of the incubation period, the soils were extracted with ice-cold 1 M NaCl (200 rev min^{-1}, 15 min), centrifuged (24,000 g, 15 min, 4°C) and the ^{14}C in the supernatant measured by liquid scintillation counting as described above (Rousk and Jones, 2010). Glucose was used as the C substrate due to its almost ubiquitous use by soil microorganisms and it represents the largest C input to soil in a polymeric form (Gunina and Kuzyakov, 2015).

2.4. 16S amplicon sequencing

Fresh soil was placed into a MoBio PowerMag Soil DNA Isolation Bead Plate (MoBio Laboratories Inc., Carlsbad, CA). DNA was extracted following MoBio's instructions on a KingFisher Flex robot (Thermo Fisher Scientific Corp, Waltham, MA). Bacterial 16S rRNA genes were PCR-amplified with dual-barcoded primers targeting the V4 region (515F 5'-GTGCCAGCMGCCGCGGTAA-3', and 806R 5'-GGACTACHVGGGTWTCTAAT-3'), as per the protocol of Kozich et al. (2013). Amplicons were sequenced with an Illumina MiSeq using the 300-bp paired-end kit (v.3) (Illumina Inc., San Diego, CA). Sequences were denoised, taxonomically classified using Silva (v. 138) as the reference database, and clustered into 97%-similarity operational taxonomic units (OTUs) with the mothur software package (v. 1.44.1) (Schloss et al., 2009).

The potential for contamination was addressed by co-sequencing DNA amplified from samples and from template-free controls (negative control) and extraction kit reagents processed the same way as the samples, a positive control, was also included. OTUs were considered putative contaminants (and were removed) if their mean abundance in controls reached or exceeded 25 % of their mean abundance in the samples. OTUs were filtered if they had fewer than 3 counts and occurred in fewer than 10% of the samples.

2.5. Statistics and data analysis

To describe glucose mineralization, a double first order kinetic decay equation model was fitted to the loss of ^{14}C from the soil (S_{min}; i.e. the inverse of ^{14}CO$_2$ accumulation) where

$$S_{\min} = (P_1 \times \exp^{-k_1 \times t}) + (P_2 \times \exp^{-k_2 \times t}) \tag{1}$$

and where P_1 describes the amount of ^{14}C allocated to the first mineralization pool and k_1 is the rate constant for P_1. Similarly, P_2 is the proportion partitioned into the second slower C mineralization pool described by rate constant k_2. The equation was fitted to the experimental data using a least squares iterative model in SigmaPlot v12.3 (Systat Software Inc., San Jose, CA). Dependency values for each model parameter were used to indicate whether the parameter values were strongly dependent on one another. To critically evaluate which decay model best described the experimental data, the following criteria were employed: An r^2 value of 0.90 was deemed acceptable for assessing the fit of the model to the experimental data. To check for model over-fitting, a dependency value cut-off of 0.98 was selected. The half-life ($t_{1/2}$) for the mineralization pool P_1 was calculated as follows:

$$t_{1/2} = \ln(2)/k_1 \tag{2}$$

Further details of the modelling approach and its assumptions are provided in Glanville et al. (2016). Microbial C use efficiency for glucose (CUE_{mic}) was calculated according to Jones et al. (2018) where

$$CUE_{\mathrm{mic}} = P_2 / (P_1 + P_2) \tag{3}$$

where P_1 is the amount of ^{14}C allocated to catabolic processes and P_2 is the amount of ^{14}C allocated to anabolic processes. Differences in substrate half-life and CUE_{mic} between location treatments was tested using an ANOVA model followed by a posthoc Tukey HSD test.

From the 16S data, alpha diversity was calculated using the Shannon index on raw OTU abundance tables after filtering out contaminants, as described in section 2.4. The significance of diversity differences between location treatments was tested using an ANOVA model followed by a posthoc Tukey HSD test.

To obtain the overall variance in microbial composition, the similarities in microbial diversity across samples and location treatments were visualized by nonmetric multidimensional scaling (NMDS) ordinations based on Bray-Curtis dissimilarity. Ellipses for each location treatment were calculated using the 'veganCovEllipse' function. Significant environmental variables ($p < 0.05$) based on permutated data were selected and fitted onto the NMDS ordination space using the 'envfit' function in the '*vegan*' R package (Oksanen et al., 2020), significances of correlations were tested with 999 permutations. The NMDS results were quantitively evaluated with permutational multivariate analysis of variance

(PERMANOVA) using the 'adonis 'function in '*vegan*', followed by posthoc pairwise comparisons to evaluate microbial diversity differences between location treatments with the function "pairwise.perm.manova" from '*RVAideMemoire*' (Hervé, 2021). Although a PERMDISP test conducted with the 'betadisper' function in '*vegan*' identified non-homogenous dispersion between location treatments ($F = 3.15$, $p = 0.038$) PERMANOVA was performed as it is robust against non-homogeneous dispersions with balanced designs (Anderson, 2017).

Normality and homoscedasticity of the data were first checked using Anderson Darling and Levene's tests, respectively. Above-ground biomass was subjected to natural log transformation to ensure normality was met. Mixed-effect models were performed for each measured variable with the '*lme4*' package (Bates et al., 2018). The models included the fixed factor EC (as a proxy for salt stress) and patch number as a random factor to account for spatial variation. Predicted fitted values from the mixed-effect model were calculated with *predictInterval* with the '*merTools*' package (Knowles et al., 2020). The statistical significance cut-off for all analysis was set at $p < 0.05$.

3. Results

3.1. Impact of salinity on glucose mineralization rate and microbial CUE

Overall, a double exponential kinetic model fitted well to the $^{14}CO_2$ mineralization data for both the low ($r^2 > 0.985$; mean dependency 0.73 ± 0.01) and high glucose treatments ($r^2 > 0.980$; mean dependency 0.90 ± 0.01). Exploring the ^{14}C-labelled glucose mineralisation rates across the saline gradient showed variation with both patch location (i.e., salinity) and glucose (low vs. high) dose (Fig. 3). Under low glucose addition (100 µM), the rate of mineralisation was more rapid in the control soil samples than the samples with the highest salinity ($F_{(3,8)} = 20.2$, $p < 0.001$; Fig. 3). This was evidenced by the shorter half-life for the fast catabolic C pool (P_1) in the control treatment ($t_{1/2} = 0.70 \pm 0.03$ h) relative to those with highest salt concentrations ($t_{1/2} = 1.00 \pm 0.05$ h)($p = 0.012$). No major effect of salt was seen on the rate of processing of ^{14}C through the slow metabolic pool (P_2) attributable to the turnover of the microbial biomass ($F_{(3,8)} = 4.1$, $p = 0.051$). Under the low ^{14}C-glucose-C availability, salt stress slightly increased soil microbial CUE from 0.749 ± 0.003 in the control treatment to 0.809 ± 0.008 in the highest salinity treatment ($p < 0.05$) (Table S1).
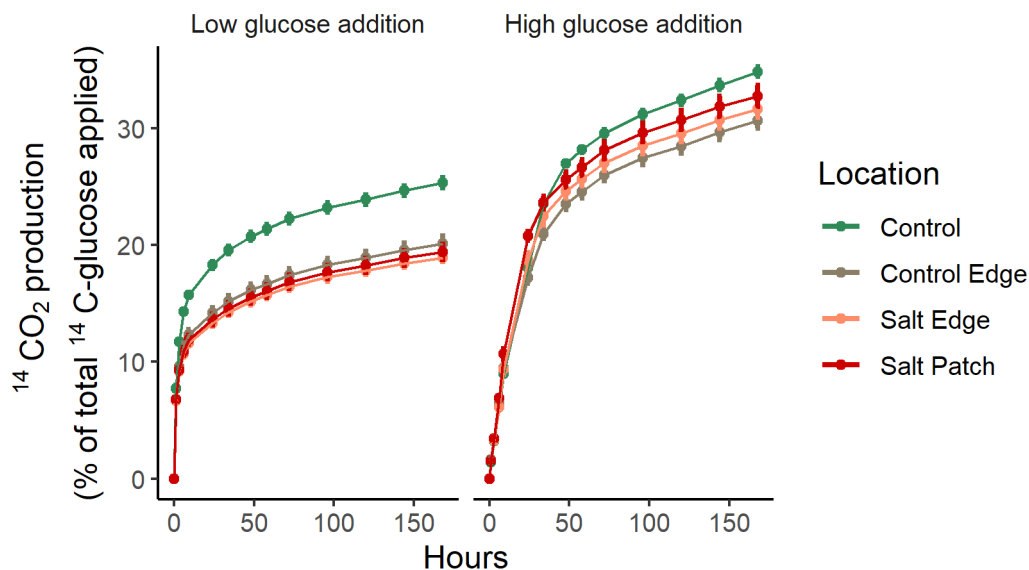
Figure 3. *A comparison of the ^{14}C-lablelled glucose mineralisation rates of soils across a saline gradient dosed with either 100 µM (CUE Low) or 100 mM (CUE High). Points represent cumulative ^{14}C respired over time (n = 9), error bar bars represent SEM. This data was used to calculate CUE presented in Table 1 using the double first order kinetic decay equation model described in section 2.5.*

Under high glucose addition (100 mM), a greater proportion of glucose was mineralised to $^{14}CO_2$ relative to the low glucose treatment (paired t-test $p < 0.001$). In addition, the half-life for the fast catabolic C pool (P_1) was much slower under the high glucose treatment ($t_{½} = 14.1 \pm 1.2$ h) relative to the low glucose treatment, however, soil salt content had no effect on the turnover rate of this pool ($F_{(3,8)} = 3.0$, $p = 0.095$). Salinity had less impact on the final cumulative amount of glucose-C respired, although the control samples exhibited the highest cumulative respiration rates (Fig. 3). In contrast to the low-glucose treatment, soil salinity status had no effect on CUE in the high glucose treatment ($p = 0.73$) or on the rate of turnover of the microbial biomass, Pool P_2 ($F_{(3,8)} = 0.95$, p = 0.462).

3.2. Impact of salinity on 16S bacterial community structure

In total, 10442 bacterial operational taxonomic units (OTUs) were identified across all 16S rRNA gene reads. There was some variation in the proportional abundance of OTUs

between location treatments, however, generally Alphaproteobacteria (Gram-negative) and Bacilli (Gram-positive) were the most abundant classes (Fig. 4, Fig. S3). Shannon diversities also differed between location treatments as tested by ANOVA ($F_{(3,32)} = 7.41$, $p < 0.01$). Subsequent posthoc tests revealed that diversity was significantly higher in the Salt Edge ($p < 0.01$) and Salt Patch ($p < 0.05$) compared to the Control treatment.
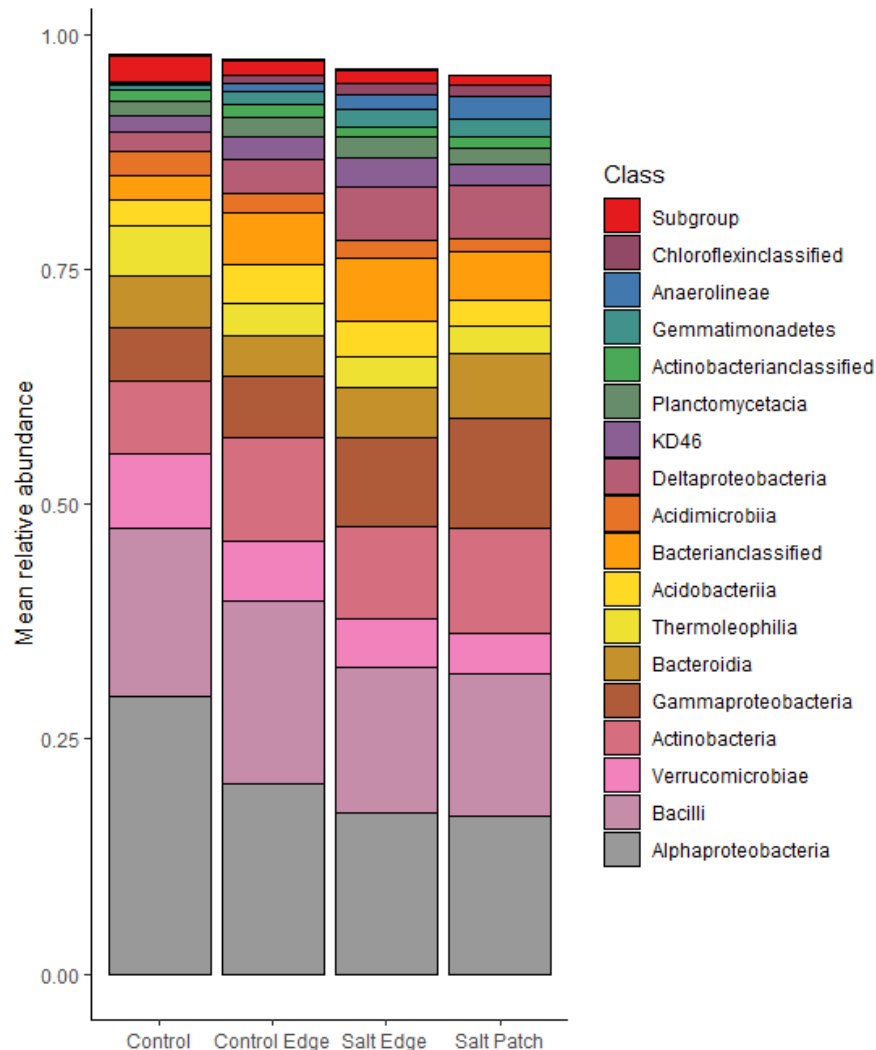
Figure 4. *Mean relative abundance of the dominant soil bacteria at the class taxonomic level (> 1%) within each salt gradient treatment.*

Based on the relative abundance of taxa ordered by class, beta-diversity analysis by NMDS and variance comparison of Bray-Curtis distances also determined differences in soil microbial diversity between location treatments (Fig. 5, PERMANOVA; $F = 11.07$, $p = 0.001$). Pairwise comparisons of the microbial diversity revealed significant differences between all locations along the salinity gradient (Table S2). The salt stress gradient also correlated strongly

with the ordination, as indicated by the fitted vectors of environmental variables (Fig. 5). Here, soil EC, a direct measure of salt stress, had the highest squared correlation coefficients (r^2) compared to other environmental variables (Table S3).

Figure 5. Non-metric dimensional scaling ordination (stress = 0.07) of bacterial community diversity across salt gradient treatments denoted by colours. Results of PERMANOVA (F = 11.07, p = 0.001) and dispersion of variances of groups (F = 3.15, p = 0.031) were significant.
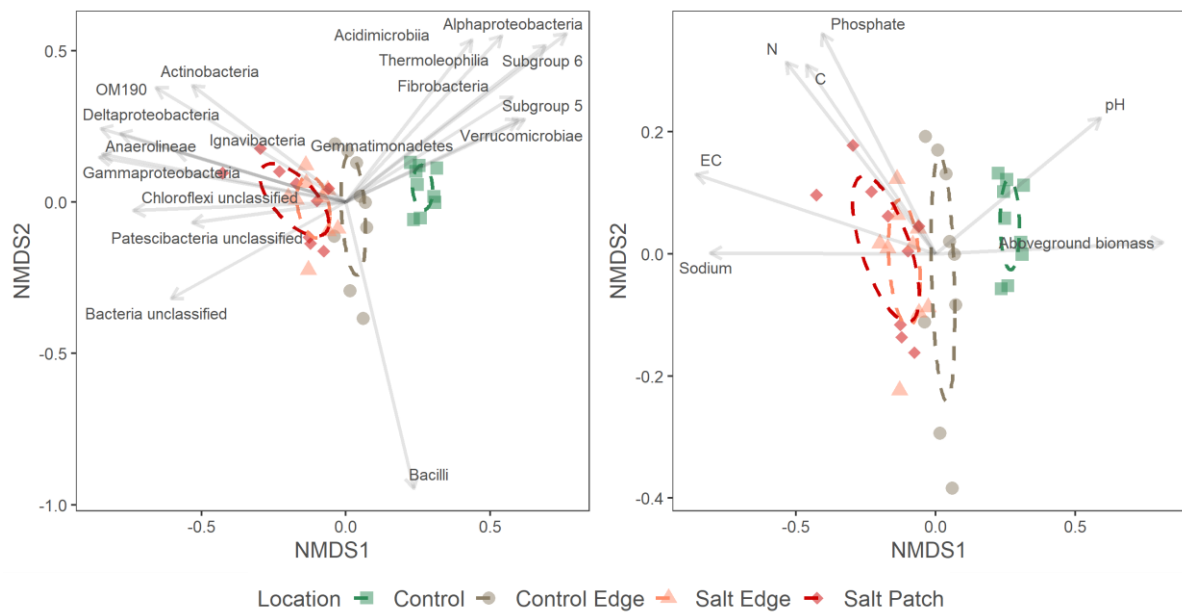
3.3. Soil physicochemistry

Exploring relationships through mixed effects models (Fig. 6) shows that salt stress significantly reduces aboveground biomass ($p < 0.005$), where the mean aboveground biomass difference between samples collected from the control locations and the salt patch locations was 96% (Table S4). Overall, we found that soil nutrient availability increased along the salt stress gradient as evidenced by the positive soil carbon, nitrogen, ammonium, nitrate, and phosphate relationship with salt stress (Fig. 6 and Table S5). As an artifact of both total soil C and N % increasing with increased salt stress and N having a slightly stronger relationship than C (*t* value for N = 3.99 ad *t* value for C = 3.12), we found a weak negative relationship between soil C:N ratios and salt stress ($t = -2.36$, $p = 0.03$). Soils also became more acidic and compact with increased salt stress, as demonstrated by the negative soil pH relationship ($t = -4.98$, $p < 0.05$) and positive bulk density relationship ($t = 3.55$, $p < 0.001$) with salt stress, respectively.
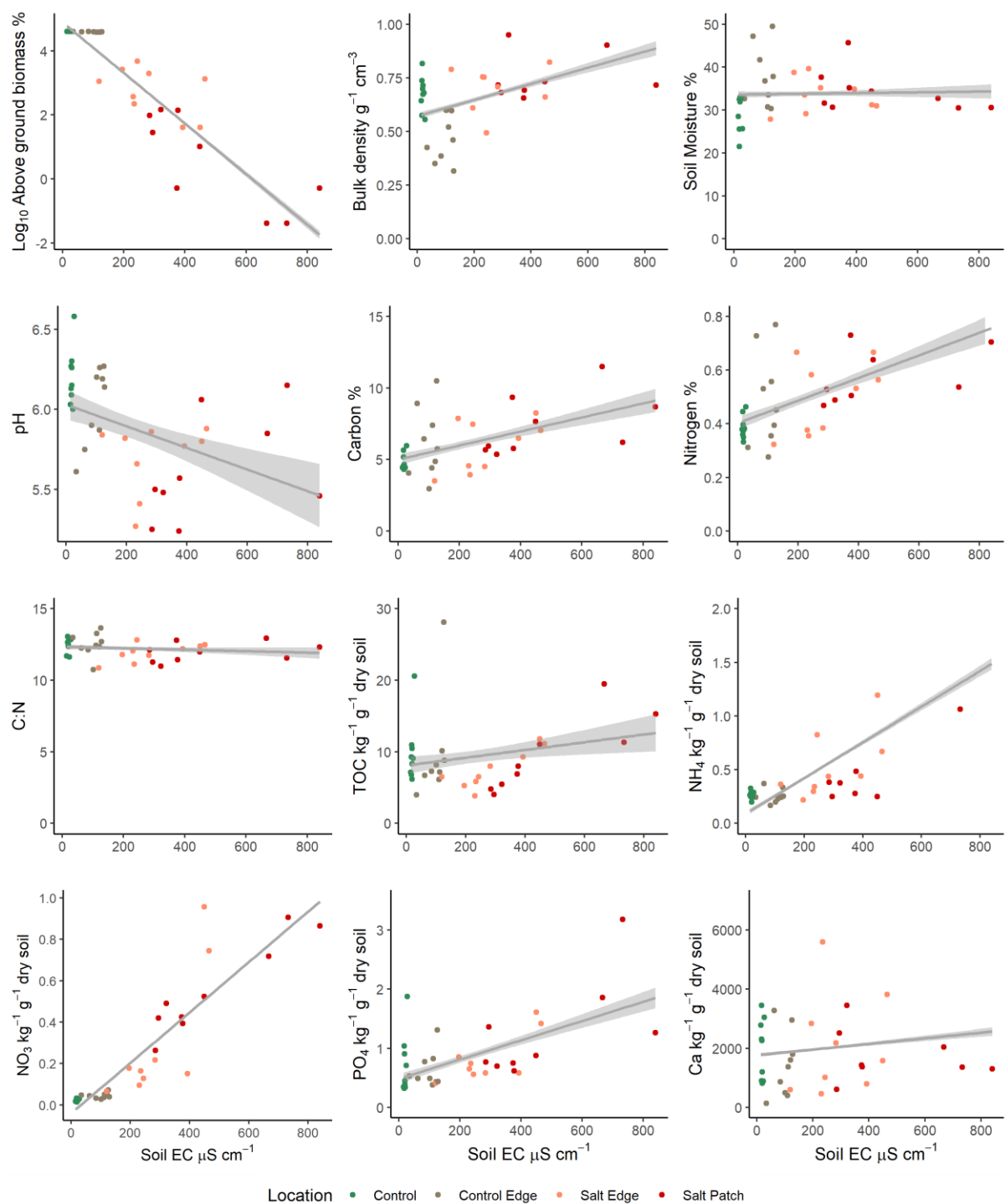
Figure 6. Trends in above ground biomass, soil bulk density, pH, carbon, nitrogen, C: N ratio, total organic carbon (TOC), ammonium, nitrate and phosphate across a saline gradient. Points represent individual sampling points where colour denotes sampling location to ensure a saline gradient was captured. The trend lines represent the predictive fitted ratio change values based

on the mixed effects models, where coloured shaded areas represent 95% upper and lower confidence intervals of the mean.

4. Discussion

4.1. CUE along a soil salinity gradient

Bacterial growth and respiration have both been shown to decrease with higher salinity, particularly in soils under agricultural management (Rath et al., 2019b; Rath and Rousk, 2015). Here, we used a low concentration (100 µM) of ^{14}C-labelled glucose to measure the ability of the intrinsic community to metabolise labile substrate, i.e., maintenance. As well as a high concentration (100 mM) of ^{14}C-labelled glucose to assess the ability of the community to grow under unlimited substrate, i.e., growth. There was no significant relationship between CUE under the high ^{14}C-labelled glucose application and soil EC (Table 1), suggesting that fast growing members of the microbial community were not inhibited by excess salt in the soil (i.e., adaptation of the copiotrophic community, *r*-strategists) (Fierer et al., 2007). This also suggests that the community did not divert a large amount of C towards osmoprotectant production or the operation of Na^+ efflux pumps. However, there was a significant positive correlation between CUE under low ^{14}C-labelled glucose application and soil EC (Fig. 3), providing evidence to suggest that metabolic activity was shifted under higher salt stress. We speculate that while the high glucose dose only targets the *r*-strategists, the low glucose dose provides a more representative view across the whole community (i.e., includes slow growing *K*-strategists) (Fierer et al., 2007). This CUE pattern under high and low glucose doses was contrary to our expectations as we hypothesized that the fast-growing community would experience greater disturbance to salt stress, resulting inreduced glucose uptake rate (P_1, k_1) and CUE (Luo et al., 2020). We also hypothesized that C turnover through the microbial biomass (i.e. P_2, k_2) would be slower in the salt-affected soils due to a reduction in mesofaunal abundance and microbial grazing. Comparison of the CUE values in the low and high glucose doses may therefore indicate that the *K*-strategists are less adapted to salt stress.

The structure of the soil microbiome and its intrinsic CUE underpins the ability of a soil to store C, as well as its functional ability to cycle and retain nutrients. In this study we clearly showed that storm surge-induced coastal inundation resulted in a major shift in bacterial community structure and functioning in terms of C turnover. Metabolic C partitioning in the

microbial biomass is fundamentally controlled by a range of environmental factors; determining the level of community stress, and soil nutrient stoichiometry (Manzoni et al., 2012). Increasing the microbial CUE of agricultural soils is seen as beneficial, potentially increasing C storage while reducing C system losses (Kallenbach et al., 2019). Increasing CUE is also likely to enhance the retention of N and P through stoichiometric balance with C. Previously, it has been shown that soil communities under high abiotic stress have a lower CUE, as more C is respired as organisms attempt to maintain normal cell function while producing stress mitigation compounds e.g., osmolytes (Empadinhas and da Costa, 2008; Manzoni et al., 2012), or to repair stress-induced damage (Jones et al., 2019; Xu et al., 2018). In this soil we have previously shown that osmotic stress induces the transitory production of osmoprotectants by the microbial biomass (Miura et al., 2020), which would be consistent with a low CUE and a decreased potential for long term C storage and sequestration (Sinsabaugh et al., 2013). This is also supported by the CUE data presented here for the low glucose additions.

In this study, while soil total C increased with salinity, there was no strong statistical relationship between total organic C and salinity ($p = 0.8$; Fig. 6 and Table S5). Elevated soil salinity has been shown to have mixed effects on soil C, either leading to an increase (Chambers et al., 2013; Servais et al., 2019) or decrease in C mineralisation (Ardón et al., 2018; Herbert et al., 2018). The relative change appears to depend on several factors including experimental location and prevailing environmental conditions. As previously discussed by de la Reguera and Tully (2021), soil moisture fluctuations will be a major control on C flux, determining the anaerobicity of soils and speed of decomposition rates. Similarly, the rate at which salt is removed from the soil profile by rainfall and plant uptake is also likely to be an important determinant (Isyenkov et al., 2019; Li et al., 2018; Munns et al., 2015). CUE in this experiment was performed on field-moist soil, to be as representative as possible of field conditions on collection. However, it is highly likely that the CUE of the soil across the saline gradient explored here is dynamic and will vary with soil moisture i.e., the degree of inundation and subsequent rainfall (Stark et al., 2019). It is therefore recommended that future research explores the relationship between frequency and degree of saline and brackish water inundation and soil CUE further.

4.2. Changes in the 16S bacterial community across the saline gradient

Salt significantly affected the structure (Fig. 4, Fig. 5, Table S2, and Table S3) and likely also the function of the soil bacterial community. We ascribe this change to salt toxicity, as well as physical changes to soil structure and a reduction or change in plant primary productivity changing the dynamic of rhizosphere-associated bacteria (Rath and Rousk, 2015). Indeed, several studies have previously concluded that salinity negatively affects the diversity and community composition of microorganism in soils and sediments, consistently across coastal ecosystems (Behera et al., 2017; Kim et al., 2019; Zhao et al., 2020).

In this study, where saline inundation is relatively infrequent, but its legacy is long lasting (i.e. salt deposition and salinisation; Fig. 1, Fig. S2), the importance of soil ecological resistance (ability to withstand disturbance) and resilience (ability to recover from disturbance) must be considered (Bardgett and Caruso, 2020; Griffiths and Philippot, 2013). We know from field observations that our field site has experienced unprecedented and repeated storm-surge saline intrusions in recent years. Although the dynamics of salt accumulation have not been measured, we know that surface salt accumulation has been a progressive process providing time for the microbial community to adapt. In contrast, the plant community has not been able to adapt leading to the loss of vegetation. We assume that creating a new quasi-stable state essentially driving a shift in community with higher salt tolerances, as seen by the significant difference in beta-diversity between the Control and Salt patch sampling treatments. Further research is required to understand the effects of salt exposure on the temporal dynamics of the soil microbial community and the effect on C cycling as it is highly likely that the frequency and severity of these events will increase in the future due to climate change (Met Office, 2018a).

Plants are soil-ecosystem engineers, with their root exudates creating nutrient hotspots within the soil, and consequently influencing the soil microbial community (Berendsen et al., 2012; Pathan et al., 2020). The grassland soil studied here can essentially all be classed as rhizosphere due to the high intrinsic root density (~25 cm root cm^{-3} soil (0 – 10 cm)). In comparison to bulk soil, rhizosphere-associated bacterial communities are denser, have larger cells (Lopes et al., 2016) and increased microbial activity (Reinhold-Hurek et al., 2015). Plants use their rhizosphere to select for (generally beneficial) microbial communities (Dawson et al., 2017; Yin et al., 2021), for example plant root exudate cocktails having been shown to encourage bacteria with matching substrate uptake preferences (Zhalnina et al., 2018). This pre-selection has often been associated with a decrease in species richness and evenness (Peiffer et al., 2013; Shi et al., 2015). Here we saw a significant reduction in plant aboveground

biomass with increasing salinity (Fig. 1, Fig. 6) as the species within the grassland were not halotolerant and had senesced within the saline patches. With the death of plants and therefore loss of the rhizospheric pre-selection of the bacterial community, changes in bacterial alpha- and beta-diversity occurred (Fig. 5).

4.3. Soil chemistry as affected by soil salinity

As plant biomass decreased, as a result of salinity induced ion toxicity and osmotic stress, (Fig. 6, Table S5) (Bidalia et al., 2019; Shrivatava and Kumar, 2015), uptake of soil available nutrients is impaired (particularly P, NH_4^+ and NO_3^-), leading to significant accumulation in soil porewater. With a reduction in root- biomass and perturbation, and the likely flocculation of clay particles (Imadi et al., 2016), we found bulk density to increase under saline conditions (Fig. 6). Equally, Na and other saltwater cations can desorb NH_4^+ from soil exchange sites (Jun et al., 2013; Weston et al., 2010), resulting in dissimilatory nitrate reduction in which NO_3^- is reduced to NH_4^+ (Giblin et al., 2010), leading to further NH_4^+ accumulation. This study took place > 2 months after inundation, allowing NH_4^+ and P to accumulate relative to the control (Fig. 6) (Herbert et al., 2018; Sánchez-Rodríguez et al., 2018). Additionally, microbial extracellular enzyme activity is likely to be affected by salinity with inhibitory effects having been shown on a range on enzymes (e.g. dehydrogenase, β-glucosidase, urease, protease, alkaline phosphatase, acidic phosphatase and arylsulphatase) (Rietz and Haynes, 2003; Tripathi et al., 2007; Zheng et al., 2017), potentially leading to slower nutrient cycling rates and lowering microbial growth and biomass (Singh, 2016). While soil pH is likely to increase initially after inundation, as seawater is alkaline (pH 8.1), we ascribe the decrease in pH with salinity across the gradient to anion accumulation (e.g. NO_3^- and PO_4^{3-}) and cation exchange between Na^+ and H^+ (Fig. 6) (Van Tan and Thanh, 2021).

Conclusions

Climate change is highly likely to increase the frequency and severity of coastal flooding in low lying areas. Understanding the effects of salt exposure on the soil microbial community and the associated effect on soil C cycling is therefore of high impetus, as salt stress can affect the provision of many ecosystem services, including nutrient cycling and soil fertility, which is highly likely to have an impact of agroecosystem productivity. Here, we

showed that a gradient of salinity caused by infrequent brackish water flooding to a coastal grassland significantly altered the soil bacterial community.

Despite this, the CUE of the soil biological community was relatively unchanged (i.e. functionally resilient) under 'growth' conditions and higher under 'maintenance' conditions suggesting that the microbial community adapted to and/or become more tolerant to salt stress, allowing the community to efficiently respond to relatively small labile C inputs. We suggest that the soil microbial community strategy (r vs. K) was driving differences in CUE under different C availability. Potentially showing that C storage and cycling ecosystem service provision is likely be dependent on the availability and/or quality of organic substrates as well as the level of salt stress. The most significant driver of changes across the gradient was likely to be decreased plant biomass with increasing salinity, which is likely to have influenced soil chemistry and microbial community structure. Therefore, we conclude that the observed soil microbial functioning effects from salt stress are indirect and are mediated by the lack of plant C inputs, nutrient uptake and maintenance of soil structure.

Acknowledgements

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Saltwater intrusion induces shifts in soil microbial diversity and carbon use efficiency in a coastal grassland ecosystem

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Figure S1. Sample site location, a sheep-grazed grassland agricultural field located next to the Menai Strait, which forms part of the Irish Sea and is adjacent to the Afon Rhaeadr-fawr river.

Figure S2. Visual representation of the salinity gradient at the patch edge. The white colouration is the accumulation of salt at the soil surface.

Figure S3. Heatmap of the relative abundance of the dominant soil bacteria ordered by class (> 1%) for each sample within each location treatment ($n = 9$).
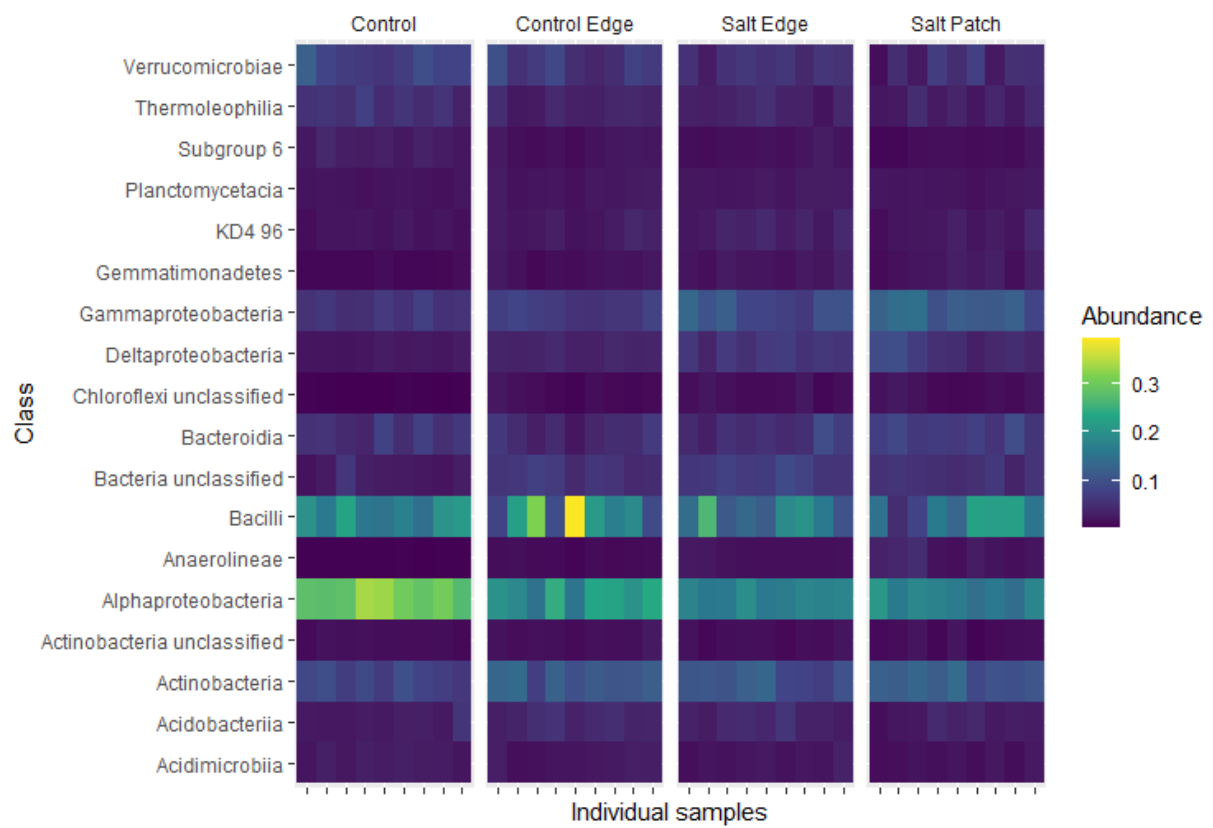
Table S1. Parameters estimates (β) and respective standard errors (SE), and significance levels of the mixed effect models explaining the variability of carbon use efficiencies with low (CUE Low) and high carbon availability (response variables).

Response Variable	Covariates	β	SE	t-value	*p*-value
CUE Low	(Intercept)	0.77	0.008	86.62	<0.005
	EC	0.00007	0.00002	3.71	**<0.05**
CUE High	(Intercept)	0.67	0.008	83.24	<0.005
	EC	0.00007	0.00002	0.35	0.728

Table S2. Comparisons of group averages of bacterial diversity at class level among saline treatments using pairwise PERMANOVA. Benjamini-Hochberg adjustment was used for p value correction. Overall tests revealed significant differences of group average among saline treatments ($F = 11.07$, $p = 0.001$).

Pairwise comparison			F value	Adjusted p value
Control	v	Control edge	10.8	**0.002**
Control	v	Salt edge	29.3	**0.002**
Control	v	Salt patch	26.5	**0.002**
Control edge	v	Salt edge	3.39	**0.02**
Control edge	v	Salt patch	5.6	**0.002**
Salt patch	v	Salt edge	2.4	**0.03**

Table S3. Correlations of environmental variables with the NMDS ordination of microbial diversity and the significance of the correlation based on the 'envfit' function (999 permutations).

Environmental variable	r^2	p value
Electrical conductivity	0.75	<0.001
Aboveground biomass	0.66	<0.001
Sodium	0.65	<0.001
Potassium	0.57	<0.001
Nitrate	0.56	<0.001
Ammonium	0.42	<0.001
pH	0.40	<0.001
Total N	0.38	<0.01
Total C	0.31	<0.01
Phosphate	0.30	<0.01
TOC	0.14	0.09
Bulk density	0.09	0.24
Soil moisture	0.07	0.32
C:N ratio	0.05	0.45
Calcium	0.01	0.90

Table S4. Mean values ± SEM for each of the variables measured across the saline gradient.

	Location along salinity gradient			
	Control	Control edge	Salt edge	Salt patch
CUE Low	0.75 ± 0.004	0.80 ± 0.006	0.81 ± 0.003	0.81 ± 0.005
CUE High	0.65 ± 0.004	0.69 ± 0.007	0.68 ± 0.007	0.67 ± 0.009
Aboveground biomass (%)	99.9 ± 0.04	98.7 ± 0.29	19.4 ± 3.98	3.7 ± 1.2
Bulk density (g cm^3)	0.68 ± 0.03	0.47 ± 0.04	0.70 ± 0.04	0.78 ± 0.04
Soil moisture (%)	29.3 ± 1.40	37.8 ± 2.34	33.5 ± 1.36	34.3 ± 1.64
pH	6.20 ± 0.06	6.02 ± 0.08	5.70 ± 0.07	5.62 ± 0.11
Total carbon (%)	4.84 ± 0.20	6.14 ± 0.81	5.95 ± 0.61	7.35 ± 0.70
Total nitrogen (%)	0.39 ± 0.01	0.49 ± 0.06	0.49 ± 0.05	0.61 ± 0.05
C:N ratio	12.5 ± 0.17	12.5 ± 0.28	11.9 ± 0.21	11.9 ± 0.23
DOC (mg C kg^{-1})	9.86 ± 1.44	9.60 ± 2.38	7.56 ± 0.90	9.57 ± 1.73
Ammonium (mg N kg^{-1})	0.27 ± 0.01	0.25 ± 0.02	0.53 ± 0.10	0.80 ± 0.25
Nitrate (mg N kg^{-1})	0.02 ± 0.002	0.05 ± 0.004	0.30 ± 0.11	0.56 ± 0.07
Phosphate (mg P kg^{-1})	0.71 ± 0.17	0.63 ± 0.10	0.82 ± 0.14	1.26 ± 0.28
Calcium (mg Ca kg^{-1})	1962 ± 342	1436 ± 369	2099 ± 576	2351 ± 653
Sodium (mg Na kg^{-1})	37 ± 6	234 ± 40	336 ± 57	432 ± 71
Potassium (mg K kg^{-1})	64.2 ± 3.9	88.4 ± 12.8	152 ± 14.5	178 ± 19

Table S5. Parameters estimates (β) and respective standard errors (SE), and significance levels of the mixed effect models explaining the variability of soil physiochemical parameters (response variables).

Response Variable	Covariate	β	SE	t-value	*p* value
Aboveground biomass (%)	(Intercept)	4.93	0.22	22.25	<0.005
	EC	-0.008	0.0006	-13.64	**<0.005**
Bulk density (g cm^{-3})	(Intercept)	6.11	0.16	39.08	<0.005
	EC	-0.001	0.0002	-4.98	**<0.005**
pH	(Intercept)	6.11	0.16	39.08	<0.005
	EC	-0.001	0.0002	-4.98	**<0.05**
Carbon (%)	(Intercept)	5.20	0.71	7.27	<0.05
	EC	0.004	0.001	3.12	**<0.005**
Nitrogen (%)	(Intercept)	0.42	0.048	8.57	<0.005
	EC	0.004	0.00009	3.99	**<0.005**
C:N ratio	(Intercept)	12.47	0.33	38.00	<0.005
	EC	-0.001	0.0005	32.86	**<0.05**
DOC (mg C kg^{-1})	(Intercept)	8.95	2.22	4.02	<0.05
	EC	0.0008	0.003	0.26	0.80
Ammonium (mg N kg^{-1})	(Intercept)	0.11	0.08	1.35	0.25
	EC	0.002	0.0002	7.23	**<0.005**
Nitrate (mg N kg^{-1})	(Intercept)	-0.05	0.03	-1.44	0.16
	EC	0.001	0.0009	13.20	**<0.05**
Phosphate (mg P kg^{-1})	(Intercept)	0.55	0.18	3.15	0.054
	EC	0.001	0.004	3.86	**<0.005**