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Soil proteases: widening the bottleneck of the terrestrial nitrogen cycle

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Soil proteases: widening the bottleneck of the terrestrial nitrogen cycle

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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.

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Thesis summary

Nitrogen (N) is a major nutrient needed for plant growth. Around 40% of N is considered unavailable for plant use in the form of proteins. Proteins can be broken down into constituents available for plants by extracellular proteases released by microorganisms. Therefore, understanding soil proteases and their role in organic matter breakdown could provide a mechanism for increasing organic N acquisition that would improve N supply in soil leading to increased plant productivity. However, there are ongoing discussions over the lack of standardisation of assays used to measure soil protein and protease activity. The overall aims of the thesis were to a) critically evaluate the methods used to extract proteins from soil and measure soil protease activity and b) determine the factors that influence protein breakdown in the soil-plant-microorganism system. First, I critically evaluated methods to extract proteins from soil by comparing the ability of common extractants to recover soluble proteins from three soil types. I found that the dependence of protein recovery on both extractant and soil type prevents direct comparison of studies using different recovery methods, particularly if no extraction controls are used. Secondly, I investigated how topsoil and subsoil properties affect protein breakdown along a grassland altitude and primary productivity gradient that contained a range of soil types. I concluded that protein breakdown was not regulated by a small number of factors but a wide range of interacting factors which were site specific. Furthermore, I suggested that differences in soil N cycling and the generation of ammonium are more related to the rate of protein supply rather than limitations in protease activity and protein turnover. I then determined whether plants actively secrete proteases to enhance the breakdown of soil protein or are they functionally reliant on soil microorganisms to undertake this role? The results indicated that plant uptake of organic N is only functionally significant when soil protein is in direct contact with root surfaces. The lack of protease upregulation under N deficiency suggests that root protease activity is unrelated to enhanced soil N capture. Next, I determined the spatial distribution of protease activity in the rhizosphere of barley (*Hordeum vulgare* L.) using *in situ* zymography. I analysed the effect of root hairs and soluble protein addition on rhizosphere protease activity. The results showed protease activity was highest in the barley genotype with root hairs and with protein addition suggesting that plants with root hairs have a greater advantage in accessing protein hotspots in the soil indirectly via microbial-derived proteases. Lastly, I conducted a systematic review and meta-analysis of the common methods used to measure soil protease activity globally. I collected data on environmental and methodological factors to determine the variation of protease activity in soil. From this, I found soil protease activity to vary widely due to study-biases and observed a lack of reporting of key assay conditions by studies. Together, this research provides a more detailed understanding of protein mineralisation and protease activity in soil. Going forward, comprehensive reporting of enzyme assay conditions is essential to increase the accuracy and reliability of interpreting soil protease activity dynamics in the soil-plant-microorganism system.

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Lastly, to my parents and sister. Thank you for always believing in me and being proud of my achievements. You have encouraged me to go for anything and keep going even when times seem hard.

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Abbreviations

AMC – 7-amino-4-methlycoumarin

ANOVA – Analysis of variance

BAA – N-benzoyl arginine amide

BSA – Bovine serum albumin

CEC – Cation exchange capacity

CUE – Carbon use efficiency

DOC – Dissolved organic carbon

DON – Dissolved organic nitrogen

EC – Electrical conductivity

MIT – Mineralisation-immobilisation-turnover

MW – Molecular weight

NH_3 – Ammonia

NH_4^+ – Ammonium

NO_3^- – Nitrate

NO_2^- – Nitrite

N_2O – Nitrous oxide

NPP – Net primary productivity

SDS-PAGE – Sodium dodecyl sulphate - polyacrylamide gel electrophoresis

SEM – Standard error of the mean

SIMS – Secondary ion mass spectrometry

SOM – Soil organic matter

Z-phe-leu – N-[(Benzyloxy)carbonyl] phenylalanyl leucine

Chapter 1

Introduction

Soil protease activity: Challenges and the need for research

1.1. Soil protease activity: Challenges and the need for research

Nitrogen (N) availability represents one of the key regulators of primary productivity in terrestrial ecosystems. In soil, ca. 40% of N is present as protein and considered unavailable to plants with the predominant forms of N taken up by plants considered to be ammonium (NH_4^+) and nitrate (NO_3^-) (Schulten and Schnitzer, 1997). However, since the 1990s, evidence has shown that plants can also directly acquire organic N (e.g. oligopeptides and amino acids) (Schimel and Bennett, 2004). Proteases are catalysts for the breakdown of protein into peptides and amino acids which plants can utilise for N nutrition. Protein breakdown is the initial step of N mineralisation in soil and said to occur at a slower rate than amino acid mineralisation leading to a bottleneck in the N cycle (Jan et al., 2009; Weintraub and Schimel, 2005). The ability to expand this bottleneck could increase N acquisition by plants. Plant N acquisition is particularly important in agricultural systems where there is a need to improve N use efficiency. Inorganic N fertilisers used in conventional farming systems, are added in higher quantities than used by crops, contribute to greenhouse gas emissions (e.g. N_2O), reduce air quality (e.g. due to NH_3 volatilisation), leach into surrounding watercourses causing eutrophication and contaminate groundwater (e.g. NO_3^-) (Dungait et al., 2012). Understanding soil proteases and their role in organic matter breakdown could identify mechanisms for increasing organic N acquisition that could improve plant productivity in agricultural systems and mitigate the environmentally detrimental effects of inorganic N fertilisers. However, to be able to reliably measure soil protease activity, standardised methods must be established.

Soil exoproteomics, the study of extracellular proteins in soil, and protein quantification rely on the extraction of proteins from soil for analysis. However, a standardised extraction method is lacking. Currently, there are numerous extractants, molarities and pH values used to recover proteins from soil (Keiblinger et al., 2012). However, these protocols tend to be optimised to single unrepresentative proteins, single soils, or use quantification methods that suffer from interference e.g. co-extraction of humic substances (Kanerva et al., 2013; Roberts and Jones, 2008). Soil properties can differ greatly depending on a range of intrinsic pedogenic and local management factors (e.g. organic matter content, clay content, pH and land use). These properties directly affect the degree to which proteins move in soil, interact and bond with soil surfaces (Bastida et al., 2009). Thus, in order to fully understand the

roles proteins play across different soil ecosystems we need to be able to reliably and accurately extract proteins for identification and quantification.

There are numerous methods for measuring soil protease activity but currently no standard protocol has been adopted. Protease assays are usually measured using colorimetric or fluorimetric techniques with substrates that are chromo- or fluorophores. Within these analytical methods, a wide range of soil pre-processing, substrates and assay conditions are used which determine the protease type targeted (e.g. endo- or exopeptidases). This means inter- and even intra-study comparisons lack accuracy (Nannipieri et al., 2017). Furthermore, key environmental factors such as temperature and pH are not always properly accounted for in enzyme assay protocols despite the many reviews that state the need for standardisation and documentation of key environmental variables (e.g. Dick, 2011; German et al., 2011; Nannipieri et al., 2017).

Many environmental factors affect protease activity, including temperature (Brzostek and Finzi, 2012), atmospheric CO_2 concentration (Kandeler et al., 2006), soil water content (Sardans and Peñuelas, 2005) and C and N availability (Geisseler and Horwath, 2008). Variations in these factors can induce changes to protease activity in the soil. However, previous studies have not examined in depth how these edaphic and climatic factors interact to regulate protease activity. The heterogenous nature of soil means interacting factors that regulate protease activity are likely to change depending on environmental conditions (e.g. soil type, land use, temperature and precipitation). An improved mechanistic knowledge of the factors regulating protease activity could therefore be used to identify management options to regulate and optimise N available for plants and reduce N losses to the wider environment.

Typically, protease activity is measured as potential *ex situ* activity rather than *in situ* activity of the soil. Studies have characterised total protease activity in plant-microorganism-soil systems (Spohn et al., 2013; Vagnerova and Macura, 1974) and microorganism-soil systems (Ladd and Butler, 1972; Watanabe and Hayano, 1996). Yet, it has not been examined the individual contribution of plants and microorganisms to total soil protease activity or if this differs between crop species. It is also not clear whether extracellular plant root protease activity is a) an active process in order to obtain N for nutrition, b) a passive process as a result of root exudation or c) indirectly from another functional mechanism e.g. the regulation of proteins in response to developmental and environmental cues (Tornkvist et al., 2019; Vranová et al., 2013).

The ability of plants to actively increase root protease activity to obtain N would allow plants to be more competitive for organic N with microorganisms. It would also create the potential for targeted genetic manipulation or conventional breeding to optimise N capture from organic N compounds by enhancing protease activity in the rhizosphere.

Microbial activity is highest in the rhizosphere due to the high exudation of a variety of compounds (e.g. carbohydrates, amino acids, enzymes, proteins and phenols) from plant roots which provide a source of C and N for microorganisms (Koo et al., 2005; Rovira, 1969). Many studies have found C and N exudation to enhance enzyme activity in the rhizosphere (Brzostek et al., 2013; Kandeler et al., 1994). Root morphology plays a major role in determining the quality, quantity and distribution of exudates along the root surface and, thus, enzyme activity. In addition, inputs from C and N also come from variety of other sources e.g. plant litter, root biomass and decaying micro- and macrofauna and functional proteins released into the soil by plants and microorganisms to carry out specific functions (Rillig et al., 2007). However, few studies have investigated the effect of external protein addition in combination with root morphology.

Uncertainties and knowledge gaps presented above have motivated the need for further research undertaken in this thesis. I will use a combination of experimental work and a meta-analysis to elucidate the fundamental mechanisms driving protein mineralisation and protease activity in soil.

1.2. Thesis aims and objectives

This section details the main aims and objectives of the thesis, followed by a brief description of the relevant chapters and experimental work referring to each objective. This thesis is divided into 5 chapters as a series of 4 experimental chapters and one meta-analysis. A list of the experimental chapter titles is presented in section 1.3. Individual hypotheses and objectives are described in each of the prepared manuscripts. Figure 1.1 describes the thesis journey from the initial (3) to the last experimental chapter (7) and the questions that arose from each chapter to lead into the next.

1.2.1. Thesis aims

The work I present in this PhD thesis aims to firstly critically evaluate the methods used to extract proteins from soil and measure soil protease activity.

Secondly, I aim to determine the factors that influence protein mineralisation and protease activity in the soil-plant-microorganism system.

Objective 1

Determine the efficiency of different methods for recovering protein from soil.

In Chapter 3, three contrasting, sterilised soils were incubated with ^{14}C-labelled tobacco leaf protein. Eleven extractants including distilled water were investigated at four different concentrations to determine how efficient different extractants are at recovering protein from soil. The amount of ^{14}C-labelled tobacco leaf protein that was extracted from the soil was measured using liquid scintillation counting. I hypothesised that the choice of extractant and soil type will affect the amount of protein that could be recovered from soil.

Objective 2

Investigate the soil properties that affect organic N breakdown.

In Chapter 4, I conducted a laboratory-scale experiment, investigating how soil properties affect organic N mineralisation. Soils were collected at two depths at ten points along an altitudinal and productivity gradient in Abergwyngregyn, UK to provide a range of soil properties. Rates of protein and amino acid mineralisation were measured by adding ^{14}C-labelled plant protein and a ^{14}C-labelled amino acid mixture, respectively, and measuring $^{14}CO_2$ evolution over a two-month period. Rates of mineralisation along the sequence were correlated with various soil properties. I hypothesised that 1) key regulators (NH_4^+, NO_3^-, protein, amino acid, microbial biomass-C, pH, CEC, N mineralisation, sorption and primary productivity) will predict protein mineralisation rates as these drive or limit degradation processes; 2) The rate of protein mineralisation will decrease along the grassland altitudinal gradient (from low to high altitude) as primary productivity, pH and C and N availability reduce microbial activity, and 3) Protein mineralisation is negatively correlated with depth as protein inputs and microbial biomass C decreases in the subsoil relative to the topsoil.

Objective 3

Determine whether plants use root-derived proteases as a mechanism for N nutrition.

In Chapter 5, I conducted a series of experiments to determine possible mechanisms to produce plant root-derived proteases. I used a fluorimetric assay to

measure protease activity as well as ^{14}C-labelled plant protein to determine the uptake of protein breakdown products by plants. I then compared plant root-derived protease activity to total rhizosphere activity to determine its contribution to soil protease activity. I hypothesised that 1) plants will both secrete proteases from their roots but also retain surface-bound protease activity to maximise protein-N capture from soils and 2) protease activity from rhizosphere soil will be proportionally higher than for roots as it is more energetically favourable for the soil microbial community to use the products of protein hydrolysis rather than inorganic N

Objective 4

Determine the effect of protein addition and root morphology on protease activity in the rhizosphere.

In Chapter 6, I used zymography to determine the spatial distribution of leucine aminopeptidase activity in the rhizosphere of barley (*Hordeum vulgare* L.) with and without root hairs. Soluble protein was added in bands along the soil and root surface to determine whether soluble protein addition increased the magnitude and extent of protease activity and whether this is enhanced by the presence of root hairs. I hypothesise that 1) protein addition would increase leucine aminopeptidase activity in the rhizosphere due to an increase in substrate, 2) root hairs would accelerate leucine aminopeptidase activity in the rhizosphere by providing a higher surface area and more root exudates, and 3) root hairs would increase the rhizosphere extent of leucine aminopeptidase activity due to the larger surface area of the root.

Objective 5

Determine the variability of soil protease activity on a global scale and whether environmental and methodological conditions can explain the variation.

In Chapter 7, I conducted a meta-analysis of colorimetric and fluorimetric methods used to measure soil protease activity. Data was collected on environmental (e.g. mean annual temperature, latitude, soil pH) and methodological aspects (e.g. assay temperature, substrate, pH) to investigate the variation between and within studies. I hypothesised that pH and temperature will explain the variation of protease activity at a global scale as they are key factors in limiting protease activity.

1.3. Experimental chapter information

The experimental chapters of the current thesis have been prepared in the style of journal article manuscripts. The title page of each experimental chapter includes details of the authors, author contributions to the manuscript and the current progress of each manuscript (e.g. published / accepted / submitted / not yet submitted). The thesis consists of four experimental chapters and one meta-analysis chapter, located in Chapters 3-7 of the current document. For continuity and clarity, the experimental chapters will be referred to as they appear in this thesis. The titles of the experimental chapters are as follows:

Chapter 3: Methodological bias associated with soluble protein recovery from soil

Chapter 4: Is soluble protein mineralisation and protease activity in soil regulated by supply or demand?

Chapter 5: Do plants use root-derived proteases to promote the uptake of soil organic nitrogen?

Chapter 6: Protein addition to soil increases rhizosphere extent and promotes protease activity

Chapter 7: Critical review and meta-analysis of methods used to determine soil protease activity

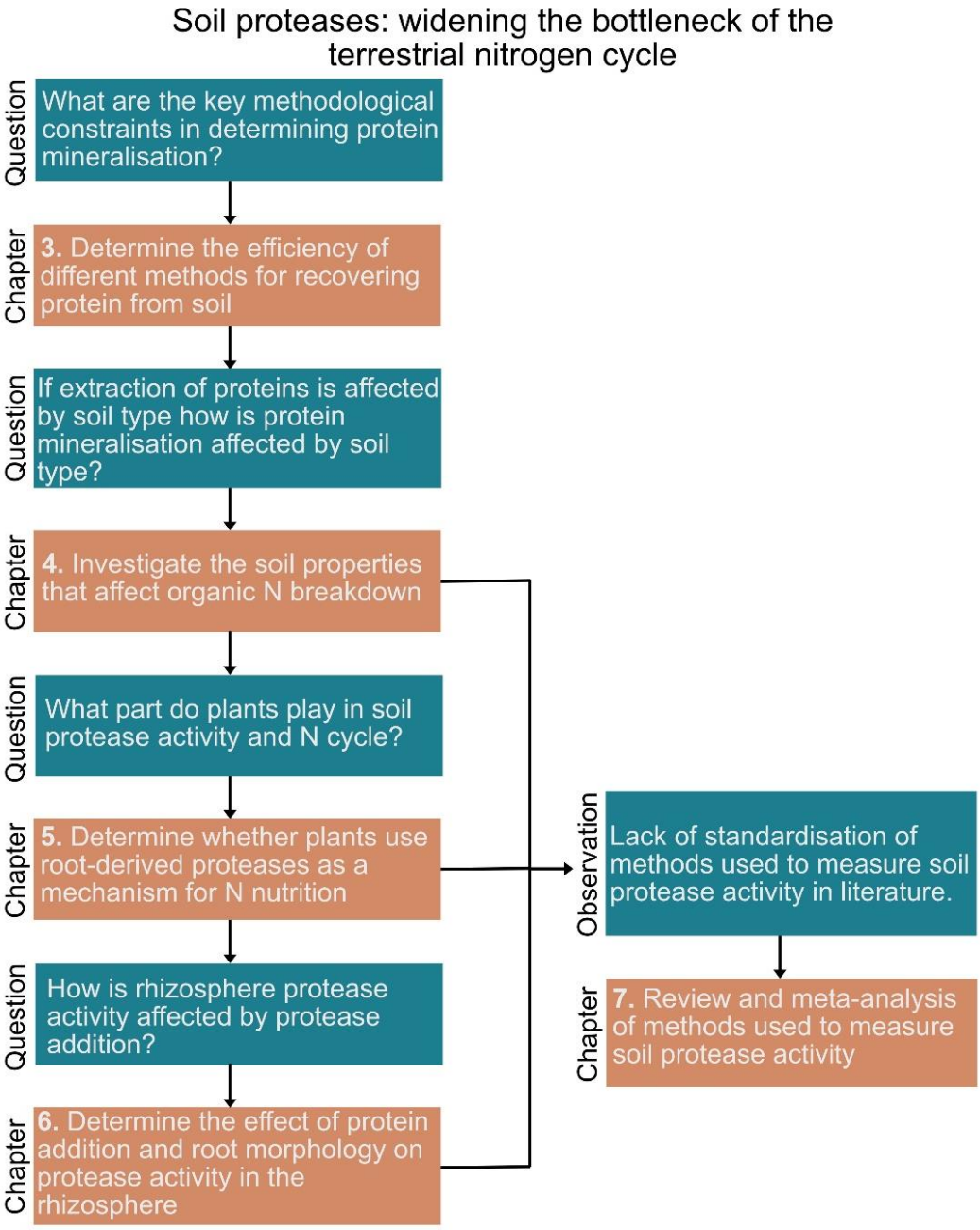

Figure 1.1. A schematic diagram of the thesis journey

1.4. References

Bastida, F., Moreno, J.L., Nicolás, C., Hernández, T., García, C., 2009. Soil metaproteomics: A review of an emerging environmental science. Significance, methodology and perspectives. European Journal of Soil Science 60, 845–859.

Brzostek, E.R., Finzi, A.C., 2012. Seasonal variation in the temperature sensitivity of proteolytic enzyme activity in temperate forest soils. Journal of Geophysical Research: Biogeosciences 117, 1–10.

Dick, W.A., 2011. Development of a soil enzyme reaction assay, in: Dick, R.P. (Ed.), Methods of Soil Enzymology. Soil Science Society of America, Madison, WI, USA, pp. 71–84.

Dungait, J.A.J., Cardenas, L.M., Blackwell, M.S.A., Wu, L., Withers, P.J.A., Chadwick, D.R., Bol, R., Murray, P.J., Macdonald, A.J., Whitmore, A.P., Goulding, K.W.T., 2012. Advances in the understanding of nutrient dynamics and management in UK agriculture. Science of the Total Environment 434, 39–50.

Geisseler, D., Horwath, W.R., 2008. Regulation of extracellular protease activity in soil in response to different sources and concentrations of nitrogen and carbon. Soil Biology & Biochemistry 40, 3040–3048.

German, D.P., Weintraub, M.N., Grandy, A.S., Lauber, C.L., Rinkes, Z.L., Allison, S.D., 2011. Optimization of hydrolytic and oxidative enzyme methods for ecosystem studies. Soil Biology & Biochemistry 43, 1387–1397.

Jan, M.T., Roberts, P., Tonheim, S.K., Jones, D.L., 2009. Protein breakdown represents a major bottleneck in nitrogen cycling in grassland soils. Soil Biology & Biochemistry 41, 2272–2282.

Kandeler, E., Mosier, A.R., Morgan, J.A., Milchunas, D.G., King, J.Y., Rudolph, S., Tscherko, D., 2006. Response of soil microbial biomass and enzyme activities to the transient elevation of carbon dioxide in a semi-arid grassland. Soil Biology & Biochemistry 38, 2448–2460.

Kanerva, S., Smolander, A., Kitunen, V., Ketola, R.A., Kotiaho, T., 2013. Comparison of extractants and applicability of MALDI-TOF-MS in the analysis of soil proteinaceous material from different types of soil. Organic Geochemistry 56, 1–9.

Keiblinger, K.M., Wilhartitz, I.C., Schneider, T., Roschitzki, B., Schmid, E., Eberl, L., Riedel, K., Zechmeister-Boltenstern, S., 2012. Soil metaproteomics -

Comparative evaluation of protein extraction protocols. Soil Biology & Biochemistry 54, 14–24.

Ladd, J.N., Butler, J.H.A., 1972. Short-term assays of soil proteolytic enzyme activities using proteins and dipeptide derivatives as substrates. Soil Biology & Biochemistry 4, 19–30.

Nannipieri, P., Trasar-Cepeda, C., Dick, R.P., 2017. Soil enzyme activity: a brief history and biochemistry as a basis for appropriate interpretations and meta-analysis. Biology & Fertility of Soils 54, 1–9.

Rillig, M.C., Caldwell, B.A., Wösten, H.A.B.B., Sollins, P., 2007. Role of proteins in soil carbon and nitrogen storage: Controls on persistence. Biogeochemistry 85, 25–44.

Roberts, P., Jones, D.L., 2008. Critical evaluation of methods for determining total protein in soil solution. Soil Biology & Biochemistry 40, 1485–1495.

Sardans, J., Peñuelas, J., 2005. Drought decreases soil enzyme activity in a Mediterranean Quercus ilex L. forest. Soil Biology & Biochemistry 37, 455–461.

Schimel, J.P., Bennett, J., 2004. Nitrogen Mineralization: Challenges of a Changing Paradigm. Ecology 85, 591–602.

Schulten, H.-R., Schnitzer, M., 1997. The chemistry of soil organic nitrogen: a review. Biology & Fertility of Soils 26, 1–15.

Spohn, M., Carminati, A., Kuzyakov, Y., 2013. Soil zymography – A novel in situ method for mapping distribution of enzyme activity in soil. Soil Biology & Biochemistry 58, 275–280.

Tornkvist, A., Liu, C., Moschou, P., 2019. Proteolysis and Nitrogen: emerging insights. Journal of Experimental Botany 70, 2009–2019.

Vagnerova, K., Macura, J., 1974. Relationships between plant roots, proteolytic organisms and activity of protease. Folia Microbiologica 19, 525–535.

Vranová, V., Rejsek, K., Formanek, P., 2013. Proteolytic activity in soil: A review. Applied Soil Ecology 70, 23–32.

Watanabe, K., Hayano, K., 1996. Seasonal variation in extracted proteases and relationship to overall soil protease and exchangeable ammonia in paddy soils. Biology & Fertility of Soils 21, 89–94.

Weintraub, M.N., Schimel, J.P., 2005. Seasonal protein dynamics in Alaskan arctic tundra soils. Soil Biology & Biochemistry 37, 1469–1475.

Chapter 2

Literature Review

2.1. Introduction

Nitrogen (N) availability is one of the key regulators of primary productivity in terrestrial ecosystems (Robertson and Groffman, 2007). Plants can utilise N in the form of ammonium (NH_4^+), nitrate (NO_3^-), amino acids and oligopeptides. Evidence that plants can acquire organic N was well established since the 1990s. Yet, the factors that regulate organic N availability in the soil are poorly understood compared to inorganic N. Improving our knowledge of organic N availability is vital for increasing the efficiency of sustainable agricultural systems.

Around 40% of the total soil N is in proteinaceous form (Schulten and Schnitzer, 1997). However, plants do not have direct access to this N source. First protein must be broken down into oligopeptides and amino acids by protease enzymes. Most extracellular proteases are produced by microorganisms to obtain their own C and N nutrition. Proteases are thought to be catalysts for the rate-limiting step in N mineralisation that determines the availability of plant N (Jan et al., 2009; Weintraub and Schimel, 2005). The ability to expand this bottleneck could increase N acquisition by plants. In agricultural systems, inorganic N fertilisers are typically added to soil to increase crop yields, yet these can have detrimental environmental effects. Thus, widening the bottleneck of N plant acquisition is particularly important for increasing the efficiency of low input and organic systems. Understanding soil proteases and their role in organic matter break down could, therefore, provide a mechanism for increasing organic N plant acquisition.

A range of methodological approaches are used to characterise and measure the activity of soil proteases, however, there is no consensus on the best protocol. The accuracy and development of methods is currently hindered by limitations in protein extraction and measurement. The distribution of proteins in soil is variable as well as their affinity to be adsorbed to soil surfaces making it difficult to access the proteins in question (Bastida et al., 2009). Protein assays, with the addition of substrates containing chromo- and fluorophores, are widely used to determine protease activity in soil systems but their accuracy is reduced due to the interference of humic substances and metals present in the soil solution (Roberts and Jones, 2008). *In situ* zymography is an approach used to assess the spatial distribution of protease activity that avoids plant-soil system disturbance (Spohn et al., 2013). Stable isotopes, namely ^{13}C and ^{15}N, are used extensively to trace molecules through the C and N cycles. Radioisotopes (e.g. ^{14}C) can be used to determine microbial breakdown and

mineralisation of soil organic matter (SOM) and advances of position-specific labelling provide insights into specific soil processes (Apostel et al., 2013; Dippold and Kuzyakov, 2013).

This review aims to synthesise and examine the literature on soil proteases and the methodological approaches used to determine protein mineralisation and protease activity in soils.

2.2. Nitrogen in the soil system

2.2.1. Soil N cycle

N is a key element in the Earth's biogeochemistry; it is present in the atmosphere, hydrosphere, lithosphere, and biosphere. The physicochemical mobility of N means it can be cycled between pools taking on many forms (Mengel and Kirby, 1978; Robertson and Groffman, 2007). Figure 2.1 shows a simplified schematic of the soil N cycle explained in more detail in the following section. N is fixed from the atmosphere, where it is present predominantly as N_2, by nitrogen-fixing microorganisms. These diazotrophs can be free-living or occur in a symbiotic association with plants. They produce NH_3 that is subsequently converted into organic compounds, such as glutamine, because NH_3 is toxic to most organisms (Bottomley and Myrold, 2007).

N mineralisation is the conversion of organic N compounds into inorganic N compounds (namely NH_4^+) by microorganisms, released as a by-product of metabolism. First, high molecular weight (MW) compounds are converted into low MW compounds in the rate-limiting step of N mineralisation catalysed by protease enzymes. Protein degradation to amino acids is approximately 20 times slower than amino acid mineralisation (Jan et al., 2009). In cases when the N contained in organic compounds is not enough to sustain a microorganism's metabolic needs the microorganism retains N. Therefore, N becomes immobilised in the microbial biomass and less N is available for plant use. The rates of mineralisation and immobilisation depend on the quantity of N in organic compounds and the demands of specific microorganisms (Hopkins and Dungait, 2010). When substrates have a low C:N ratio (<25:1; Myrold and Bottomley, 2008) mineralisation occurs but when substrates have a high C:N ratio immobilisation is dominant in order to assimilate more N.

Nitrification is the oxidation of NH_3 to less reduced forms i.e. NO_3^-, NO_2^- by microorganisms. It is a two-step process carried out predominately by autotrophic bacteria, but also some heterotrophic bacteria and fungi. Nitrification is determined predominately by NH_4^+ concentration unless limited by other factors e.g. soil pH, aeration, tillage, atmospheric N deposition and fertilisation (Robertson and Groffman, 2007). NO_3^- is an anion and, therefore, more mobile than the ammonium ion which has a positive charge and so can be adsorbed onto negatively charged SOM and clay mineral surfaces. NO_3^- can easily be leached out of the soil by water and converted into N_2 and N_2O gas by microorganisms through denitrification.

Denitrification is the reduction of NO_3^- to N gases i.e. NO, N_2O and N_2 by heterotrophic bacteria. This process mainly occurs in low O_2 conditions because NO_3^- is used by the bacteria as a terminal electron acceptor in respiration, but it is less efficient than oxygen (Robertson and Groffman, 2007). NO_3^- can also be reduced during nitrate ammonification, or dissimilatory nitrate reduction to NH_4^+, NO_2^- and N_2O by soil-isolated nitrate-ammonifying bacteria with the end product being determined by the C:NO_3^- ratio (Stremińska et al., 2012).

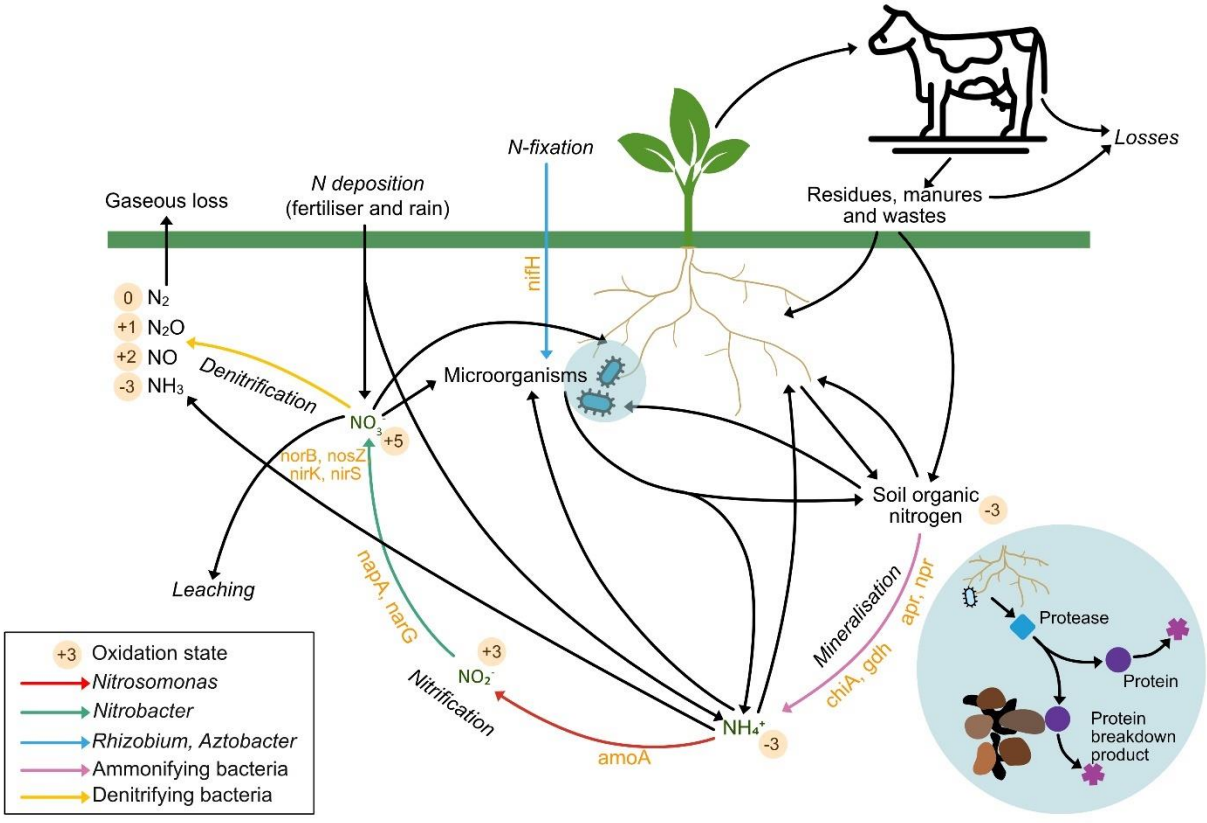

Figure 2.1. Schematic diagram of a simplified soil N cycle. Genes coding particular N conversion processes are in orange.

2.2.2. Organic nitrogen

SOM (e.g. proteins, peptides and amino acids) represents the largest pool of N in terrestrial ecosystems at approximately 88% of the total terrestrial N pool (170 billion tonnes; Powlson, 1993). Schulten and Schnitzer (1997) stated proteinaceous N to be 40% of total soil N. However, the specific distribution in N varies depending on climate, soil type, land use and vegetation (Sowden et al., 1977). The efficiency of organic N extraction is far from 100% and, therefore, these figures are likely to be an underestimation of the true fraction. This is due to the complex nature of organic N and the variety of forms found in the soil meaning different extraction methods target different compounds (Lehmann and Kleber, 2015; Nannipieri et al., 1980).

Amino acids are organic compounds that contain amide ($-NH_2$) and carboxyl (-COOH) functional groups along with a side chain (-R group) specific to each amino acid. The properties of the amino acid depend on the R group e.g. acidic, neutral, basic, hydrophilic, hydrophobic, polar or non-polar. Though there are over 500 amino acids only 20 are biologically used to create peptides and proteins (Lehninger et al., 1993). Amino acid sorption to soil particles e.g. clay minerals tends to be by electrostatic interactions that vary with mineral type and pH of the amino acid (Hedges and Hare, 1987). Free amino acids in the soil solution are reported to have concentrations of up to 100 µM in surface horizons (Owen and Jones, 2001). The neutral amino acids alanine and glycine are the most common amino acids in soil (Senwo and Tabatabai, 1998). In a study of ten soils across Iowa, USA, amino acid N was found to be 32-50% of total soil N and positively correlated with organic carbon and clay content (Senwo and Tabatabai, 1998).

Peptides are polymers of amino acids connected by peptide bonds formed between the carboxyl group of one amino acid and the amide group of another. This bond is formed through a condensation reaction which keeps all of the amino acid R groups on the same side (Lodish et al., 2000). Short chain oligopeptides are 2-10 residues long whilst polypeptides can be up to 4000 residues long (Lodish et al., 2000). The arrangement of hydrophilic and hydrophobic residues and the polarity of residues within a peptide can affect their solubility and interaction with soil particles. However, the structure may be different in each environment as the soil pH can affect the charge of amino acid residues (Niena, 2019).

Proteins are peptides with at least 40 amino acids residues. This cut off merges with polypeptides resulting in the two terms sometimes being used interchangeably. Proteins have numerous biological functions including providing structural rigidity to cells, controlling the flow of compounds though membranes and catalysing chemical reaction (enzymes) (Lodish et al., 2000). The function of a protein is determined by its three-dimensional (3D) structure which is determined by the amino acid residues it contains (Lodish et al., 2000). The primary structure of a protein is the sequence of amino acid residues that make up the peptide chain. The secondary structure is the localised organisation of parts of the peptide chain. This organisation can occur randomly or stabilised by hydrogen bonds between certain residues that cause the peptide backbone to fold into an α helix (spiral) or β sheet (planar structure) (Lodish et al., 2000). The tertiary structure is the 3D arrangement of the amino acid residues and is stabilised by hydrophobic interactions of nonpolar side chains or if sulfhydryl groups are present in the -R group as disulphide bonds (Lodish et al., 2000). When proteins contain two or more peptide chains have a quaternary structure. Peptide chains a bonded by noncovalent bonds (Lodish et al., 2000). Proteins can be split into high MW (>100 kDa) and low MW (<20 kDa). Due to the smaller size of low MW proteins they can fit through root pores (Paungfoo-Lonhienne et al., 2008). Proteins bind to soil surfaces by a range of different bonds depending on the compound e.g. proteins bond to mineral surfaces by electrostatic interactions (Quiquampoix et al., 1995). When not bound in the soil, proteins are rapidly depolymerised to oligopeptides and amino acids by proteases released by microorganisms resulting in short turnover times (Kögel-Knabner, 2006; Lipson and Näsholm, 2001).

2.3. Microbial and plant utilisation of N

2.3.1. Microbial assimilation of N

The assimilation of N by microorganisms is thought to occur by two strategies; 1) directly by assimilation of low MW substrates e.g. amino acids that are then deaminated inside the cell and excess ammonium released, or 2) mineralisation-immobilisation-turnover (MIT) where organic N is mineralised outside the cell to produce ammonium that is then assimilated by microorganisms (Barraclough, 1997).

To determine the dominant method of N assimilation in microorganisms, Hadas et al. (1992) added equal concentrations of L-alanine and $(NH_4)_2SO_4$ labelled with ^{15}N

to two different soil types and incubated them for up to 1.2 days. Microbial N biomass was determined by chloroform-fumigation extraction and the $^{14/15}N$ ratio by emission spectroscopy. The results showed that assimilation of alanine and ammonium happened simultaneously suggesting that there is no dominant method of N substrate for acquisition. In contrast, Barraclough (1997) and Drury et al. (1991) found that the MIT mechanism was favoured whilst Barak et al. (1990) showed evidence of the direct assimilation of amino acids using similar methodologies.

Mycorrhizal fungi are microorganisms that have a symbiotic relationship with plants either in living within the root tissues (arbuscular) or on the surface (ectomycorrhizal) (Read et al., 1988). Ectomycorrhizal fungi can assimilate organic N not available to the root and increase ammonium uptake in exchange for carbohydrates e.g. glucose from the plant (Miller and Cramer, 2005). The symbiotic relationship has been shown to increase plant growth compared to non-mycorrhizal plants (Abuzinadah et al., 1989).

2.3.2. Plant assimilation of N

Up until the 1990s, it was largely accepted that plants acquired only inorganic forms of N (NH_4^+ and NO_3^-). These inorganic N compounds are formed by the microbial decomposition of organic N compounds by mineralisation (Fig. 2.2a) (Schimel and Bennett, 2004). In order for a particular N compound to be utilised as a N source it must a) be available to the plant in the soil solution, b) have a mechanism for direct or indirect uptake system, and c) be metabolised in the plant for use (Näsholm et al., 2009). NH_4^+ and NO_3^- are actively transported into the plant cell by proton gradients that power movement across the epidermal root cells through specific transporters (Miller and Cramer, 2005). A similar mechanism has now been found for amino acid uptake. A proton gradient is maintained by H^+-ATPase that moves protons out of the cell membrane allowing the movement of amino acid into the cell by H^+-cotransport (Jones et al., 1994; Miller and Cramer, 2005).

The ability for a plant to utilise both inorganic and organic N sources has formed a new paradigm for N cycling in the soil (Fig. 2.2b) whereby N uptake is dependent on substrate availability and plant N demand. NH_4^+ is energetically more favourable, but it can be toxic to plants in high concentrations and thus cannot be used as the sole source of N by a plant. Martins-Loução and Cruz (1999) found that plant growth was inhibited by 55% when nutrition was solely provided by ammonium compared to nitrate

on equi-molar concentrations. NO_3^- is very mobile in soil and even soils with a high cation exchange capacity can leach NO_3^- leading to lower concentrations in the soil solution. Access to both organic and inorganic N sources improves the potential of plants to achieve their N demand and promotes growth (Fig. 2.2b).

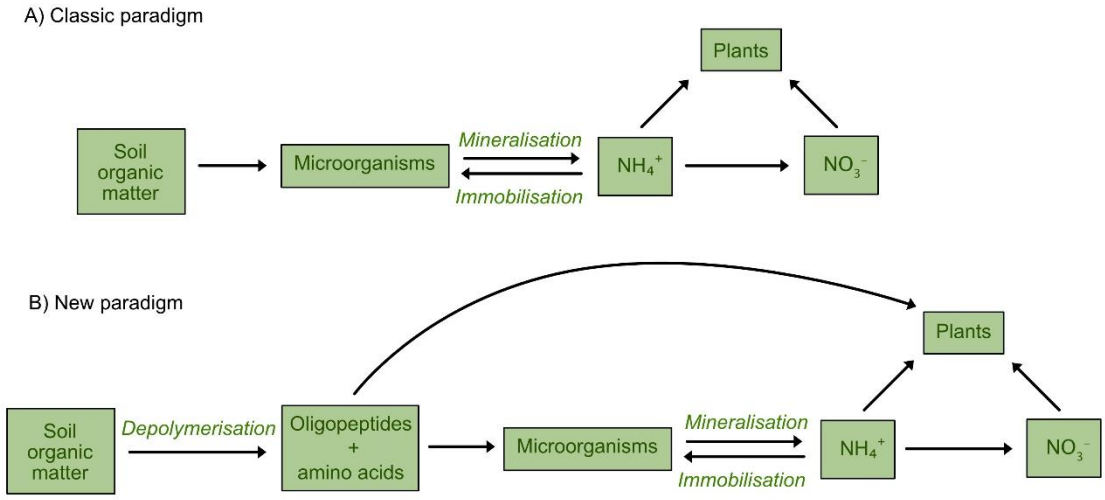

Figure 2.2. The changing paradigm of the soil N cycle. (A) The dominant paradigm of N cycling up through the middle 1990s. (B) The paradigm as it developed in the late 1990s. Adapted from Schimel and Bennett (2004).

It is accepted that plant roots can adsorb amino acids (Jones and Darrah, 1994; Wright, 1962), but whether plants utilise this transport pathway for N nutrition or can compete with microorganisms for organic N is unclear. For example, in Arctic ecosystems, annual plant N demand is 2-6 times larger than the annual pool of inorganic N (Kielland, 2001). Yet, inorganic N nutrition by plants remains the focus of understanding because of the extensive use of inorganic N fertilisers and role of inorganic N in agricultural systems (Näsholm et al., 2009).

Proteins have also been found to be available for N nutrition by plants. For example, Paungfoo-Lonhienne et al. (2008) studied two non-mycorrhiza plants, the heathland plant *Hakea acites* and the brassica ruderal *Arabidopsis thaliana*. Seedlings were grown in a sterile culture of 1) no added nitrogen, 2) with protein (BSA), 3) with inorganic nitrogen, and 4) with both N sources in the same quantities for 12 weeks. Protease activity was determined by SDS-PAGE and fluorescence imaging: protease hydrolysis of BSA releases protein fragments with fluorophores that are dequenched.

Therefore, intact protein uptake can be determined by the presence of quenched protein. The results showed that protein could be acquired intact, likely by endocytosis, but this was limited to those roots with hairs. The presence of intact protein in the cell suggests it was not assimilated rapidly. One possible reason for specificity of intact protein uptake to root hairs could be related to areas which are active in mineral N acquisition or to the recovery of exuded amino acids (Gilroy and Jones, 2000; Jones and Darrah, 1994). In cultures of exclusively protein, plant growth was far less than with inorganic N addition as well. This suggests that there is a limitation on protease production when inorganic N is limiting. Proteases are proteins and, therefore, their production requires N. Thus, when the plant is grown under inorganic N-limited conditions the plant cannot afford to use vital N resources for protease production.

Plant N limitation causes decreased root growth, lateral root initiation suppression, increase in plant C:N ratio, photosynthesis reduction and early leaf senescence (Kant et al., 2011 and references therein). Due to this many mechanistic studies with N limiting treatments use seedlings or young plants to avoid the effect of N limitation masking mechanistic processes in N dynamics in plant roots (e.g. Paungfoo-Lonhienne et al., 2008; Godlewksi and Adamczyk, 2007).

Agricultural systems are considered to have higher N mineralisation rates compared to natural ecosystems. This is likely due to plants generally being poor competitors for organic N against microorganisms in agricultural systems (Bardgett et al., 2003; Näsholm et al., 2000). Näsholm et al. (2000) measured glycine uptake (using ^{13}C- and ^{15}N-labelled glycine) and compared it with ^{15}N-labelled NO_3^- and NH_4^+ uptake in four agricultural grass species that were transferred to pots after one year in the field. Labelling plants with ^{13}C- and ^{15}N-glycine allowed them to determine if amino acids were acquired by the plants intact or mineralised. The results showed that 19-23% of glycine was taken up intact across all four pasture species. In contrast, in a boreal forest ecosystem, intact amino acid uptake was 45-61% of glycine nutrition suggesting that organic N uptake is more important in these forest systems (Näsholm et al., 1998). However, the uptake of glycine was lower than NO_3^- and NH_4^+ for the four species. The species used were ectomycorrhizal and, therefore, ectomycorrhizal fungi may have contributed to the uptake of intact amino acids. As the study offered no comparison against non-mycorrhizal associated plant species it is impossible to say whether mycorrhiza are the dominant drivers of organic N uptake. Several studies have observed organic N uptake in species grown under non-mycorrhizal conditions

(Abuzinadah and Read, 1986; Jones and Darrah, 1994; Stoelken et al., 2010). Although there is a great debate on whether intact uptake of organic N is in fact intact (Dion et al., 2018 and references therein), studies have shown variations in the flux of uptake (e.g. Näsholm et al., 2000; Bardgett et al., 2003) suggesting that uptake depends on species, N conditions in the soil and the importance of microbial competition for N.

2.3.3. Plant and microbial competition for N

Both plants and microorganisms use inorganic and organic N sources for nutrition, thus, there is competition between them for these N sources. This competition is difficult to measure because of a) the complex nature of the N cycle that generates various pathways in which plants and microorganisms can obtain N, b) the symbioses that occur between some plants and microorganisms (Hodge et al., 2000a), and c) the different timescales at which competition occurs.

Timescale is an important factor when discussing the competition for N. The short microbial lifespan means that N can by cycled many times through microbial biomass allowing plants multiple opportunities to compete for and accumulate N owing to their relative longevity (Hodge et al., 2000a; Kaye and Hart, 1997). It is generally accepted that plants utilise N that has been previously assimilated by microorganisms (Hodge et al., 2000a). Hodge et al. (2000b) carried out an isotope pool dilution experiment of dual-labelled (^{15}N and ^{13}C) lysine and ^{15}N-labelled urea. After 49 days, plants acquired 45-54% of ^{15}N compared to 7-13% by the microbial biomass. C was not enriched in the plant, but the microbial biomass was ^{13}C-enriched suggesting that microorganisms assimilated the N sources first and released excess N as inorganic N that was then utilised by plants.

On the other hand, over a single competition event plants cannot use their longevity to an advantage and, therefore; microorganisms are superior because of their high growth rates and proximity to N in the soil (Hodge et al., 2000a). Jackson et al. (1989) demonstrated this in the addition of ^{15}N-NO_3^- and ^{15}N-NH_4^+ over 24 h to a grassland soil. Microorganisms assimilated ^{15}N-NH_4^+ five times faster and ^{15}N-NO_3^- twice as fast as plants. This demonstrates that plants compete more effectively for nitrate than NH_4^+. NO_3^- is more mobile than NH_4^+ and so interception with the sparsely distributed plant roots is more likely (Schimel et al., 1989).

Plants are considered to compete ineffectively with microorganisms for organic N. The rate of amino acid diffusion in the soil is slow and combined with spatial limitation of plant roots it is more likely that microorganisms will assimilate the amino acids by mycorrhizal associations (Miller and Cramer, 2005). This was supported by Owen and Jones (2001) who found that microorganisms out competed *Lolium perenne* and *Cynsurus cristatus* for ^{14}C-labelled glutamate, glycine and lysine and mineralised 80% of total amino acids added. Plants have been shown to acquire amino acids from axenic cultures and that roots can reacquire up to 90% of amino acids previously exuded by the root (Jones and Darrah, 1993). However, within the rhizosphere, transport rates of amino acids are similar for plants and microorganisms leading to intense competition for amino-N (Jones and Hodge, 1999). The larger use of amino acids by microorganisms seen by Owen and Jones (2001) may be due to microorganisms occupying greater areas of the soil, therefore, in closer proximity to the amino acids. Plant-microbial competition for N is important to understand with agricultural systems where it is estimated that <50% of applied fertiliser is utilised by the crop (Galloway et al., 2004).

2.4. Anthropogenic perturbation of the global nitrogen cycle

The influence of human activities on Earth system functioning is profound leading to a new era the “Anthropocene” (Crutzen and Stoermer, 2000). Human modification of the N cycle has resulted in human activities converting more atmospheric N_2 into reactive forms than all terrestrial processes (Rochström et al., 2009). This has resulted in the biochemical N cycle becoming one of the original seven planetary boundaries: a concept used to “estimate a safe operating space for humans within the functioning of Earth’s systems’ processes” (Rochström et al., 2009, p. 1). The biggest anthropogenic use of global N is agriculture at ca. 85% (Galloway et al., 2008). The human manipulation of N in agriculture, along with advances in irrigation systems and crop breeding, resulted in significant increases in crop production between 1960-1990 known as the Green Revolution which saw food production to double in certain areas (Evans and Lawson, 2020).

2.4.1. Nitrogen in agricultural systems

In addition to phosphorus and potassium, N is one of the nutrients commonly applied to agricultural systems as fertiliser. The application of N fertilisers has helped

agricultural crop and forage production keep pace with population growth and the associated increased demand for food (Robertson et al., 2009). In the 19th century, N fertilisers were predominately sourced from mining potassium nitrate and guano. However, due to the increased demand of these limited resources at the beginning of the 20th century and new technological advances, sources switched to industrially synthesised fertiliser namely by the Haber-Bosch process (Vandermeer, 2011). Nowadays N fertilisers are predominantly in the form of inorganic N synthesised via the Haber-Bosch process. This process of making NH_3 for fertilisers is energy intensive with a large quantity of this energy supply coming from fossil fuels (Galloway et al., 2008; Gruber and Galloway, 2008). Large pulse additions of mineral N results in increased denitrification, NH_3 volatilisation and nitrous oxide production (N_2O) – a greenhouse gas (Snyder et al., 2009). The reactive N is responsible for many environmental problems including eutrophication of nearby water courses; ecosystem acidification; photochemical smog; greenhouse gas production e.g. N_2O and stratospheric ozone loss (Gruber and Galloway, 2008; Erisman et al., 2013).

Inorganic N fertilisers (e.g. NH_3, NH_4NO_3) and simple organic N fertilisers (e.g. urea) are generally applied at a higher rate than that which plants can utilise resulting in excess N in the agricultural system (Dungait et al., 2012). It is estimated that only half of N applied to crops is incorporated into the plant biomass (Campbell et al., 2017). This is because, despite the expense of applying such high quantities of N to fields, the high risk of yield losses from under-applying leads to many overcompensating (Miller and Cramer, 2005). As N fertilisers are made to be mobile for greater plant availability, they are highly susceptible to transformation, leaching and volatilisation leading to more variable concentrations in the soil (Miller and Cramer, 2005). Also, the addition of NH_4^+-based and urea fertilisers and growth of legumes are some of the major causes of soil acidification in agricultural land (Goulding, 2016). It occurs when NH_4^+ is nitrified to NO_3^-, which is then leached from the soil, causing the loss of H^+ ions. To combat this, lime is used to buffer the acidification by reacting with the H^+ ions to produce CO_2 and H_2O and increase the pH. However, liming has an additional economic cost on top of the initial fertiliser application as well as producing CO_2 (a greenhouse gas) (Goulding, 2016; Rice and Herman, 2012). The myriad of environmental problems as a result of the Green Revolution has led to a push for increased N fertiliser use efficiency to improve agricultural production and sustainability (Evans and Lawson, 2020; Han et al., 2020).

Regenerative agriculture combines a wide range of agricultural practices including conversation agricultural, agroforestry, restoration of soil health and soil carbon sequestration (Lal, 2020). One possible solution to the excessive use of synthetic mineral N fertilisers is organic N fertilisers. Organic N fertilisers (e.g. manure, straw, slurry and sewage sludge) are less favoured in intensive farming systems. Due to their greater complexity and higher MW, the release of inorganic N into the soil from organic N fertilisers is slower than inorganic N fertilisers which can more quickly meet crop growth demands (Gutser et al., 2005). Also by their organic nature, organic N fertilisers could increase carbon content of agricultural soils and contribute to improving soil health. Soil enzymes, such as proteases, are catalysts that increase the breakdown rate of organic N compounds, into readily available oligopeptides and amino acids. Sustainable agricultural practices promote a move to low-input, organic systems (Paterson, 2003) but this is dependent on understanding the process in which plants can utilise organic N and even accelerate turnover for increased acquisition. One such process is proteolysis.

2.5. Soil proteases

Proteases, also known as proteinases, peptidases and proteolytic enzymes, are enzymes that break down proteins, by hydrolysing peptide bonds, into oligopeptides and amino acids. Plant proteases perform four major functions: 1) enhancing availability of N for nutrition; 2) defence against plant pathogenic organisms; 3) root cell expansion; and 4) regulation of proteins and peptides in response to developmental and environmental cues (Wilkesman and Kurz, 2009). They are generally secreted into the soil solution by microorganisms, plants and animals (Vranová et al., 2013). However, whether protease release is a passive or active process in plants remains unclear. The cleavage of peptide bonds can occur at different sites along a protein, which categorises proteases into four classes determined by their active site: cysteine, serine, aspartic and metalloproteases (Wilkesman and Kurz, 2009; van der Hoorn, 2008, Table 2.1, Fig. 2.3). In aspartic and metalloproteases, a water molecule is activated by the acidic active site and acts as a nucleophile to hydrolyse the peptide bond. In cysteine and serine proteases, the active site of the enzyme acts as a nucleophile forming an intermediate covalent bond with the amine group of the peptide bond whilst the other half of the bond is broken off. A water molecule is then activated to hydrolyse the covalent bond and the second part

of the peptide bond is released (Schaller, 2004). Exopeptidases cleave C and N terminal bonds at the end of a peptide chain resulting in an amino acid and a shortened peptide chain (Wilkesman and Kurz, 2009; van der Hoorn, 2008; Vranova et al., 2013) (Fig. 2.4). Certain exopeptidases can cleave either one (aminopeptidases), two (dipeptidyl-peptidases) and/or three (tripeptidyl-peptidases) from the N terminal (Landi et al., 2011). Carboxypeptidases and peptidyl-dipeptidases cleave one and two amino acids respectively from the C-terminal (Breddam, 1986). Endopeptidases hydrolyse bonds within the peptide chain e.g. trypsin (Fig. 2.4). Some proteases are substrate-specific whereas other proteases catalyse the hydrolysis of a broad range of proteins.

Table 2.1. Classification and characteristics of proteases. Adapted from Loll and Bollag (1983).

Protease	pH optima	Molecular weight (kDa)	Metal requirement	Example
Aspartic	1-5	35	-	Pepsin
Cystine	4-8	20-50	-	Papain
Metallo-	7-8	35-45	Zn, Mg, Co, Fe, Mn, Ni	Carboxypeptidase A
Serine	9-11	26-34	-	Trypsin

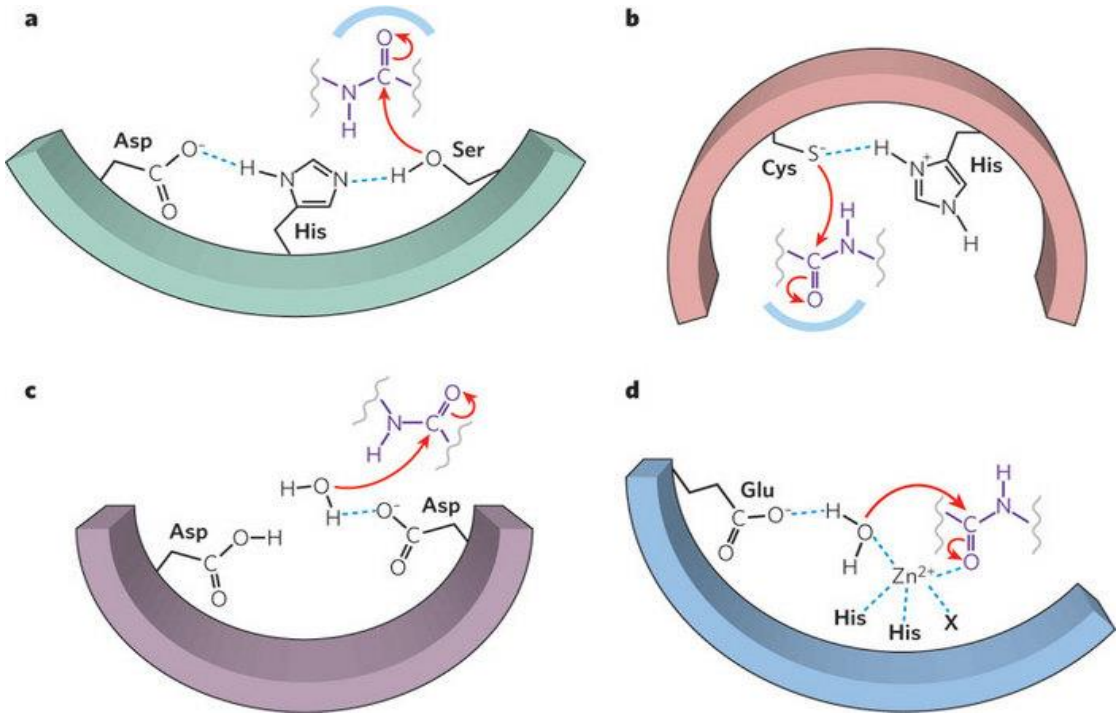

Figure 2.3. Schematic diagram of the hydrolytic cleavage of peptide bonds by a) serine proteases, b) cysteine proteases, c) aspartic proteases, and d) metalloproteases. Source: Erez et al. (2009)

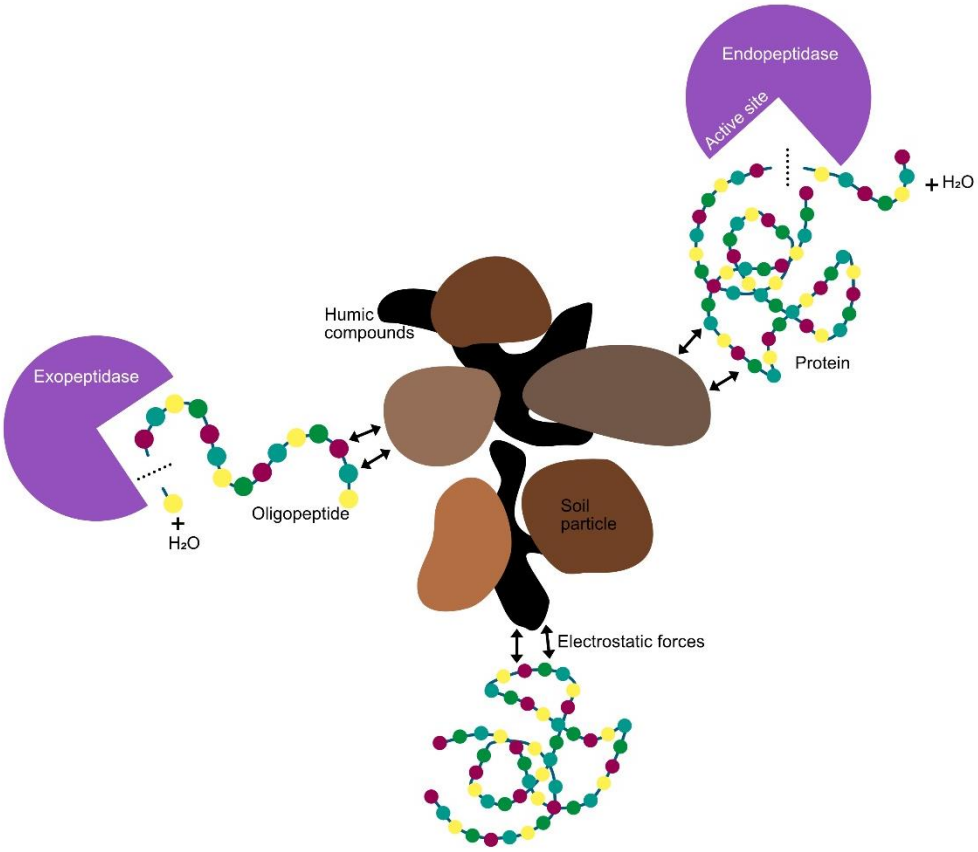

Figure 2.4. Schematic diagram of the mode of attack of different proteases on protein and peptide chains and their interaction with soil particles.

2.5.1 Protease activity in soils

Protease activity (or proteolysis) is the hydrolysis of peptide bonds by proteases. It has been found to be an important process within the soil as it plays a key role in soil N cycling (Vranova et al., 2013 and references therein).

The origin of soil protease activity is considered to be predominantly from microorganisms (Vranová et al., 2013). However, studies have not quantified the contribution of microorganisms, plants and animal faeces to total protease activity in soil. This is likely because early studies measuring extracellular protease activity from plants assumed root contribution to be negligible (e.g. Chang and Bandurski, 1964; Vágnerová and Macura, 1974). However, a more recent study by Godlewski and Adamczyk (2007) reported 15 different agricultural and wild plant species to secrete proteases into soil solution. The differences in results could be due to the different methods used to measure protease activity and plant growth conditions (German et al., 2011). Whether plants themselves exude root proteases as a mechanism for N

nutrition is unclear. Eick and Stöhr (2009) found no change in membrane-bound protease activity under N deficient conditions, whilst Adamczyk et al. (2009) suggested that protease activity could increase free amino acids in the soils. Again, differing results could be attributable to methodological bias. I am to contribute to this knowledge gap by determining whether plants use root-derived proteases as a mechanism for N nutrition in Chapter 5 and whether protein addition increases rhizosphere protease activity in Chapter 6.

Protease activity is spatially heterogeneous and interacts with various soil components. The main locations of protease activity are: 1) in microbial, plant and animal cells associated with metabolism; 2) attached to the outer surfaces of cells; 3) in the soil solution after exudation from living cells; 4) in proliferating cells e.g. fungal spores and plant seeds; 5) leaked from lysed cells that then become part of the soil solution but perform a function inside living cells; 6) adsorption, co-polymerisation or entrapment with humic compounds; 7) adsorbed onto clay minerals or within lattice of 2:1 layer silicates but may prevent proteolytic activity; and 8) soluble or insoluble enzyme substrate complexes (Burns, 1982). Studies have found that protease activity is highest at the root-soil interface correlated with microbial biomass specifically at actively growing root tips compared to mature roots (Badalucco et al., 1996; DeAngelis et al., 2008). As a protein, proteases in the soil solution tend to be metabolised quickly, but proteases can also easily bind to soil surfaces e.g. organic materials and clay minerals (Vranová et al., 2013). Marx et al. (2005) found leucine aminopeptidase activity was highest in the clay fraction of a sandy loam which correlated with the highest concentration of leucine. It is suggested that this is because clay fractions concentrate proteins on their surface increasing rates of hydrolysis (Estermann and McLaren, 1961). Fuka et al. (2008) also found highest protease activity in soil with the highest clay fraction and lowest in sandy soils that was further correlated with amino acid concentration. However, other studies suggest that the binding of enzymes and humic substances inhibits protease activity (Loll and Bollag, 1983 and references therein). The numerous locations of proteases complicates the determination of the lifetime of a protease and its activity, which has not yet been quantified.

2.5.2. Regulation of soil protease activity

In this section, I outline the factors that regulate soil protease activity. A summary of this is represented schematically in Figure 2.4.

Nutrient availability & SOM

Enzyme production requires both N and energy and, therefore, production occurs at the expense of growth and metabolism (Allison and Vitousek, 2005). Proposed mechanisms that would promote protease synthesis and secretion are high quantities of substrates and low quantities of nutrients e.g. C, N, P and S. Repression could be as a result of high quantities of easily metabolised nutrients e.g. NH_4^+ and NO_3^- (Geisseler and Horwath, 2008; Kalisz, 1988). Allison and Vitousek (2005) found that NH_4^+ suppressed glycine aminopeptidase over a 28-day incubation and protein addition increased protease activity. Similarly, Geisseler and Horwath (2008) found that NH_4^+ did not affect protease activity up to two weeks after addition yet casein increased activity within the first 5 days. An increase in soil C:N ratio and microbial biomass was positively correlated with protease activity. Nannipieri et al. (1983) also found that a high C:N ratio stimulated protease activity following addition of a C source (glucose or ryegrass residues) and NO_3^-, whereas NO_3^- alone did not alter activity. Ryegrass induced protease activity was ca. twice as high as glucose suggesting that the composition of SOM (e.g. C:N ratio, complexity and protein content) influences activity. The increase in activity was rapid for 5 days then lowered. By excluding plants from the experiments, the influence of plant inputs (e.g. root exudates) on protease activity is not measured. An increase in activity would be expected in planted soils due to a higher microbial biomass in the rhizosphere. However, Jan et al. (2009) measured substrate addition in both planted and unplanted soil but observed no difference in the response of protease activity to substrate addition. This could be due to the microbial community of the unplanted soil, collected from a site that was fallow for 3 years, adapted specifically to the substrate availability at this site. Also, it could be due to the availability of the substrate to the exoenzyme i.e. unavailable due to substrate sorption to soil particles (Dungait et al., 2012). This highlights the potential influence of nutrient availability on protease activity which varies between studies and could be due to other factors exercising a greater regulating effect.

Soil pH

Most studies of proteases in the soil have found that maximum protease activity occurred at neutral or alkaline conditions. Kamimura and Hayano (2000) studied acidic (pH 3.4) tea fields in Japan and found a peak of activity at pH 5.5 and a higher peak at pH 9.5 found to be from two different proteases by 2-D zymography. The acidic

proteases were characterised as serine carboxypeptidase, alkaline metalloproteases and metallocarboxypeptidase meaning they had different substrate preferences. Puissant et al. (2019) observed the pH optima for proteases to differ between soil types, suggesting that microbial communities were adapted to their soil environment. Therefore, soil pH is a crucial factor in determining protease activity.

Texture

It has long been established that proteins adsorb onto clay mineral surfaces (Frankenberger and Johanson, 1982; Quiquampoix, 2008; Quiquampoix et al., 1995). However, the specific mechanisms and conditions under which proteins do this are complex and poorly understood. The interaction between soil pH and the isoelectric point (IEP) of a protein determines whether a protein is 1) unfolded on the clay surface; 2) adsorbed with little modification of the protein structure; and 3) in the soil solution. It is generally assumed that the first results in inhibition of enzyme activity when the protein in question is an enzyme (Quiquampoix et al., 1993). However, with potentially thousands of different proteins occurring in a single gram of soil, determining the status of each protein is currently impossible (Nannipieri and Smalla, 2006). A study of differing clay contents on different enzyme activities including protease, showed protease activity to double in 50% clay soil compared to no clay (Wei et al., 2014). This suggests that proteases are not inhibited in the presence of clay, but whether these proteases are adsorbed onto the clay surfaces or are in the soil solution is unclear. Further, as the study used an artificial soil mixture with the same amount of OM from the litter of a forest, it is uncertain how the interaction between OM and clay would affect protease activity.

Microbial community & biomass

Generally, microbial community determines the type of soil extracellular proteases and microbial biomass the quantity of proteases released into the soil solution (Hayano, 1993; Nannipieri et al., 1983). Schnecker et al. (2014) found a positive association between microbial community and biomass and protease activity in Arctic soils. Kandeler et al. (1994) measured a similar positive association between microbial biomass and protease activity across tea orchard soils. Both studies showed that site was a common factor for differing microbial communities and biomass. The variation in microbial community across sites is likely linked with environmental factors

and, thus, microbial communities are adapted to their own conditions (e.g. labile nutrient inputs and soil pH; Koranda et al., 2011; Puissant et al., 2019).

Water content

Water availability is another factor shown to affect protease activity. Sardans and Peñuelas (2005) observed protease activity to reduce by 35-45% in Inceptisols after a 3-year water runoff and rainfall exclusion. Protease activity was measured in both spring and autumn, where spring had higher temperatures, moisture and organic matter contents that linked with the higher protease activity for more optimal conditions. In contrast, Geisseler et al. (2010) found that protease activity was not affected by soil water potential. Yet, when additional substrate was added to the dry soils, the highest protease activity was similar to higher water potentials treatments, but microbial biomass was reduced. This could be due to increased access for proteases to soil particles for binding. The effect of water content on protease activity could, therefore, be a result of an indirect process e.g. by altering substrate availability and microbial community or activity. However, it is unclear which mechanisms or combination of mechanisms is at play and the effect could be highly site-specific.

Temperature

The influence of temperature on protease activity was investigated by Brzostek and Finzi (2012) in forest sites in the USA of *Acer saccarum*, *Fagus grandifolia*, *Tsuga canadenis* and *Fraxinus americana* all with mycorrhizal associations. Inceptisol soil samples (0-15 cm) were collected in the vicinity of each tree and incubated at six temperatures from 4-35°C. Maximum protease activity was between 25-35°C in soil associated with each tree species with a rough linear increase with increasing temperature. However, warmer temperatures combined with more substrate resulted in a further increase of protease activity. Protease activity was more sensitive to substrate increase alone than temperature increase. On an annual scale temperature is influential on activity because of seasonal-temporal fluctuations, but also for long term climate predictions of temperature increase. Noll et al. (2019) also measured a positive effect of temperature on arable, pasture and forest soil with protease activity almost three times higher at 25°C compared to 5°C giving a temperature coefficient (Q_{10}) value of 1.5 averaged across the land uses (lowest in forest). Both of these studies measured potential protease activity because they collected samples at

specific sites and then manipulated the temperature. Puissant et al. (2015) collected samples seasonally and measured potential protease activity at 28°C. Maximum protease activity was measured in autumn > winter > spring > summer yet mean soil temperatures were highest in the opposite order. Therefore, soils collected during autumn, assuming a Q_{10} of 2.0 (average Q_{10} in majority of studies reviewed by von Lützow and Kögel-Knabner, 2009), would have a soil protease activity six times higher at 28°C than measured at native soil temperatures. Whilst in summer, protease activity would only be doubled when measured at 28°C compared to native soil temperature resulting in similar activities to autumn. The problem with standardising assay temperatures at higher than native soil temperatures is that it would result in unrealistic optimal conditions and represent a larger proportion of the total enzyme pool. Thus, there is an important debate over whether to use field versus optimised conditions when measuring the effect of environmental conditions on protease activity.

Ambient CO_2 concentration

Atmospheric CO_2 concentrations are steadily increasing as observed by numerous long-term measuring stations (Friedlingstein et al., 2019). CO_2 is essential for plant photosynthesis and growth and thus is important for ecosystem productivity. Kandeler et al. (2006) studied the effect of ambient CO_2 (360 µmol L^{-1}) to elevated CO_2 (720 µmol L^{-1}) on Aridosol grassland with *Bouteloua gracilis*, *Pascopyrum smithii* and *Stipa comate* dominant species in chambered plots over two years. At the soil surface (0-5 cm depth) protease activity was higher under elevated than ambient CO_2, but the difference decreased with depth with no differences by 10-20 cm. The N-limited grassland is likely to have responded to elevated CO_2 levels with increased growth leading to higher N demand that is served by increased protease activity to mineralise N. Li et al. (2010) conducted a similar experiment on oak seedlings and observed increased protease activity under elevated CO_2. Again, interactions between environmental and edaphic factors appear to be crucial in regulating protease activity.

Land use

Land use has an indirect influence on soil protease activity by affecting the regulating factors discussed above. In this thesis, I focus on agricultural plant species and use farming as a specific example of how land use can affect soil protease activity. A comparison between organic and chemical fertilisers on protease activity was

conducted by Sakurai et al. (2007). Both fertilisers contained the same quantity of N, P and K applied onto an Andosol sown with lettuce for 56 days. Protease activity in the rhizosphere was highest for the organic fertiliser treatment for all time points except one. Despite organic fertiliser treatment resulting in significantly higher protease activity than chemical fertiliser, inorganic N concentrations were the same. This indicates that the flux of N from the organic N fertilisers is greater than from SOM. Phylogenetic analysis of the soil microbiota determined *Pseudomonas fluorescens* to be the dominate proteolytic bacteria secreting alkaline metalloprotease. *Bacillus megatium* was also common, secreting neutral metalloprotease. Interestingly, there was a correlation between the type of proteolytic bacteria and protease activity suggesting that proteolysis is dependent on the bacterial community. Only alkaline and neutral metalloprotease genes were determined because of the evidence that these are the main proteases for soil bacteria. However, proteases originating from bacteria could be of a different class to plants (Godlewski and Adamczyk, 2007), therefore, other proteases should not be ruled out. The effect of reduced inorganic N fertiliser was also seen in a field study comparing conventional and precision farming practices by Schloter et al. (2003). Under precision farming (lower rate of inorganic N fertiliser) protease activity was higher. The effect of high C:N ratio compounds on increased protease activity was observed in a laboratory study of field Chernozem with biofuel by-product amendment (Alotaibi and Schoenau, 2011). These studies suggest that farming practices are vital in increasing protease activity in the soils.

Interaction of regulating factors

Few studies have looked at multiple edaphic and environmental factors in the same study. Noll et al. (2019) measured protease activity across pasture, forest and arable soils. Edaphic factors measured included microbial abundance, nutrient availability (both inorganic and organic N), pH, cation exchange capacity (CEC), soil temperature and water content to be associated with protease activity. Ma et al. (2020) measured an increase in soil protease activity of a meadow under N addition, but only when precipitation increased by 15%. A suggested mechanism was that increased precipitation stimulated microbial growth and thus the production of protease enzymes. It is likely that edaphic and environmental factors have complex interactions with the mechanisms that regulate protease activity. The most limiting factor could be site-specific and, consequently, the mechanism controlling protease activity could alter

between sites as well. This knowledge gap leads to my aim for Chapter 4 to determine how key regulators described above may affect protein mineralisation rates and, thus, the limiting factors on the soil N cycle.

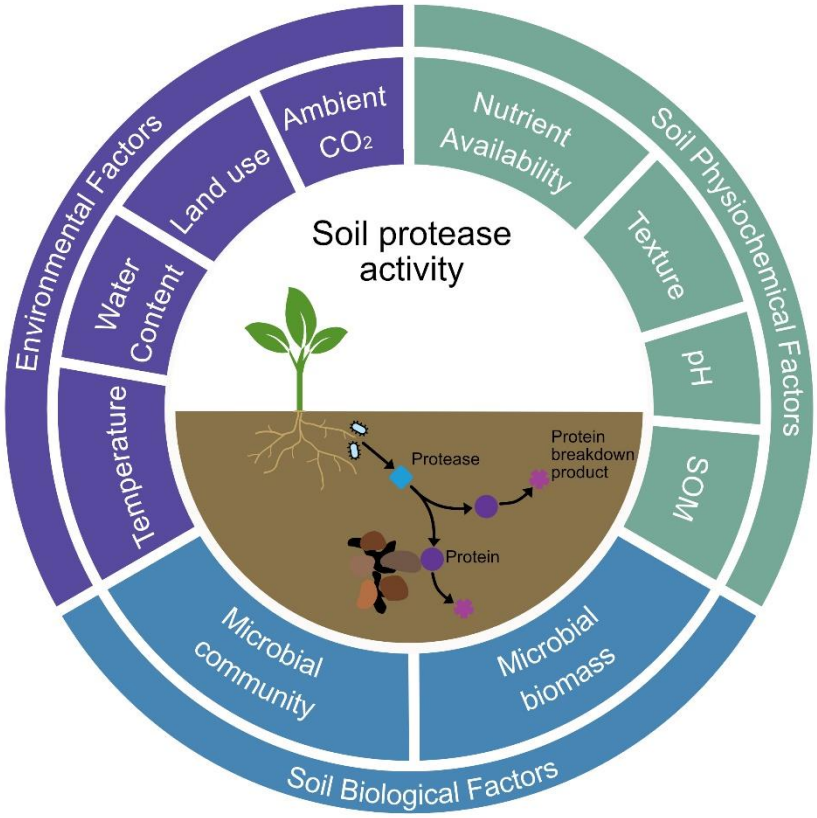

Figure 2.5. Schematic diagram of the factors that regulate soil protease activity

2.6. Protein mineralisation methods

2.6.1. Protein extraction

To characterise proteins in soil they firstly need to be separated from the soil matrix. The complex nature of soil means this is not a straightforward process. Protein distribution in soil is highly variable and separating intracellular and extracellular proteins is difficult, making the extraction of proteins from the soil matrix unreliable. Extracellular proteins are adsorbed onto the surface of clay minerals and humic substances. Intracellular proteins are found in microorganisms and plants; thus cell walls have to be broken in order to release and extract these proteins (Nannipieri and Smalla, 2006). A variety of extractants have been used to perform these tasks, each claiming to extract proteins from soil, but each with advantages and disadvantages (Bastida et al., 2009).

Bremner and Lees (1949) produced the first comprehensive comparison of protein extractants. The amount of total soil N recovered from four soil types was determined by the micro-Kjeldahl method for twenty extractants of inorganic and organic salts with a sodium cation compared with NaOH and Na_2CO_3. Out of twenty, pyrophosphate ($Na_4P_2O_7$) extracted the most N along with citrate and oxalate but these were not analysed further because they are not as preferable as pyrophosphate due to the release of potentially interfering compounds. On average, pyrophosphate extracted 9% of organic N, however, it is unknown how much this represented protein-N. For each of the further experiments on pyrophosphate (variables: pH; extraction time, temperature and shaking; ball milling; and acid leaching) the extractant was compared with NaOH which extracted greater amounts of N in all experiments. However, NaOH denatures proteins due to its high alkalinity making it unsuitable for analyses that require intact proteins. The variability in the percentage of soil N extracted could be observed between soils (a range of 9.3-22% of total soil N for pyrophosphate), yet, only the first experiment comparing all twenty extractants used all four soil types. Therefore, the observed variety in extraction of N for different soil types could have been affected by pH, extraction time etc. but this was not comprehensively tested as only two soils were assessed.

A comprehensive comparison of extractants was carried out by Criquet et al. (2002) which tested five buffers (succine, *bis*-TRIS, Na-phosphate, Na-pyrophosphate and Na-Citrate) and four salt solutions ($CaCl_2$, KCl, NaCl and Na_2SO_4) at pH 6.0. The Bradford assay (Bradford, 1976) was used to determine protein concentrations of evergreen oak litter extracted solution. However, as no tracer protein was added extractant efficiency could not be determined. Many of the extractant solutions produced a yellow-brown colour with Na-pyrophosphate and the darkest colour produced by the Na-citrate buffer, however, this was not quantitatively analysed. The presence of these colours suggest humic substances were also extracted from the soil that lead to interference of the colorimetric measurement of the proteins (Roberts and Jones, 2008). The study concluded that 0.1 M $CaCl_2$ was the only extractant to recover protein without interfering substances. The use of litter rather than soil in the experiment removes the complexities of protein adsorption to soil particles. Therefore, the results from Criquet et al. (2002) cannot directly inform extractant choice for protein extraction from soil.

From the studies that have attempted to compare protein extractants, it is apparent that a 'good' extractant requires the following properties 1) no to minimal co-extraction of interfering substances; 2) no denaturing of proteins (only applicable to analysis that require intact proteins e.g. SDS-PAGE); and 3) to work across multiple soil types (Bastida et al., 2009). Any internationally agreed standard procedure for protein extraction would need to account for these properties as well as standardising concentration of the extractant, extraction time, pH and shaking procedures. I aim to contribute to standardised procedures in protein extraction by determining the extraction efficiency of common chemicals used to recover soluble proteins from different soil types in Chapter 3.

2.6.2. Protein assays

Protein assays provide a method of quantifying protein extracted from soils. One of the most popular methods for determining protein concentration in soil is the Bradford assay (Bradford, 1976) which was developed using Coomassie Brilliant Blue dye (triphenylmethane dye) based on the binding to protein that shifts maximum adsorption of the dye from 465 (red) to 595 (blue) nm. The reagent does not form a complex with compounds that have a molecular weight below 3000 Da (i.e. peptides). This makes the assay sensitive to study protein concentrations and in protease assays (Sapan et al., 1999). Buroker-Kilgore and Wang (1993) used this assay to determine protease activity by adding Coomassie Brilliant Blue G-250 to the incubated mixture of casein and enzyme. The reagent only coloured casein and not derivatives, therefore, the difference in quantity of the substrate before and after can be determined. However, there is variability in the sensitivity to different proteins due to particular chemical groups binding with the dye (Sapan et al., 1999).

Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) is an electrophoresis method that allows proteins to be characterised by mass. Proteins are extracted in an appropriate buffer and heated to denature them then loaded onto the polyacrylamide gel. The gel acts like a sieve separating the proteins by mass with travel induced by a voltage applied to the gel which causes the negatively charged proteins to migrate to the positive anode. Sample proteins can be compared to proteins of known mass loaded onto the gel. Coomassie Brilliant Blue (similar to Bradford Reagent) is used to stain the gel to reveal locations of proteins. Alternatively, fluorescent substrates can be used and detected under UV light (Wilkesman and Kurz,

2009). This can be combined with matrix-assisted laser desorption ionisation time of flight (MALDI-TOF) mass spectrometry for identification (Wilkesman and Kurz, 2009). This method is time-consuming; however, it does offer characterisation of proteins on top of quantification.

Another method is acid hydrolysis of proteins to amino acids and their subsequent detection by fluorescence. Briefly, proteins are hydrolysed with a strong acid (e.g. 6 M HCl) under N_2 for 24 h at a high temperature (around 110°C). The solution is then neutralised by a strong alkaline (e.g. NaOH) and the amount of amino acids present quantified using OPAME reagent - a fluorophore (Roberts and Jones, 2008). This method does not guarantee the complete hydrolysis of soil protein and, therefore, is not 100% accurate at determining protein concentration. Also, like the methods outlined above, the reliability of protein hydrolysis in quantifying proteins is reduced due to interference of high MW compounds like humics (Roberts and Jones, 2008). However, Roberts and Jones (2008) compared common protein assays and concluded that acid hydrolysis is the best method for quantifying proteins as it is the most accurate and reliable.

2.6.3. Protease assays

Protease activity is generally measured as a potential, rather than *in situ* measurement, using substrate hydrolysis to determine the activity. There are several limitations of the methods used 1) the inability to distinguish between extracellular enzymes and those of living cells; 2) the difficulty in determining in precise locations of activity and responsible components for overall activity; 3) controlled conditions (pH, temperature, incubation time, buffer solutions); and 4) use of few substrates that are either non-specific (e.g. casein) or specific (e.g. leucine 7-amino-4-methlycoumarin) (Vranová et al., 2013). A study by Vranová et al. (2009) compared optimal conditions for protease activity (0.05 M Tris-HCl buffer at pH 8.6, 50°C, 2 h) with field protease activity conditions (average soil temperature and pH over 4 days at 5 cm depth, 72 h) in Luvisol from a mountain meadow in Czech Republic. Field conditions decreased protease activity by >98% compared to optimum conditions. Thus, it is important to consider whether to measure field or optimised conditions and that comparisons between the two measurements are not readily comparable.

Methods used to assay soil protease activity can be split into two main categories: fluorometric and colorimetric analysis. Fluorometric analysis uses

fluorophore e.g. 7-amino-4-methylcoumarin bonded to an amino acid or oligopeptide. Soil proteases then break this bond allowing the fluorochrome to emit UV light in one wavelength upon excitation by a different wavelength using a fluorometer. The enzyme activity is then quantified by measuring known quantities of the fluorophore (Bell et al., 2013). Some colorimetric analyses use chromophores e.g. p-nitroaniline, with a similar mechanism to the fluorophores, but instead the molecule changes colour and can be detected with a spectrometer. Other colorimetric analyses use chromophores that react with the breakdown products of the substrates added e.g. Folin-Ciocalteu (typically used with casein) and ninhydrin reagents (typically used with BAA). Fluorometric assays are more sensitive than their colorimetric counterparts with limit of detection around 50 pmol of substrate, however, they suffer from interference which must be controlled (Deng et al., 2013). Assays can be carried out using 'bench top' or 'microplate' based protocols with the latter allowing for larger volume of samples to be processed but incurring more measurement error e.g. pipetting (Bell et al., 2013; Deng et al., 2013). Despite key differences in protocols between colorimetric and fluorimetric techniques, both Deng et al. (2013) and Dick et al. (2018) found significant correlation between fluorimetric 4-methylumbelliferone substrates and colorimetric *p*-nitroaniline substrates. Yet, studies recommend the use of fluorimetric microplate assay because of its higher sensitivity as long as standardised protocols are used (Deng et al., 2013; Nannipieri et al., 2017). A summary of the advantages and disadvantages of both techniques is presented in Table 2.2. I aim to synthesise the common methods used to measure soil protease activity and whether a standardised protocol is adopted in Chapter 7.

Table 2.2. Summary of key advantages and disadvantages for fluorimetric and colorimetric protein assays.

Technique	**Advantages**	**Disadvantages**
Fluorimetric	Microplate assay means a high throughput	High operational error due to microliter volumes used
	Measure several enzymes on a single microplate	More controls needed for a standard curve for each soil sample to account for quenching
	Highly sensitive (>50 pmol MUF detected in one well).	Substrates more expensive
Colorimetric	Substrates more cost-effective	Interference of substances e.g. humics
	Soil solution created with assay chemicals	Methods measuring the release of NH_4^+ could also measure microbial mineralisation of NH_4^+

Zymography is an electrophoretic method for measuring protease activity generally using sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE). The gel separates proteins by their isoelectric point to give a determination of the diversity of enzymes with a specific activity (Wilkesman and Kurz, 2009). The use of zymography has increased exponentially since 1960s with over 12,500 published studies between 1960 and 2009 (Wilkesman and Kurz, 2009). *In situ* soil zymography (ISZ) is the most recent development in zymography methods for the 2-D quantification of enzyme activities within the soil. The method works by incubating a specimen, in which the desired enzyme is located, on a gel. The gel contains the enzyme's substrate that is subsequently stained allowing colorimetric visualisation of enzyme breakdown of the substrate (Spohn et al., 2013; Wilkesman and Kurz, 2009) (Fig. 2.5). ISZ was used by Spohn et al. (2013) to map the spatial distribution of protease and amylase within the rhizosphere. *Lupinus polyphyllus* was grown for four weeks in Rhizoboxes that were then sliced in half and a gel placed on the surface. Coomassie Brilliant Blue was used to stain proteins and Lugol's iodine was used to stain starch. The enzyme activity was followed by decolouration to remove the stains from areas where the substrates had been broken down by enzyme activity. Images were taken and the grey colour scale used to convert colour into enzyme activity (difference between initial substrate concentration and concentration after incubation

divided by incubation time). When compared with standard methods of enzyme assays, the enzyme activities recorded by ISZ were in the same range. The zymogram showed high levels of enzyme activity were associated with roots. The study outlined a major benefit of using the technique compared to standard assays; it does not require destruction of the soil structure for a slurry preventing overestimation by solubilisation of complexed proteins. However, the conditions used in the zymography (incubated at 22°C with specific substrate concentration and pH) are likely different from those naturally occurring in soils.

Most studies have assessed protease activity of bacterial origin or have not determined the difference between microbial and plant proteolysis. Methods that have been used either measure the decrease of initial substrate or the increase in peptide and amino acid products by detecting the MW or by measuring fluorophores or chromophores (Landi et al., 2011). While the accepted international unit for enzyme activity is U (µmol min^{-1}), very few studies have actually adopted it. This may be due to the difficulty to ascertain the substrate used to determine activity by using U and it not being applicable for studies that measured activity over 24 hours or more. Despite many studies urging a standardised protocol to be adopted, there is no standardised procedure for the pH, incubation time, buffer solution, extraction shaking rates and temperature to be used or a standard unit for protease activity (Burns et al., 2013; DeForest, 2009; Nannipieri et al., 2012).

Another indirect measurement of protease activity is measuring protein mineralisation by isotope labelling studies (detailed review in Section 2.6.2). Briefly, this method involves added a labelled tracer (e.g. protein or peptides) and measuring how much is produced as different end-products (e.g. amino acids, NH_4^+ and CO_2). However, due to complexities of N transformations (see Section 2.1) that are regulated by microbial usage it does not provide a direct measure of protease activity *per se* (Barraclough, 1997). Typically, studies that use isotope-labelling to measure protein mineralisation also measure protease activity (e.g. Geisseler et al., 2009).

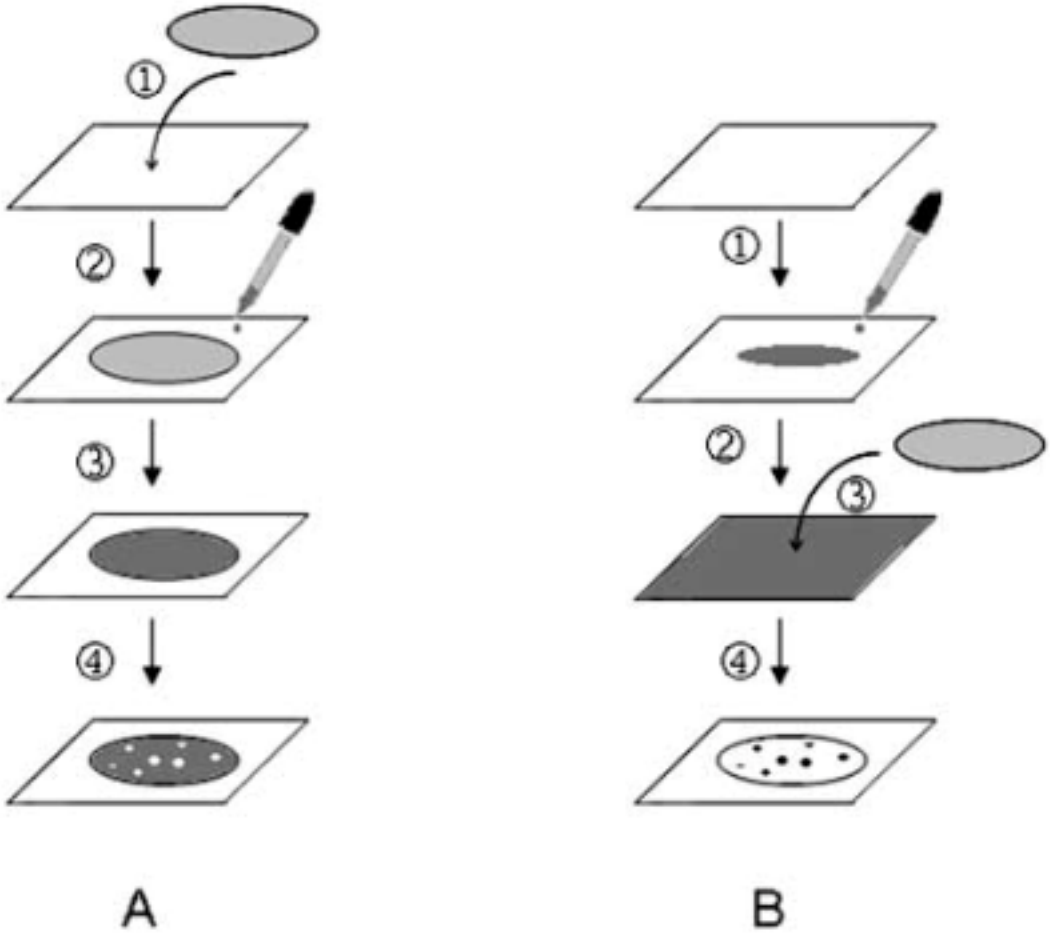

Figure 2.6. "Schematic overview of *in situ* zymography. The method may be performed using (A) a photographic emulsion, or (B) a fluorescent substrate. (A) 1. A tissue section is laid over the slide. 2. The slide is cover with photographic emulsion. 3. Slide is incubated. 4. After development and fixation, protease activity is visualized as white spots over a dark background. (B) 1. An empty slide is coated with a fluorescent labelled substrate. 2. Substrate is uniformly extended over the slide and excess is removed. 3. A tissue section is applied over the slide. 4. After incubation, protease activity is visualized as dark spots over a fluorescent background." Source: Wilkesman and Kurz (2009)

2.6.4. NanoSIMS

NanoSIMS are instruments that use high-resolution secondary ion mass spectrometry (SIMS) to enable the analysis of small-scale (nano) soil processes (Mueller et al., 2013). The NanoSIMS uses an ion source to produce a beam of ions that erode material from the sample surface which produce secondary ions that can be analysed by a mass spectrometer. Sample particles can be visualised within 50-150 nm lateral resolution (Mueller et al., 2013). This technique can be applied to the

biogeochemical processing of organic matter at the macroaggregate to single cell level. NanoSIMS have a range of applications from cosmochemistry to biology, however, their use in soil science was introduced by Hermann et al. (2007). The study showed the ability of NanoSIMS to detect the spatial location of microbial activity within a heterogeneous soil matrix. A comparison between time-of-flight SIMS and bulk sampling of $^{15}NH_4^+$ isotopic labelled soil found SIMS to detect spatial heterogeneity in N mineralisation that was not apparent in bulk samples (Cliff et al., 2007). In soil studies, NanoSIMS are often used with stable isotope labelling (typically ^{15}N) as natural abundance techniques lack the appropriate precision for accurate analysis (Mueller et al., 2013). Samples must be 1) dry, 2) stable under a high vacuum, 4) relatively flat, and 5) conductive. Whilst the detection of organic matter and microbial activity in soil samples has been shown to work well, more difficulties are faced when analysing the plant-soil-microorganism interface. Some advancement to tackle this was made by Clode et al. (2009) using 100 nm thick cross sections of ^{15}N-labelled wheat roots in a soil matrix in epoxy resin, however, this method is time-consuming and tricky. In addition, it is difficult to measure C:N ratio in SOM due to the yield of C and CN changing relative to each other during the course of analysis (Mueller et al., 2013). As plant-soil-microorganism interactions and C:N ratio are crucial measurements when trying to determine soil protein mineralisation, NanoSIMS technology is not yet at the stage for use in the application within this thesis.

2.6.5. Proteomics

Proteomics is the study of proteins to provide a comprehensive view of their structure, function and regulation in ecosystem functioning (Solaiman et al. 2007). Originally used with 2D gels (see section 2.6.2), proteomics uses mass spectrometry to identify and characterise proteins in a given sample. Before analysis, samples must be solubilised, lysed and clarified (e.g. centrifugation) with the resulting supernatant analysed. However, due to the need to extract the proteins several problems occur namely interference with humic compounds (discussed in detail in section 2.6.1). This makes the application to soils with high SOM content difficult (Kanerva et al., 2013). The proteomic approach can be further applied to protease and protease-substrates to determine the type of proteases and quality and quantity of substrates in samples using protease databases e.g. MEROPS. However, these databases are not specific to proteases and proteins in soil and thus may result in a high number of unknown

matches (López-Ottin and Overall, 2002). Overall, proteomics is a useful tool in quantifying proteins and determining their function in soil systems. However, challenges in sample preparation, specifically limiting contamination of interfering substances, need to be overcome before wide-scale application on all soil types.

2.6.6. Metagenomics

Metagenomics is the study of genetic material from environmental samples (Handelsman et al., 1998). The technique involves extracting DNA from samples and then sequencing the DNA to determine the soil microbial community and function (White et al., 2017). However, just because a gene is detected in soil does not mean that the function is expressed or that when a taxon is isolated that the gene would be expressed with other taxa present (Nannipieri et al., 2019). It also does not provide a direct link between microorganisms and biogeochemical processes but can be used as a tool to help explain soil processes. Similar to proteomics, co-extraction of interfering compouds e.g. phenols make extracting DNA difficult as well as the assumption that all microorganisms can be lysed (White et al., 2017).

2.6.7. Isotope tracing

Stable Isotopes

Since the first study by Norman and Werkman in 1943 on stable isotopes, they have been widely used to trace compounds through complex biological systems (Barraclough, 1995). Specifically, ^{13}C, $^{14}N/^{15}N$, ^{16}O and ^{18}O provide a method of studying C and N-related processes in plants and soil (Murphy et al., 2003). Broadly, the two main methodological approaches are isotope natural abundance and isotope enrichment.

Natural abundance relies on the fractionation of isotopes in a particular pool. Fractionation results from the heavier (less abundant) or usually the lighter isotope of an element being favoured in a biological reaction compared to the other causing the product of the reaction to be enriched in one more than the other. Compounds in the N cycle are transformed by biological reactions creating products that have a different isotopic fractionation to the substrate and thus, the pools of N. Coupling $\partial^{15}N$ with $\partial^{13}C$ or $\partial^{18}O$ can make measurements more accurate (Handley and Raven, 1992). Each measurement is divided by a standard, typically: $\partial^{13}C$ in the PeeDee limestone; $\partial^{15}N$ as N_2 in the atmosphere; and $\partial^{18}O$ in the Vienna Standard Mean Ocean Water (Peterson and Fry, 1987). Templer et al. (2007) used $\partial^{15}N$ to determine indicators of

N mineralisation. There was a positive correlation between $\partial^{15}N$ of SOM soil fraction and fine roots with N mineralisation and nitrification rates. This suggests that isotopic fractionation can be used to indicate the importance of different N processes. However, the determination of isotopic fractionation of each N pool requires the ability to separately measure each pool but separating N pools for measurement is difficult. Estimations of each pool can be measured using the net ∂ signature compared to the signature of the substrate added using fractionations for intermediate processes that have previously been characterised (e.g. Davidson et al., 1991). On the other hand, the use of natural isotopes avoids the high cost associated with the addition of heavy isotope-enriched compounds into the soil system.

The isotope pool dilution method is the addition of heavy isotope-enriched compounds into the soil system and then the dilution of the enriched pool is measured by the flux of unlabelled compounds into the pool. For example, Davidson et al. (1991) used the pool dilution method to measure mineralisation, by addition of $^{15}NH_4^+$, and nitrification, by addition of $^{15}NO_3^-$. A review by Murphy et al. (2003) outlined several methodological considerations that need to be taken into account when using isotope pool dilution including: 1) amount and enrichment of the stable isotope, 2) incubation period, 3) rapid consumption of $^{15}NH_4^+$, 4) injection into undisturbed soil and 5) spatial heterogeneity of soil. The use of dual-labelled compounds (e.g. ^{13}C and ^{15}N) can be used to determine the decomposition of a substrate (Jones et al., 2005). For example, Hodge et al. (2000) used dual-labelled amino acids to determine whether root uptake of N occurred as intact molecules or whether microbial decomposition of the amino acid as to a keto acid and NH_4^+ had occurred beforehand. A disadvantage of isotope pool dilution is the addition of the enriched compounds results in the addition of substrates that can affect the rate of decomposition. To avoid this fractionation effect, the total amount of enriched N added should be <50% of the pool size which the isotope is being added (Davidson et al., 1991).

Radioactive isotopes

Although a radioactive form of N is not commonly used in experimentation, dissolved organic nitrogen (DON) compounds can be labelled with radioactive isotopes (e.g. ^{14}C, ^{35}S, ^{33}P) to trace their fate in the plant-soil system. These have several advantages over stable isotopes including, that 1) experimental times can be much reduced; 2) they can be spatially imaged in samples by autoradiography and

phosphorimaging; and 3) the samples can be analysed rapidly (ca. 1 sample min^{-1}) (Table 2.2). It uses the same principles as the stable isotope pool dilution technique by the addition of radioactive-labelled compounds to a soil system, but subsequent analysis is quicker by use of liquid scintillation counting that quantifies the emission of light from the reaction of ß-particles released from the isotopes with chemiluminescent reactive organic solvents. However, radioisotopes have safety implications especially when being used in the field (Hauck and Bremner, 1976).

The most common use of radioactive isotopes in the soil system is the addition of ^{14}C-labelled compounds and measurement of the subsequent ^{14}C-CO_2 respiration to evaluate their rate of microbial processing. For example, Jan et al. (2009) measured protein mineralisation rates under different environmental conditions and directly compared this to amino acid mineralisation rates. ^{14}C-labelled protein and amino acid was added to a Cambisol from a UK grassland and the $^{14}CO_2$ respired captured in NaOH traps and measured by liquid scintillation counting. These results showed that protein mineralisation rates were much slower than for amino acids, however, the corresponding rate of N cycling was not determined. In addition, this method does not easily identify the specific processes that transform the substrates to CO_2 only the rate of the overall process. Recent advances in position-specific labelling are providing insights into this, however, the technology is still in its infancy (Apostel et al., 2013; Dippold and Kuzyakov, 2013; Moran-Zuloaga et al., 2015).

Table 2.3. Summary of key advantages and disadvantages for using radio- and stable isotope labelling to determine protein mineralisation.

Isotope	Advantages	Disadvantages
Radio	• Can be spatially imaged • Rapid analysis (ca. 1 sample min^{-1}) • Experimental times are typically shorter	• No radioactive N isotope available for use • Health & safety must be assessed
Stable	• Natural abundance studies require no isotope addition • Analysis of isotopes in liquid, solid and gas phases • Use of dual-labelled ^{13}C and ^{15}N organic compounds	• Mass spectrometers are expensive and timely to run • Care needs to be taken to avoid contamination with other compounds as different compounds have different isotope signatures

2.6.8. Protein mineralisation methods summary

To summarise, there are many methods that can be used either singularly or within a toolkit to provide comprehensive analysis on protein mineralisation in soil from rates of processes to genes that code these processes. Table 2.4 outlines the advantages and disadvantages of the methods outlined to measure protein mineralisation in soil. In this thesis, I have used enzyme assays, zymography and radioactive isotope labelling to measure protein mineralisation in soil as these methods provide efficient analysis of rates of protein mineralisation.

Table 2.4. Summary of the advantages and disadvantages of methods used to measure protein mineralisation in soil.

Method	Advantages	Disadvantages
Enzyme assays	• Determine enzyme kinetics • Determine rates	• Limited to substrates that target specific proteases
Zymography	• *In situ* sampling • Measure spatial distribution • Determine rates	• Limited to substrates that target specific proteases
NanoSIMS	• Nanoscale measurements of biogeochemical processes • Measure spatial distribution of processes	• Measurement of plant-soil-microorganisms interaction challenging • Difficult to measure C:N ratio
Proteomics	• Comprehensive analysis of protein structure, function, and regulation	• Extraction of proteins complex especially for organic soils • Costly
Metagenomics	• Determine microbial diversity and function	• No direct understanding of the link between microorganisms and biogeochemical processes • Costly
Stable isotopes	• Use in tracer studies • Determine rates • Use of dual-labelled ^{13}C and ^{15}N organic compounds	• Timely analysis • Requires knowledge of natural abundance and fractionation of pools
Radioactive isotopes	• Use in tracer studies • Rapid analysis • Determine rates	• No radioactive N isotope available for use

2.7. Conclusions

The research on protein mineralisation and protease activity in soil has been extensive but it leaves crucial gaps that need to be addressed in order to better understand the role of extracellular proteases in plant and microbial N nutrition. Investigating the gaps in soil proteases will develop a deeper understanding in how widening the bottleneck of N mineralisation created by proteases may be possible. This is particularly pertinent for agricultural systems in which the N cycle is a requisite for crop growth. A greater knowledge of protease behaviour should also aid in the design of more sustainable management systems. I have identified the following gaps which this thesis aims to contribute towards:

1) The methods used for determining protease activity in soil vary widely including the specific conditions and overall methods (i.e. colorimetric and fluorometric protease assays). The inconsistency of methods prevents comparisons between studies on top of the complexities created by the different plant-soil systems being studied. At the root of this problem is the lack of standardised methods. I aim to 1) determine efficiency of different methods for recovering protein from soil in Chapter 3, and 2) determine the variability of soil protease activity across studies and whether environmental and methodological conditions can explain the variation in Chapter 7.
2) Many studies have examined the effects of individual soil characteristics or a mixture of a few characteristics on protein mineralisation in soil (e.g. Brzostek and Finzi, 2012, Kandeler et al., 2006 and Geisseler and Horwath, 2008). However, few studies have looked at the interaction between multiple edaphic factors on protein mineralisation. I aim to investigate the soil properties that affect organic N breakdown in Chapter 4.
3) It is widely considered that microorganisms contribute the majority of extracellular soil proteases. Yet, the contribution of plant proteases to total soil proteases has not been quantified. I aim to determine whether plants use root-derived proteases as a mechanism for N nutrition in Chapter 5.
4) Root morphology plays a major role in determining the quality, quantity and distribution of exudates along the root surface. However, there has been little investigation into the effect of external protein addition (e.g. from mortality hotspots) on enzyme activity in the rhizosphere and the interaction with root morphology. I aim to determine the effect of protein addition and root morphology on protease activity in the rhizosphere in Chapter 6.

2.8. References

Abuzinadah, R.A., Read, D.J., 1986. The Role of Proteins in the Nitrogen Nutrition of Ectomycorrhizal Plants. III. Protein Utilization by Betula, Picea and Pinus in Mycorrhizal Association with *Hebeloma crustuliniforme*. New Phytologist 103, 507–514.

Abuzinadah, R.A., Read, D.J., Mary, S., 1989. The role of proteins in the nitrogen nutrition of ectomycorrhizal plants. New Phytologist 112, 55–60.

Adamczyk, B., Godlewski, M., Smolander, A., Kitunen, V., 2009. Degradation of

proteins by enzymes exuded by *Allium porrum* roots–A potentially important strategy for acquiring organic nitrogen by plants. Plant Physiology and Biochemistry 47, 919–925.

Allison, S.D., Vitousek, P.M., 2005. Responses of extracellular enzymes to simple and complex nutrient inputs. Soil Biology & Biochemistry 37, 937–944.

Alotaibi, K.D., Schoenau, J.J.., 2011. Enzymatic activity and microbial biomass in soil amended with biofuel production byproducts. Applied Soil Ecology 48, 227–235.

Apostel, C., Dippold, M., Glaser, B., Kuzyakov, Y., 2013. Biochemical pathways of amino acids in soil: Assessment by position-specific labeling and ^{13}C-PLFA analysis. Soil Biology & Biochemistry 67, 31–40.

Badalucco, L., Kuikman, P.J., Nannipleri, P., 1996. Protease and deaminase activities in wheat rhizosphere and their relation to bacterial and protozoan populations. Biology & Fertility of Soils 23, 99–104.

Barak, P., Molina, J.A.E., Hadas, A., Clapp, C.E., 1990. Mineralization of Amino Acids and Evidence of Direct Assimilation of Organic Nitrogen. Soil Science Society of America Journal 54, 769.

Bardgett, R.D., Streeter, T.C., Bol, R., 2003. Soil Microbes Compete Effectively with Plants for Organic-Nitrogen Inputs to Temperate Grasslands. Ecology 84, 1277–1287.

Barraclough, D., 1997. The direct or MIT route for nitrogen immobilization: A ^{15}N mirror image study with leucine and glycine. Soil Biology & Biochemistry 29, 101–108.

Barraclough, D., 1995. ^{15}N isotope dilution techniques to study soil nitrogen transformations and plant uptake, in: Nitrogen Economy in Tropical Soils. Springer Netherlands, Dordrecht, pp. 185–192.

Bastida, F., Moreno, J.L., Nicolás, C., Hernández, T., García, C., 2009. Soil metaproteomics: A review of an emerging environmental science. Significance, methodology and perspectives. European Journal of Soil Science 60, 845–859.

Bottomley, P.J., Myrold, D.D., 2007. Biological N Inputs, in: Eldor, P. (Ed.), Soil Microbiology, Ecology and Biochemistry. Elsevier, Oxford, UK, pp. 365–387.

Bradford, M.M., 1976. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. Analytical Biochemistry 72, 248–254.

Breddam, K., 1986. Serine carboxypeptidases. A review. Carlsberg Research

Communications 51, 83–128.
Bremner, J.M., Lees, H., 1949. Studies on soil organic matter: Part II. The extraction of organic matter from soil by neutral reagents. The Journal of Agricultural Science 39, 274–279.
Brzostek, E.R., Finzi, A.C., 2012. Seasonal variation in the temperature sensitivity of proteolytic enzyme activity in temperate forest soils. Journal of Geophysical Research: Biogeosciences 117, 1–10.
Burns, R.G., 1982. Enzyme activity in soil: Location and a possible role in microbial ecology. Soil Biology & Biochemistry 14, 423–427.
Burns, R.G., DeForest, J.L., Marxsen, J., Sinsabaugh, R.L., Stromberger, M.E., Wallenstein, M.D., Weintraub, M.N., Zoppini, A., 2013. Soil enzymes in a changing environment: Current knowledge and future directions. Soil Biology & Biochemistry 58, 216–234.
Buroker-Kilgore, M., Wang, K.K.W., 1993. A Coomassie Brilliant Blue G-250-Based Colorimetric Assay for Measuring Activity of Calpain and Other Proteases. Analytical Biochemistry 208, 387–392.
Campbell, B.M., Beare, D.J., Bennett, E.M., Hall-Spencer, J.M., Ingram, J.S., Jaramillo, F., Ortiz, R., Ramankutty, N., Sayer, J.A. and Shindell, D., 2017. Agriculture production as a major driver of the Earth system exceeding planetary boundaries. Ecology and Society, 22(4), 8.
Chang, C.W., Bandurski, R.S., 1964. Exocellular Enzymes of Corn Roots. Plant Physiology 39, 60–64.
Cliff, J.B., Bottomley, P.J., Gaspar, D.J., Myrold, D.D., 2007. Nitrogen mineralization and assimilation at millimeter scales. Soil Biology & Biochemistry 39, 823-826.
Clode, P.L., Kilburn, M.R., Jones, D.L., Stockdale, E.A., Cliff, J.B., Hermann, A.M., Murphy, D.V., 2009. In situ mapping of nutrient uptake in the rhizosphere using nanoscael secondary ion mass spectrometry. Plant Physiology, 151, 1751-1757.
Criquet, S., Farnet, A.M., Ferre, E., 2002. Protein measurement in forest litter. Biology & Fertility of Soils 35, 307–313.
Crutzen, P. J., Stoermer, E.F., 2000. Global change newsletter. Anthropocene 41, 17-18.
Dastagiri, M.B., Venkata, N.S., Suresh, R., Mude, R.N., 2020. Agriculture, economics, ecology and trade: A way forward for better world. World Food Policy wfp2.12019.

Davidson, E.A., Hart, S.C., Shanks, C.A., Firestone, M.K., Shanks C. A., Firestone, M.K., 1991. Measuring gross nitrogen mineralization, and nitrification by ^{15}N isotopic pool dilution in intact soil cores 42, 335–349.

DeAngelis, K.M., Lindow, S.E., Firestone, M.K., 2008. Bacterial quorum sensing and nitrogen cycling in rhizosphere soil. FEMS Microbiology Ecology 66, 197–207.

DeForest, J.L., 2009. The influence of time, storage temperature, and substrate age on potential soil enzyme activity in acidic forest soils using MUB-linked substrates and l-DOPA. Soil Biology & Biochemistry 41, 1180–1186.

Deng, S., Popova, I.E., Dick, L., Dick, R., 2013. Bench scale and microplate format assay of soil enzyme activities using spectroscopic and fluorometric approaches. Applied Soil Ecology 64, 84–90.

Dick, R.P., Dick, L.K., Deng, S., Li, X., Kandeler, E., Poll, C., Freeman, C., Jones, T.G., Weintraub, M.N., Esseili, K.A., Saxena, J., 2018. Cross-laboratory comparison of fluorimetric microplate and colorimetric bench-scale soil enzyme assays. Soil Biology & Biochemistry 121, 240–248.

Dion, P.-P., Jämtgård, S., Bertrand, A., Pepin, S., Dorais, M., 2018. Organic Nitrogen Uptake and Assimilation in Cucumis sativus Using Position-Specific Labeling and Compound-Specific Isotope Analysis. Frontiers in Plant Science 9, 1596.

Dippold, M.A., Kuzyakov, Y., 2013. Biogeochemical transformations of amino acids in soil assessed by position-specific labelling. Plant and Soil 373, 385–401.

Drury, C.F., Voroney, R.P., Beauchamp, E.G., 1991. Availability of NH_4^+-N to microorganisms and the soil internal N cycle. Soil Biology & Biochemistry 23, 165–169.

Dungait, J.A.J., Cardenas, L.M., Blackwell, M.S.A., Wu, L., Withers, P.J.A., Chadwick, D.R., Bol, R., Murray, P.J., Macdonald, A.J., Whitmore, A.P., Goulding, K.W.T., 2012. Advances in the understanding of nutrient dynamics and management in UK agriculture. Science of the Total Environment 434, 39–50.

Dungait, J.A.J., Hopkins, D.W., Gregory, A.S., Whitmore, A.P., 2012. Soil organic matter turnover is governed by accessibility not recalcitrance. Global Change Biology 18, 1781–1796.

Eick, M., Stöhr, C., 2009. Proteolysis at the plasma membrane of tobacco roots: Biochemical evidence and possible roles. Plant Physiology and Biochemistry 47, 1003–1008.

Erez, E., Fass, D., Bibi, E., 2009. How intramembrane proteases bury hydrolytic reactions in the membrane. Nature 459, 371–378.

Erisman, J.W., Galloway, J.N., Seitzinger, S., Bleeker, A., Dise, N.B., Petrescu, A.R., Leach, A.M. and de Vries, W., 2013. Consequences of human modification of the global nitrogen cycle. Philosophical Transactions of the Royal Society B: Biological Sciences, 368(1621), 20130116.Estermann, E.F., McLaren, A.D., 1961. Contribution of rhizoplane organisms to the total capacity of plants to utilize organic nutrients. Plant and Soil 15, 243–260.

Evans, J.R. and Lawson. T., 2020. From green to gold: agricultural revolution for food security. Journal of Experimental Botany, 71(7), 2211-2215.

Frankenberger, W.T., Johanson, J.B., 1982. Effect of pH on enzyme stability in soils. Soil Biology & Biochemistry 14, 433–437.

Friedlingstein, P., Jones, M.W., O'Sullivan, M., Andrew, R.M., Hauck, J., Peters, G.P., Peters, W., Pongratz, J., Sitch, S., Le Quéré, C., Bakker, D.C.E., Canadell, J.P., 2019. Global Carbon Budget 2019. Earth System Science Data 11, 1783–1838.

Fuka, M.M., Engel, M., Gattinger, A., Bausenwein, U., Sommer, M., Munch, J.C., Schloter, M., 2008. Factors influencing variability of proteolytic genes and activities in arable soils. Soil Biology & Biochemistry 40, 1646–1653.

Galloway, J.N., Dentener, F.J., Capone, D.G., Boyer, E.W., Howarth, R.W., Seitzinger, S.P., Asner, G.P., Cleveland, C.C., Green, P.A., Holland, E.A., Karl, D.M., Michaels, A.F., Porter, J.H., Townsend, A.R., Vörösmarty, C.J., 2004. Nitrogen cycles: Past, present, and future. Biogeochemistry 70, 153–226.

Galloway, J.N., Townsend, A.R., Erisman, J.W., Bekunda, M., Cai, Z., Freney, J.R., Martinelli, L.A., Seitzinger, S.P., Sutton, M.A., 2008. Transformation of the nitrogen cycle: recent trends, questions and potential solutions. Science 320, 889–892.

Geisseler, D., Horwath, W.R., 2008. Regulation of extracellular protease activity in soil in response to different sources and concentrations of nitrogen and carbon. Soil Biology & Biochemistry 40, 3040–3048.

Geisseler, D., Horwath, W.R., Doane, T.A., 2009. Significance of organic nitrogen uptake from plant residues by soil microorganisms as affected by carbon and nitrogen availability. Soil Biology & Biochemistry 41, 1281–1288.

Geisseler, D., Horwath, W.R., Scow, K.M., 2010. Soil moisture and plant residue

addition interact in their effect on extracellular enzyme activity. Pedobiologia 54, 71–78.

German, D.P., Weintraub, M.N., Grandy, A.S., Lauber, C.L., Rinkes, Z.L., Allison, S.D., 2011. Optimization of hydrolytic and oxidative enzyme methods for ecosystem studies. Soil Biology & Biochemistry 43, 1387–1397.

Gilroy, S., Jones, D.L., 2000. Through form to function: Root hair development and nutrient uptake. Trends in Plant Science 5, 56–60.

Godlewski, M., Adamczyk, B., 2007. The ability of plants to secrete proteases by roots. Plant Physiology and Biochemistry 45, 657–664.

Goulding, K.W.T., 2016. Soil acidification and the importance of liming agricultural soils with particular reference to the United Kingdom. Soil Use and Management, 32(3), 390-399.

Gruber, N., Galloway, J.N., 2008. An Earth-system perspective of the global nitrogen cycle. Nature 451, 293–296.

Gutser, R., Ebertseder, T., Weber, A., Schraml, M., Schmidhalter, U., 2005. Short-term and residual availability of nitrogen after long-term application of organic fertilizers on arable land. Journal of Plant Nutrition and Soil Science 168, 439–446.

Hadas, A., Sofer, M., Molina, J.A.E., Barak, P., Clapp, C.E., 1992. Assimilation of nitrogen by soil microbial population: NH_4^+ versus organic N. Soil Biology & Biochemistry 24, 137–143.

Han, X., Wu, K., Fu, X., Liu, Q., 2020. Improving coordination of plant growth and nitrogen metabolism for sustainable agriculture. ABIOTECH 1–21.

Handelsman, J., Rondon, M.R., Brady, S.F., Clardy, J., Goodman, R.M., 1998. Molecular biological access to the chemistry of unknown soil microbes: a new frontier for natural products. Chemistry & Biology 5(10), 245-249

Handley, L.L., Raven, J.A., 1992. The use of natural abundance of nitrogen isotopes in plant physiology and ecology. Plant, Cell and Environment 15, 965–985.

Hauck, R.D., Bremner, J.M., 1976. Use of Tracers For Soil And Fertilizer Nitrogen Research. Advances in Agronomy 28, 219–266.

Hayano, K., 1993. Protease activity in a paddy field soil: Origin and some properties. Soil Science and Plant Nutrition 39, 539–546.

Hedges, J.I., Hare, P.E., 1987. Amino acid adsorption by clay minerals in distilled water. Geochimica et Cosmochimica Acta 51, 255–259.

Hermann, A.M., Clode, P.L., Fletcher, I.R., Nunan, N., Stockdale, E.A., O'Donnell, A.G., Murphy, D.V., 2006. A novel method for study of the biophysical interface in soils using nano-scale secondary ion mass spectrometry. Rapid Communications in Mass Spectrometry 21(1), 29-34.

Hodge, A, Robinson, D., Fitter, A., 2000. Are microorganisms more effective than plants at competing for nitrogen? Trends in Plant Science 5, 304–308.

Hodge, A., Stewart, J., Robinson, D., Griffiths, B.S., Fitter, A.H., 2000. Competition between roots and soil micro-organisms for nutrients from nitrogen-rich patches of varying complexity. Journal of Ecology 88, 150–164.

Hopkins, D.W., Dungait, J.A.J., 2010. Soil microbiology and nutrient cycling, in: Soil Microbiology and Sustainable Crop Production. Springer Netherlands, pp. 59–80.

Jackson, L.E., Schimel, J.P., Firestone, M.K., 1989. Short-term partitioning of ammonium and nitrate between plants and microbes in an annual grassland. Soil Biology & Biochemistry 21, 409–415.

Jan, M.T., Roberts, P., Tonheim, S.K., Jones, D.L., 2009. Protein breakdown represents a major bottleneck in nitrogen cycling in grassland soils. Soil Biology & Biochemistry 41, 2272–2282.

Jones, D.L., Darrah, P.R., 1994. Amino-acid influx at the soil-root interface of *Zea mays* L. and its implications in the rhizosphere. Plant and Soil 163, 1–12.

Jones, D.L., Darrah, P.R., 1993. Influx and efflux of amino acids from *Zea mays* L. roots and their implications for N nutrition and the rhizosphere, in: Barrow, N.J. (Ed.), Plant Nutrition — from Genetic Engineering to Field Practice. Developments in Plant and Soil Sciences, Vol 54. Springer, Dordrecht, pp. 91–94.

Jones, D.L., Edwards, A.C., Donachie, K., Darrah, P.R., 1994. Role of proteinaceous amino acids released in root exudates in nutrient acquisition from the rhizosphere. Plant and Soil 158, 183–192.

Jones, D.L., Healey, J.R., Willett, V.B., Farrar, J.F., Hodge, A., 2005. Dissolved organic nitrogen uptake by plants—an important N uptake pathway? Soil Biology & Biochemistry 37, 413–423.

Jones, D.L., Hodge, A., 1999. Biodegradation kinetics and sorption reactions of three differently charged amino acids in soil and their effects on plant organic nitrogen availability. Soil Biology & Biochemistry 31, 1331–1342.

Kalisz, H.M., 1988. Microbial proteinases, in: Enzyme Studies. Springer-Verlag, Berlin/Heidelberg, pp. 1–65.

Kamimura, Y., Hayano, K., 2000. Properties of protease extracted from tea-field soil. Biology & Fertility of Soils 30, 351–355.

Kandeler, E., Eder, G., Sobotik, M., 1994. Microbial biomass, N mineralization, and the activities of various enzymes in relation to nitrate leaching and root distribution in a slurry-amended grassland. Biology & Fertility of Soils 18, 7–12.

Kandeler, E., Mosier, A.R., Morgan, J.A., Milchunas, D.G., King, J.Y., Rudolph, S., Tscherko, D., 2006. Response of soil microbial biomass and enzyme activities to the transient elevation of carbon dioxide in a semi-arid grassland. Soil Biology & Biochemistry 38, 2448–2460.

Kanerva, S., Smolander, A., Kitunen V., Ketola, R., Kotiaho, T., 2013. Comparison of extractants and applicability of MALDI-TOF-MS in the analysis of soil proteinaceous material from different types of soil. Organic Geochemistry 56, 1-9.

Kant, S., Bi, Y-M., Rothstein, S.J., 2011. Understanding plant response to nitrogen limitation for the improvement of crop nitrogen use efficiency. Journal of Experimental Botany 62(4), 1499-1509.

Kaye, J.P., Hart, S.C., 1997. Competition for nitrogen between plants and soil microorganisms. Trends in Ecology & Evolution 12, 139–143.

Kielland, K., 2001. Short-Circuiting the Nitrogen Cycle: Ecophysiological Strategies of Nitrogen Uptake in Plants from Marginal Environments, in: Ae, N., Arihara, J., Okada, K., Srinivasan, A. (Eds.), . Springer Japan, Tokyo, pp. 376–398.

Kögel-Knabner, I., 2006. Chemical Structure of Organic N and Organic P in Soil, in: Nannipieri, P., Smalla, K. (Eds.), Nucleic Acids and Proteins in Soil. Springer-Verlag, Berlin Heidelberg, pp. 23–48.

Koranda, M., Schnecker, J., Kaiser, C., Fuchslueger, L., Kitzler, B., Stange, C.F., Sessitsch, A., Zechmeister-Boltenstern, S., Richter, A., 2011. Microbial processes and community composition in the rhizosphere of European beech - The influence of plant C exudates. Soil Biology & Biochemistry 43, 551–558.

Lal, R., 2020. Regenerative agriculture for food and climate. Journal of Soil and Water Conservation, 75(5), 123-124.

Landi, L., Renella, G., Giagnoni, L., Nannipieri, P., 2011. Activities of Proteolytic Enzymes, in: Dick, R.P. (Ed.), Methods of Soil Enzymology. Soil Science

Society of America, Madison, USA, pp. 247–260.

Lehmann, J., Kleber, M., 2015. The contentious nature of soil organic matter. Nature 528, 60–68.

Lehninger, A.L. Nelson, D.L., Cox, M.M., 1993. Principles of biochemistry. 2nd edition. Worth Publishers, New York.

Li, X., Han, S., Guo, Z., Shao, D., Xin, L., 2010. Changes in soil microbial biomass carbon and enzyme activities under elevated CO_2 affect fine root decomposition processes in a Mongolian oak ecosystem. Soil Biology & Biochemistry 42, 1101–1107.

Lipson, D., Näsholm, T., 2001. The unexpected versatility of plants: organic nitrogen use and availability in terrestrial ecosystems. Oecologia 128, 305–316.

Loll, M.J., Bollag, J.-M., 1983. Protein Transformation in Soil. Advances in Agronomy 36, 351–382.

López-Ottin, C., and Overall. C.M., 2002. Protease degradomics: a new challenge for proteomics. Molecular Cell Biology 3, 509-519.

Lodish, H., Berk, A., Zipursky, S.L., 2000. Section 3.1, Hierarchical Structure of Proteins, in: Freeman, W.H. (Eds.), Molecular Cell Biology. 4th edition. New York, https://www.ncbi.nlm.nih.gov/books/NBK21581/

Ma, W., Li, J., Gao, Y., Xing, F., Sun, S., Zhang, T., Zhu, X., Chen, C., Li, Z., 2020. Responses of soil extracellular enzyme activities and microbial community properties to interaction between nitrogen addition and increased precipitation in a semi-arid grassland ecosystem. Science of the Total Environment 703, 134691.

Martins-Loução, M.A., Cruz, C., 1999. Role of nitrogen source in carbon balance, in: Srivastava, H.S., Singh, R.P. (Eds.), Nitrogen Nutrition and Plant Growth. IBH Publishing Co. Pvt. Ltd., New Delhi, pp. 1282–1286.

Marx, M.-C., Kandeler, E., Wood, M., Wermbter, N., Jarvis, S.C., 2005. Exploring the enzymatic landscape: distribution and kinetics of hydrolytic enzymes in soil particle-size fractions. Soil Biology & Biochemistry 37, 35–48.

Mengel, K., Kirby, E.A., 1978. Principles of Plant Nutrition. International Potash Institute, Bern, Switzerland.

Miller, A.J., Cramer, M.D., 2005. Root Nitrogen Acquisition and Assimilation. Plant and Soil 274, 1–36.

Moran-Zuloaga, D., Dippold, M., Glaser, B., Kuzyakov, Y., 2015. Organic nitrogen

uptake by plants: reevaluation by position-specific labeling of amino acids. Biogeochemistry 125, 359–374.

Mueller, C.W., Weber, P.K., Kilburn, M.R., Hoeschen, C., Kleber, M., Pett-Ridge, J., 2013. Chapter One – Advances in the Analysis of Biogeochemical Interfaces: NanoSIMS to Investigate Soil Microenvironments. Advances in Agronomy 121, 1-46.

Murphy, D.., Recous, S., Stockdale, E.., Fillery, I.R.., Jensen, L.., Hatch, D.., Goulding, K.W.., 2003. Gross nitrogen fluxes in soil: theory, measurement and application of ^{15}N pool dilution techniques. Advances in Agronomy 79, 69–118.

Myrold, D.D., Bottomley, P.J., 2008. Nitrogen Mineralization and Immobilization, in: Schepers, J.S., Raun, W.R. (Eds.), Nitrogen in Agricultural Systems. Soil Science Society of America, Madison, USA, pp. 157–172.

Nannipieri, P., Ascher-Jenull, J., Ceccherini, M.T., Pietramellara, G., Renella, G., Schloter, M., 2019. Beyond microbial diversity for predicting soil functions: A mini review. Pedosphere 30(1), 5-17.

Nannipieri, P., Ceccanti, B., Cervelli, S., Matarese, E., 1980. Extraction of Phosphatase, Urease, Proteases, Organic Carbon, and Nitrogen from Soil. Soil Science Society of America Journal 44, 1011–1016.

Nannipieri, P., Giagnoni, L., Renella, G., Puglisi, E., Ceccanti, B., Masciandaro, G., Fornasier, F., Moscatelli, M.C., Marinari, S., 2012. Soil enzymology: classical and molecular approaches. Biology & Fertility of Soils 48, 743–762.

Nannipieri, P., Muccini, L., Ciardi, C., 1983. Microbial biomass and enzyme activities: Production and persistence. Soil Biology & Biochemistry 15, 679–685.

Nannipieri, P., Smalla, K., 2006. Nucleic Acids and Proteins in Soil. Springer, Berlin, Germany.

Nannipieri, P., Trasar-Cepeda, C., Dick, R.P., 2017. Soil enzyme activity: a brief history and biochemistry as a basis for appropriate interpretations and meta-analysis. Biology & Fertility of Soils 54, 1–9.

Näsholm, T., Ekblad, A., Nordin, A., Giesler. R, Hogberg, M., Hogberg, P., 1998. Boreal forest plants take up organic nitrogen. Nature 392, 914–916.

Näsholm, T., Huss-Danell, K., Hogberg, P., 2000. Uptake of Organic Nitrogen in the Field by Four Agriculturally Important Plant Species. Ecology 81, 1155–1161.

Näsholm, T., Kielland, K., Ganeteg, U., 2009. Uptake of organic nitrogen by plants. New Phytologist 182, 31–48.

Neina, D., 2019. The role of soil pH in plant nutrition and soil remediation. Applied and Environmental Soil Science 2019, 5794869.

Noll, L., Zhang, S., Zheng, Q., Hu, Y., Wanek, W., 2019. Wide-spread limitation of soil organic nitrogen transformations by substrate availability and not by extracellular enzyme content. Soil Biology & Biochemistry 133, 37–49.

Owen, A.G., Jones, D.L., 2001. Competition for amino acids between wheat roots and rhizosphere microorganisms and the role of amino acids in plant N acquisition. Soil Biology & Biochemistry 33, 651–657.

Paterson, E., 2003. Importance of rhizodeposition in the coupling of plant and microbial productivity. European Journal of Soil Science 54, 741–750.

Paungfoo-Lonhienne, C., Lonhienne, T.G.A., Rentsch, D., Robinson, N., Christie, M., Webb, R.I., Gamage, H.K., Carroll, B.J., Schenk, M., Schmidt, S., 2008. Plants can use protein as a nitrogen source without assistance from other organisms. PNAS 105, 4524–4529.

Peterson, B.J., Fry, B., 1987. Stable isotopes in ecosystem studies. Annual Review of Ecology and Systematics 18, 293–320.

Powlson, D.S., 1993. Understanding the soil nitrogen cycle. Soil Use and Management 9, 86–94.

Puissant, J., Cécillon, L., Mills, R.T.E., Robroek, B.J.M., Gavazov, K., De Danieli, S., Spiegelberger, T., Buttler, A., Brun, J.J., 2015. Seasonal influence of climate manipulation on microbial community structure and function in mountain soils. Soil Biology & Biochemistry 80, 296–305.

Puissant, J., Jones, B., Goodall, T., Mang, D., Blaud, A., Gweon, H.S., Malik, A., Jones, D.L., Clark, I.M., Hirsch, P.R., Griffiths, R., 2019. The pH optimum of soil exoenzymes adapt to long term changes in soil pH. Soil Biology & Biochemistry 138, 1076012.

Quiquampoix, H., 2008. Enzymes and Proteins, Interactions with Soil-Constituent Surfaces, in: Chesworth, W. (Ed.), Encyclopedia of Soil Science. Springer, Dordrecht, The Netherlands, pp. 210–215.

Quiquampoix, H., Abadie, J., Baron, M.H., Leprince, F., Matumoto-Pintro, P.T., Ratcliffe, R.G., Staunton, S., 1995. Mechanisms and Consequences of Protein Adsorption on Soil Mineral Surfaces, in: Horbett, T.., Brash, J.L. (Eds.), Proteins at Interfaces II. Fundamentals and Applications. American Chemical Society, Washington, DC, pp. 321–333.

Quiquampoix, H., Staunton, S., Baron, M.-H., Ratcliffe, R.G., 1993. Interpretation of the pH dependence of protein adsorption on clay mineral surfaces and its relevance to the understanding of extracellular enzyme activity in soil. Colloids and Surfaces A: Physicochemical and Engineering Aspects 75, 85–93.

Read, D.J., Leake, J.R., Langdale, A.R., 1988. The nitrogen nutrition of mycorrhizal fungi and their host plants, in: Boddy, L., Marchant, R., Read, D.J. (Eds.), Nitrogen, Phosphorous and Sulphur Utilization by Fungi. Cambridge University Press, Cambridge, U.K., pp. 181–202.

Rice, K.C. and Herman, J.S., 2012. Acidification of Earth: An assessment across mechanisms and scales. Applied Geochemistry, 27(1), 1-14.

Roberts, P., Jones, D.L., 2008. Critical evaluation of methods for determining total protein in soil solution. Soil Biology & Biochemistry 40, 1485–1495.

Robertson, G.P., Groffman, P., 2007. Nitrogen transformations, in: Eldor, P. (Ed.), Soil Microbiology, Ecology and Biochemistry. Elsevier, Oxford, UK, pp. 341–362.

Robertson, G.P., Vitousek, P.M., Kellogg, W.K., 2009. Nitrogen in Agriculture: Balancing the Cost of an Essential Resource. Annual Review of Environment and Resources 34, 97–125.

Rockström, J., Steffen, W., Noone, K., Persson, Å., Chapin III, F.S., Lambin, E., Lenton, T.M., Scheffer, M., Folke, C., Schellnhuber, H.J. and Nykvist, B., 2009. Planetary boundaries: exploring the safe operating space for humanity. Ecology and Society, 14(2), 32.

Sakurai, M., Suzuki, K., Onodera, M., Shinano, T., Osaki, M., 2007. Analysis of bacterial communities in soil by PCR–DGGE targeting protease genes. Soil Biology & Biochemistry 39, 2777–2784.

Sapan, C. V., Lundblad, R.L., Price, N.C., 1999. Colorimetric protein assay techniques. Biotechnology and Applied Biochemistry 29, 99–108.

Sardans, J., Peñuelas, J., 2005. Drought decreases soil enzyme activity in a Mediterranean *Quercus ilex* L. forest. Soil Biology & Biochemistry 37, 455–461.

Schaller, A., 2004. A cut above the rest: the regulatory function of plant proteases. Planta 220, 183–197.

Schimel, J.P., Bennett, J., 2004. Nitrogen Mineralization: Challenges of a Changing Paradigm. Ecology 85, 591–602.

Schimel, J.P., Jackson, L.E., Firestone, M.K., 1989. Spatial and temporal effects on

plant-microbial competition for inorganic nitrogen in a california annual grassland. Soil Biology & Biochemistry 21, 1059–1066.

Schloter, M., Bach, H.-J., Metz, S., Sehy, U., Munch, J.C., 2003. Influence of precision farming on the microbial community structure and functions in nitrogen turnover. Agriculture, Ecosystems & Environment 98, 295–304.

Schnecker, J., Wild, B., Hofhansl, F., Eloy Alves, R.J., Bárta, J., Čapek, P., Fuchslueger, L., Gentsch, N., Gittel, A., Guggenberger, G., Hofer, A., Kienzl, S., Knoltsch, A., Lashchinskiy, N., Mikutta, R., Šantrůčková, H., Shibistova, O., Takriti, M., Urich, T., Weltin, G., Richter, A., 2014. Effects of Soil Organic Matter Properties and Microbial Community Composition on Enzyme Activities in Cryoturbated Arctic Soils. PLoS ONE 9, e94076.

Schulten, H.-R., Schnitzer, M., 1997. The chemistry of soil organic nitrogen: a review. Biology & Fertility of Soils 26, 1–15.

Senwo, Z.N., Tabatabai, M.A., 1998. Amino acid composition of soil organic matter. Biology & Fertility of Soils 26, 235–242.

Snyder, C.S., Bruulsema, T.W., Jensen, T.L., Fixen, P.E., 2009. Review of greenhouse gas emissions from crop production systems and fertilizer management effects. Agriculture, Ecosystems and Environment 133, 247–266.

Solaimen, Z., Kashem, M., Matsumoto, I., 2007. Environmental Proteomics: Extraction and Identification of Protein in Soil. Advanced Techniques in Soil Microbiology 11, 155-166.

Sowden, F.J., Chen, Y., Schnitzer, M., 1977. The nitrogen distribution in soils formed under widely differing climatic conditions. Geochimica et Cosmochimica Acta 41, 1524–1526.

Spohn, M., Carminati, A., Kuzyakov, Y., 2013. Soil zymography – A novel in situ method for mapping distribution of enzyme activity in soil. Soil Biology & Biochemistry 58, 275–280.

Stoelken, G., Simon, J., Ehlting, B., Rennenberg, H., 2010. The presence of amino acids affects inorganic N uptake in non-mycorrhizal seedlings of European beech (*Fagus sylvatica*). Tree Physiology 30, 1118–1128.

Stremińska, M.A., Felgate, H., Rowley, G., Richardson, D.J., Baggs, E.M., 2012. Nitrous oxide production in soil isolates of nitrate-ammonifying bacteria. Environmental Microbiology Reports 4, 66–71.

Templer, P.H., Arthur, M.A., Lovett, G.M., Weathers, K.C., 2007. Plant and soil

natural abundance $\delta^{15}N$: indicators of relative rates of nitrogen cycling in temperate forest ecosystems. Oecologia 153, 399–406.

Vágnerová, K., Macura, J., 1974. Determination of protease activity of plant roots. Folia Microbiologica 19, 322–328.

Vandermeer, J.H., 2011. The ecology of agroecosystems. John and Bartlett Publishers, UK.

von Lützow, M., Kögel-Knabner, I., 2009. Temperature sensitivity of soil organic matter decomposition-what do we know? Biology and Fertility of Soils 46, 1–15.

Vranová, V., Formánek, P., Rejšek, K., 2009. Impact of Land-use Change on Proteolytic Activity of Mountain Meadows. Soil and Water Research 3, 122–125.

Vranová, V., Rejsek, K., Formanek, P., 2013. Proteolytic activity in soil: A review. Applied Soil Ecology 70, 23–32.

Wei, H., Guenet, B., Vicca, S., Nunan, N., Asard, H., AbdElgawad, H., Shen, W., Janssens, I.A., 2014. High clay content accelerates the decomposition of fresh organic matter in artificial soils. Soil Biology & Biochemistry 77, 100–108.

Weintraub, M.N., Schimel, J.P., 2005. Seasonal protein dynamics in Alaskan arctic tundra soils. Soil Biology & Biochemistry 37, 1469–1475.

Wilkesman, J., Kurz, L., 2009. Protease Analysis by Zymography: A Review on Techniques and Patents. Recent Patents on Biotechnology 3, 175–184.

White, R., Rivas-Ubach, A., Borkum, M., Köberl, M., Bilbao, A., Colby, S., Hoyt, D., Bingol, K., Kim, Y., Wendler, J., 2017. The state of rhizospheric science in the era of multi-omics: A practical guide to omics technologies. Rhizosphere 3, 212–221.

Wright, D.E., 1962. Amino acid uptake by plant roots. Archives of Biochemistry and Biophysics 97, 174–180.

Chapter 3

Methodological bias associated with soluble protein recovery from soil

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LG, DJ and PH conceived the experiment. LG conducted the experiment and wrote the manuscript. LG, DJ, PH, EP and EB reviewed the manuscript.

Abstract

Proteins play a crucial role in many soil processes, however, standardised methods to extract soluble protein from soil are lacking. The aim of this study was to compare the ability of different extractants to quantify the recovery of soluble proteins from three soil types (Cambisol, Ferralsol and Histosol) with contrasting clay and organic matter contents. Known amounts of plant-derived ^{14}C-labelled soluble proteins were incubated with soil and then extracted with solutions of contrasting pH, concentration and polarity. Protein recovery proved highly solvent and soil dependent (Histosol > Cambisol > Ferralsol) and no single extractant was capable of complete protein recovery. In comparison to deionised water (10-60% of the total protein recovered), maximal recovery was observed with NaOH (0.1 M; 61-80%) and Na-pyrophosphate (0.05 M, pH 7.0; 45-75% recovery). We conclude that the dependence of protein recovery on both extractant and soil type prevents direct comparison of studies using different recovery methods, particularly if no extraction controls are used. We present recommendations for a standard protein extraction protocol.

Keywords: Dissolved organic carbon, Exoenzymes, Humic substances, Soluble nitrogen

3.1. Introduction

Protein represents the dominant form of organic nitrogen (N) entering soil ecosystems and frequently the bottleneck in soil N cycling (Jan et al., 2009). Further, based on the number of proteins contained in plants and microorganisms, it can be expected that a single gram of soil may contain thousands of different proteins (Haas et al., 2005; Ishihama et al., 2008). As proteins play a key role in many soil processes, there is increasing interest in the extraction, separation, identification and quantification of proteins as indicators of soil function. However, the development of exoproteomic approaches are currently limited by the lack of standard protocols and the difficulty of recovering proteins from soil.

Extractants that have commonly been used for soil protein recovery include simple salts (e.g. K_2SO_4, Na-pyrophosphate, Na-phosphate), bases (e.g. NaOH), organic acids (e.g. Na-citrate) and surfactants (e.g. Tris-SDS) (Appendix 1, Table 1). Although previous studies have examined a range of protein extraction methods, these have been largely restricted to single unrepresentative proteins (e.g. BSA),

single soils or have used quantification methods known to suffer from severe interference by the co-extraction of humic substances (Criquet et al., 2002; Kanerva et al., 2013; Roberts and Jones, 2008). In addition, many of these studies have lacked the appropriate controls, preventing determination of protein extraction efficiency or have focused on the whole soil metaproteome.

Soil type has a large influence on protein recovery. Some studies suggest that organic matter and clay content are the key soil properties which affect protein recovery (Bastida et al., 2018; Chen et al., 2009; Kanerva et al., 2013) whilst other studies suggest soil pH is also important (Halvorson and Gonzalez, 2006; Haney et al., 2001; Masciandaro et al., 2008). Organic matter content, clay content and pH influence the adsorption of protein in soil and, therefore, affects the ease to which it can be extracted.

Our aim was to focus on soluble proteins and to compare the recovery of a mixture of ^{14}C-labelled plant proteins from soil using 39 different extractants. Our secondary aim was to evaluate the influence of soil type on protein recovery.

3.2. Materials and methods

3.2.1. Soils used in the study

We evaluated protein recovery from three soils with contrasting organic matter and Fe contents: (1) a Eutric Cambisol obtained from a temperate *Lolium perenne* L. grassland in Abergwyngregyn, Gwynedd, UK (53°14'N, 4°00'W); (2) a Fibric Histosol obtained from a temperate *Calluna vulgaris* (L.) Hull moorland in Abergwyngregyn, Gwynedd, UK (53°22'N, 4°01'W), and (3) a Rhodic Ferralsol obtained from a *Saccharum officinarum* L. plantation in Piracicaba, Brazil (22°32'S, 49°20'W) (IUSS Working Group WRB, 2015). In all cases, replicate batches of soil (n = 3) were collected from a depth of 0-15 cm, sieved (<2 mm) and kept at 4°C until required. The main soil properties are shown in Table 3.1. Soil pH and electrical conductivity (EC) were measured in 1:5 (w/v) soil:H2O extracts. Total C and N were determined with a TruSpec® analyser (Leco Corp., St Joseph, MI). Soil texture was determined with a LS1330 Particle size analyser (Beckman Coulter, Brea, CA). Cation exchange capacity (CEC) was measured by saturation with an index cation (Rhoades, 1982). Soluble protein in water extracts was measured using the Coomassie Blue method (Bradford, 1976) and was used to calibrate the rate of ^{14}C-labelled protein addition (Appendix 1, Table S3). This method, however, cannot be used with extractants other

than water due to bias from interfering substances (Bastida et al., 2014; Roberts and Jones, 2008).

Table 3.1. Major characteristics of the three soils used in the extraction trial. Values represent means ± SEM (Standard error of the mean) (n = 3). Different letters indicate significant differences between soils at the $p < 0.05$ level.

	Cambisol	**Ferralsol**	**Histosol**
pH	6.07 ± 0.02^a	4.75 ± 0.22^b	4.48 ± 0.17^b
EC (µS cm^{-1})	21.8 ± 3.3^a	140.7± 52.9^a	47.5 ± 28.4^a
Organic C (%)	2.43 ± 0.02^a	1.45 ± 0.08^a	23.2 ± 0.5^b
Total N (%)	0.21 ± 0.00^a	0.12 ± 0.00^b	1.12 ± 0.04^c
Sand (%)	40.7 ± 3.2^a	31.0 ± 1.8^a	90.7 ± 2.4^b
Silt (%)	46.0 ± 2.7^a	35.1 ± 2.2^b	8.0 ± 1.9^c
Clay (%)	13.3 ± 0.5^a	33.8 ± 0.7^b	1.3 ± 0.5^c
Cation exchange capacity (mmol kg^{-1})	145 ± 6^a	90 ± 8^a	334 ± 57^b

3.2.2. Protein extraction solutions

The extractants tested were based on previously published methods (Appendix 1, Table S1) and included: deionised water, Na-pyrophosphate (0.01, 0.05, 0.1 M; pH 7.0), Na-citrate (0.01, 0.05, 0.1, 0.5 M; pH 8.0), Tris-SDS (0.01, 0.05, 0.1 M SDS with 0.05 M Tris; pH 7.0), K-phosphate buffer (0.01, 0.05, 0.1, 0.5 M; pH 6.0 and 8.0), $CaCl_2$, NaOH and K_2SO_4 (0.01, 0.05, 0.1, 0.5 M), methanol and ethanol (25%, 50%, 75%, 100% v/v). Extractants with no pH value stated were not adjusted and their values are presented in Appendix 1, Table S4.

3.2.3. Protein addition and recovery from soil

Soil (1 g) was placed in individual 20 ml polypropylene vials and heat-sterilised (80°C, 1 h) immediately prior to experimentation (Mariano et al., 2016). This sterilisation procedure was not found to affect the CEC of the soils (Appendix 1, Table S2). In addition, it also proved effective at killing the microbial community preventing bias from microbial breakdown/immobilisation of the added protein (Appendix 1, Fig. 1). Although free soil protease activity was not completely eliminated by heat sterilisation, the exoenzyme activity was extremely low compared to the amount of protein added to the soil and was therefore not expected to bias our findings (Appendix 1, Table S5). Purified, ^{14}C-uniformly labelled soluble protein from *Nicotiana tabacum*

L. leaves (100 µl; 0.860 mg ml^{-1}; 1.2 kBq ml^{-1}; purified to >3 kDa by ultra-filtration; custom synthesised by American Radiolabeled Chemicals, St Louis, MO) was added to each soil, shaken to mix and incubated for 30 min at 20°C. An incubation time of 30 min was deemed appropriate based on initial pilot studies of protein sorption and recovery from soil at incubation times varying from 0.5 to 24 h (Appendix 1, Table S6). The time is therefore sufficient to obtain high rates of sorption while minimising the chances of proteolysis or microbial regrowth. Soluble plant proteins were chosen as they represent one of the major forms of dissolved organic N added to soil. Based on extractant methods from previous studies, the soils were subsequently shaken with 5 ml of each extractant (30 min; 200 rev min^{-1}) (Jones and Willett, 2006; Rousk and Jones, 2010), then a 1.5 ml aliquot was pipetted into 1.5 ml microfuge tubes and centrifuged (18 000 *g*; 60 s) and the supernatant recovered. The centrifugation time of 60 s allowed complete phase separation of the soil particles and supernatant (Appendix 1, Table S7). The amount of ^{14}C-label recovered in degradations per minute (DPM) of supernatant was determined using a Wallac 1414 scintillation counter (60 s) and Wallac Optiphase HiSafe3 scintillation fluid (PerkinElmer Inc., Waltham, MA). Baseline ^{14}C-labelled protein was determined by counting 100 µl of ^{14}C-labelled protein. Extraction efficiency was calculated by equation (1).

$$Extraction\ efficiency\ (\%) = \frac{^{14}C\ in\ extractant\ supernatant\ (DPM)}{baseline\ ^{14}C\ (DPM)} \times 100 \quad (1)$$

Humic acids and organic solvents had no effect on ^{14}C counting efficiency (Appendix 1, Table S8 and S9). To estimate the amount of humic substances co-extracted with the protein, the colour of the extracts was determined at 254 and 400 nm in UV-transparent plastic 96-well plate using a PowerWave HT Spectrophotometer (BioTek Inc., Winooski, VT).

3.2.4. Statistical analysis

All experiments were performed in triplicate. All statistical analysis was performed using R 3.4.1 and work was carried out in base R unless stated (R Core Team, 2018). Data was declared to be normally distributed by Shapiro-Wilk normality test ($p > 0.05$) and have equal variances across groups by Bartlett test ($p > 0.05$). Graphs were created using the R package ggplot2 (Wickham, 2016). Differences in soil properties between soil types were analysed by one-way ANOVA with TukeyHSD post-hoc testing using $p < 0.05$ as the cut-off for statistical significance. Differences in

protein recovery between treatments and soils were analysed by two-way ANOVA with TukeyHSD post-hoc testing using $p < 0.05$ as the cut-off for statistical significance. Chemical speciation modelling to estimate the net valency of each extractant was performed with Geochem-EZ (Shaff et al., 2010).

3.3. Results and discussion

3.3.1. Protein recovery from soil by water

Here we aimed to evaluate methods of soluble protein recovery. This is relevant to studies investigating the potential behaviour of isotopically labelled proteins in soil (sorption, biodegradation) or for recovering the plant or microbial exoproteome. Overall, we found significant differences in protein extraction efficiency between the different extractants ($F_{10, 318} = 118.5$; $p < 0.001$; Fig. 3.1) and soils (Histosol > Cambisol > Ferralsol) ($F_{2, 318} = 148.4$; $p < 0.001$; Fig. 3.1). As the soil was sterilised to limit microbial activity (Mariano et al., 2016), ^{14}C measured is assumed to represent intact ^{14}C-protein, therefore we refer to extraction efficiency as protein recovered. Protein recovery by deionised water varied from 10-60% between soil types. As the water is expected to recover mainly free, unbound protein, we assume the remainder became bound to the solid phase or coagulated/precipitated on entering the soil (Coen et al., 1995; Shih et al., 1992). Proteins are known to readily sorb to the surface of clay minerals, Fe/Al oxyhydroxides and humic materials in soil (Nielsen et al., 2006; Quiquampoix et al., 1995; Yu et al., 2013). Therefore, extractants should be able to displace proteins bound to surfaces during the extraction process or to solubilise the binding surfaces themselves. Ferralsols had the lowest protein recovery probably because of the higher clay and Fe-oxide fraction, compared to the Histosol and Cambisol ($p < 0.001$; Table 3.1), resulting in more protein being strongly bound to the solid phase. In comparison, the higher humic content of the Histosol ($p < 0.001$; Table 1) may have resulted in the extraction of protein as soluble humic-protein complexes (Bonmatí et al., 2009). In our soils, a complete recovery of the added ^{14}C-protein was not achieved for any soil, with ca. 25% not recoverable by any extractant. This is likely to be even higher in soils where proteins have been stabilised for long periods.

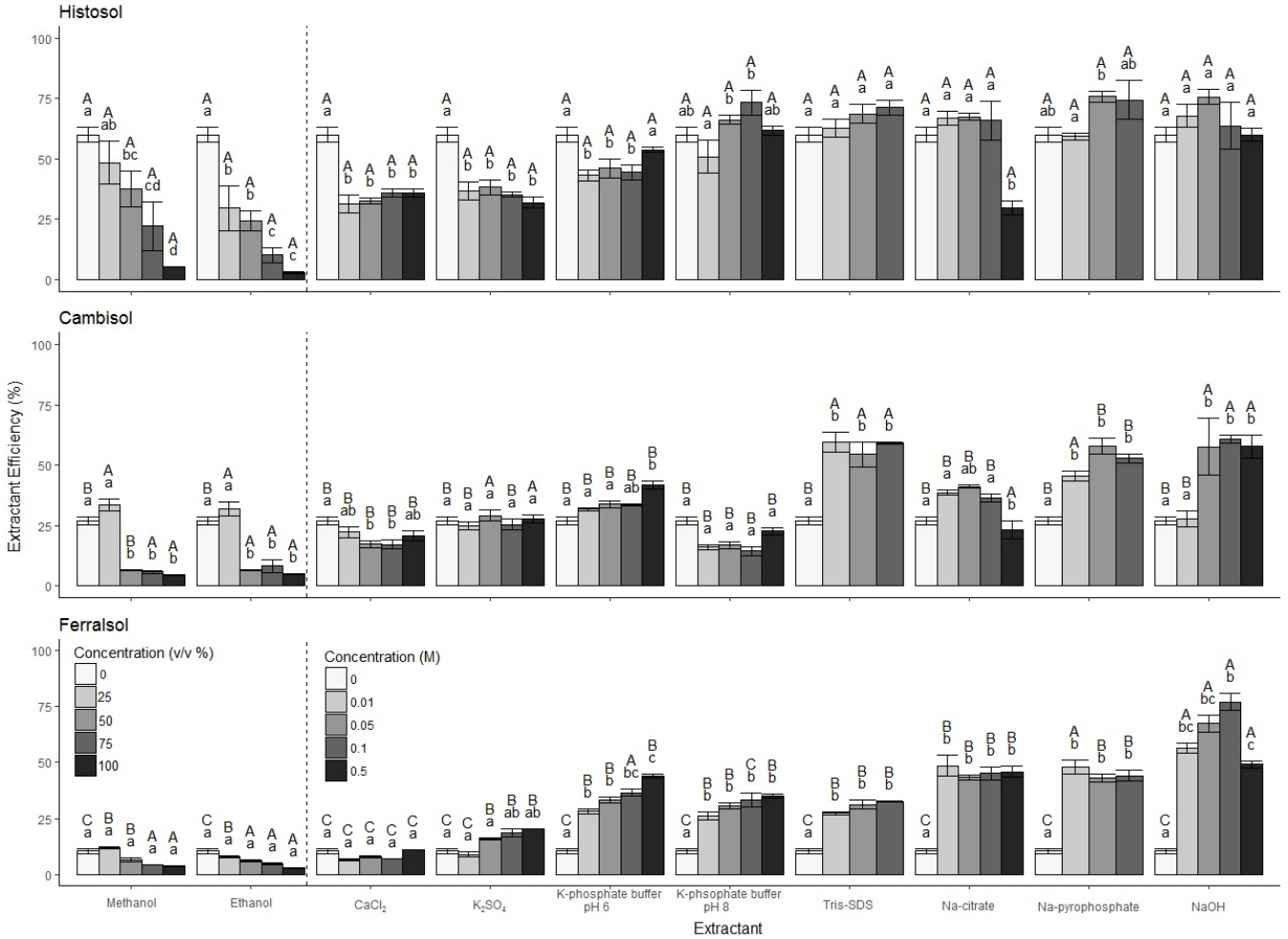

Figure 3.1. Extraction efficiency (%) of ^{14}C-labelled protein from three contrasting soils using a range of chemical extractants. The legend to the left of the dashed line refers concentrations of methanol and ethanol. For all other extractants, refer to the legend on the right of the dashed line. Different capital letters represent significant differences between soil type of the same molarity and extractant. Different letters represent significant differences between molarity of the same soil type and extractant. Values represent means ± SEM (*n* = 3).

3.3.2. Protein recovery from soil by salt extracts

For the Histosol, no significant difference was observed between deionised water and the other extractants ($p > 0.05$) except $CaCl_2$ and K_2SO_4 which lowered protein extraction compared to deionised water ($p < 0.05$). We ascribe the poor protein recovery with $CaCl_2$ and K_2SO_4 to salt-induced conformational changes in protein structure and subsequent coagulation/precipitation (Appendix 1, Table S10), a phenomenon which is well documented in the literature (Shih et al., 1992). In contrast to the Histosol, deionised water gave low protein recovery rates from the Ferralsol and Cambisol likely due to more protein adsorbed onto the clay fraction. We conclude therefore that water extracts may provide an estimate of free, unbound proteins in soil and limited information of the bound fraction. Further, while 0.5 M K_2SO_4 is frequently used as a standard extractant for dissolved organic N and for measuring soil microbial biomass-N (Joergensen and Brookes, 1990; Jones and Willett, 2006), our results suggest that the method may reduce total protein recovery.

The highest recoveries were obtained by NaOH and Na-pyrophosphate (70-76% of the total protein added), with no significant difference apparent between them ($p > 0.05$; Fig. 1). The high pH of NaOH relative to the other extractants solubilises organic matter leading to the release of protein particularly the case of the Histosol (Schnitzer, 1982). For the Ferralsol, NaOH was the most efficient extractant (49-77% compared to 43-48% by Na-citrate). NaOH also solubilises protein adsorbed to $Al(OH)_3$, resulting in protein release from the Ferralsol (Gianfreda et al., 1992).

Our results therefore suggest that the recovery of protein from soil is consistent with 1) their salting-out potential based on the Hofmeister series (Shih et al., 1992), and 2) the potential of each salt to displace bound protein from surfaces via ligand exchange, based on their net valency (i.e. $HP_2O_7^{3-}$ > $Citrate^{3-}$ > $phosphate^{1.87-}$ $_{(pH\ 8)}$ = $phosphate^{1.15-}$ $_{(pH\ 6)}$ > SO_4^{2-} > Cl^-). The exception to this was Tris-$SDS^{0.09-}$ which had a significantly higher extraction efficiency than K_2SO_4 and $CaCl_2$ ($p < 0.001$) suggesting that the presence of surfactant aids ionic displacement. Surfactants tend to gather around interfaces (e.g. the interface between the soil surface and soil solution). The surfactants compete with the protein molecules for available surface area in order for the hydrophobic tails to avoid water. Over time the SDS molecule will replace the protein molecules because the surfactant molecules are in excess (Jönsson and Jönsson, 1991; Norde, 2008).

3.3.3. Protein recovery from soil by organic solvents

The polar solvents, methanol and ethanol both proved ineffectual at recovering soluble proteins from soil likely due to the alcohol-induced precipitation of proteins (Schubert and Finn, 1981). This contrasts strongly with metabolomic studies where these extractants often yield the greatest recovery of low molecular weight organic solutes (Swenson et al., 2015; Warren, 2015).

3.3.4. Co-extraction of humic substances

NaOH caused the solubilisation of large amounts of humic substances and based on previous studies, this is likely to induce protein denaturation (Bremner, 1967; Bremner and Lees, 1949). Consequently, we would not recommend it as an extractant. However, in some analysis the structure of the protein is not important (e.g. sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and the Kjeldahl method) and NaOH can be used.

Na-pyrophosphate, NaOH, Na-citrate and both phosphate buffers extracted more humic substances in comparison to deionised water (Appendix 1, Table S11, Fig. 2) in support of previous findings (Masciandaro et al., 2008; Murase et al., 2003). Humic substances can be problematic due to their ability to bind to proteins and interfere with colorimetric procedures for quantifying protein (Bastida et al., 2009; Criquet et al., 2002). Proteins interact with humic substances to form protein-humic substance complexes (Nielsen et al., 2006). The mechanisms of the interaction are thought to consist of: a) covalent and hydrogen bonds (Rowell et al., 1973), b) ionic bonds between the functional amino group of the protein and the carboxyl or hydroxyl group of the humic substance (Sarkar, 1986), c) physically immobilised within macromolecular matrix of humic substances (Serban and Nissenbaum, 1986), and d) electron donor-acceptor complexes (Gosewinkel and Broadbent, 1986).

The co-extraction of humic substances with proteins in the protein-humic complexes results in colour in the supernatant. This interferes with colorimetric and fluorescent analysis of protein quantity (Roberts and Jones, 2008; Whiffen et al., 2007). Methods of removing interfering humic substances (e.g. PVPP (Criquet et al., 2002) and TCA precipitation (Qian and Hettich, 2017)) have been found to be ineffective (Aoyama, 2006). Therefore, NaOH, Na-pyrophosphate, Na-citrate and phosphate buffers are not ideal extractants when these types of analysis are being

used. In addition, if extracting protein from a soil with high organic matter content, more interference will occur in comparison to soils with lower organic matter contents.

3.4. Conclusions

In summary, we found that 0.1 M NaOH was the most effective extractant overall when denatured protein can be used in subsequent analysis and co-extraction of humic substances does not interfere. For analysis of intact proteins, 0.05 M Na-pyrophosphate (pH 7.0) was most effective for extracting water-soluble proteins from soil; however, it did also co-extract humic substances. Where interference of humic substances may prove problematic for subsequent analysis and intact proteins are required, deionised water is recommended. For proteomics, further analysis by LC-MS/MS will be necessary to assess the quality of the proteins extracted by each method (Bastida et al., 2014; Leary et al., 2013). In addition, although this study was limited to three soils, our results clearly indicate that soil type directly affects the amount of protein that can be recovered. This may make quantitative comparisons between soils problematic. Rarely has this been accounted for in previous studies comparing protein levels in soil. The impact of this in future studies can be evaluated by measuring the recovery of a known mixture of proteins, as undertaken here. It should also be emphasised that this study focused only on the recovery of hydrophilic proteins from soil. Similar studies are therefore required to optimise the recovery of proteins contained within the soil microbial community, especially those of a hydrophobic nature.

3.5. Acknowledgements

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3.6. References

Aoyama, M., 2006. Properties of neutral phosphate buffer extractable organic matter in soils revealed using size exclusion chromatography and fractionation with polyvinylpyrrolidone. Soil Science and Plant Nutrition 52, 378–386.

Bastida, F., Hernández, T., García, C., 2014. Metaproteomics of soils from semiarid environment: Functional and phylogenetic information obtained with different protein extraction methods. Journal of Proteomics 101, 31–42.

Bastida, F., Jehmlich, N., Torres, I.F., García, C., 2018. The extracellular metaproteome of soils under semiarid climate: A methodological comparison of extraction buffers. Science of The Total Environment 619–620, 707–711.

Bastida, F., Moreno, J.L., Nicolás, C., Hernández, T., García, C., 2009. Soil metaproteomics: A review of an emerging environmental science. Significance, methodology and perspectives. European Journal of Soil Science 60, 845–859.

Benndorf, D., Balckem, G.U., Harms, H., von Bergen, M., 2007. Functional metaproteome analysis of protein extracts from contaminated soil and groundwater. ISME Journal 1, 224–234.

Boddy, E., Roberts, P., Hill, P.W., Farrar, J., Jones, D.L., 2008. Turnover of low molecular weight dissolved organic C (DOC) and microbial C exhibit different temperature sensitivities in Arctic tundra soils. Soil Biology & Biochemistry 40, 1557–1566.

Bonmati, M., Ceccanti, B., Nannipieri, P., 1998. Protease extraction from soil by sodium pyrophosphate and chemical characterization of the extracts. Soil Biology & Biochemistry 30, 2113–2125.

Bonmatí, M., Ceccanti, B., Nannipieri, P., Valero, J., 2009. Characterization of humus–protease complexes extracted from soil. Soil Biology & Biochemistry 41, 1199–1209.

Bradford, M.M., 1976. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. Analytical Biochemistry 72, 248–254.

Bremner, J.M., 1967. Nitrogenous Compounds, in: McLaren, A.D., Peterson, G.H. (Eds.), Soil Biochemistry. Marcel Dekker Inc., New York, pp. 19–58.

Bremner, J.M., Lees, H., 1949. Studies on soil organic matter: Part II. The extraction of organic matter from soil by neutral reagents. The Journal of Agricultural Science 39, 274–279.

Busto, M.D., Perez-Mateos, M., 1995. Extraction of humic-β-glucosidase fractions from soil. Biology & Fertility of Soils 20, 77–82.

Carter, H.T., Tipping, E., Koprivnjak, J.-F., Miller, M.P., Cookson, B., Hamilton-Taylor, J., 2012. Freshwater DOM quantity and quality from a two-component model of UV absorbance. Water Research 46, 4532–4542.

Chen, S., Rillig, M.C., Wang, W., 2009. Improving soil protein extraction for metaproteome analysis and glomalin-related soil protein detection. Proteomics 9, 4970–4973.

Coen, C.J., Blanch, H.W., Prausnitz, J.M., 1995. Salting out of aqueous proteins: Phase equilibria and intermolecular potentials. American Institute of Chemical Engineers 41, 996–1004.

Criquet, S., Farnet, A.M., Ferre, E., 2002. Protein measurement in forest litter. Biology & Fertility of Soils 35, 307–313.

Edzwald, J.K., Becker, W.C., Wattier, K.L., 1985. Surrogate Parameters for Monitoring Organic Matter and THM Precursors. Research and Technology 77, 122–132.

Erez, E., Fass, D., Bibi, E., 2009. How intramembrane proteases bury hydrolytic reactions in the membrane. Nature 459, 371–378.

Friedel, K., Scheller, E., Èrgen, J., 2002. Composition of hydrolysable amino acids in soil organic matter and soil microbial biomass. Soil Biology & Biochemistry 34, 315–325.

Gianfreda, L., Rao, M.A., Violante, A., 1992. Adsorption, activity and kinetic properties of urease on montmorillonite, aluminium hydroxide and AL(OH)x-montmorillonite complexes. Soil Biology & Biochemistry 24, 51–58.

Gosewinkel, U., Broadbent, F.E., 1986. Decomplexation of phosphatase from extracted soil humic substances with electron donating reagents. Soil Science 141, 261–267.

Haas, B.J., Wortman, J.R., Ronning, C.M., Hannick, L.I., Smith Jr, R.K., Maiti, R., Chan, A.P., Yu, C., Farzad, M., Wu, D., White, O., Town, C.D., 2005. Complete reannotation of the Arabidopsis genome: methods, tools, protocols and the final release. BMC Biology 3, 1–19.

Halvorson, J.J., Gonzalez, J.M., 2006. Bradford reactive soil protein in Appalachian soils: distribution and response to incubation, extraction reagent and tannins. Plant Soil 286, 339–356.

Haney, R.L., Franzluebbers, A.J., Hons, F.M., Hossner, L.R., Zuberer, D.A., 2001. Molar concentration of K_2SO_4 and soil pH affect estimation of extractable C with chloroform fumigation-extraction. Soil Biology & Biochemistry 33, 1501–1507.

Ishihama, Y., Schmidt, T., Rappsilber, J., Mann, M., Hartl, F.U., Kerner, M.J., Frishman, D., 2008. Protein abundance profiling of the Escherichia coli cytosol. BMC Genomics 9, 1–19.

IUSS Working Group WRB, 2015. World reference base for soil resources 2014, update 2015 International soil classification system for naming soils and creating legends for soil maps. World Soil Resources Reports No. 106. Rome.

Jan, M.T., Roberts, P., Tonheim, S.K., Jones, D.L., 2009. Protein breakdown represents a major bottleneck in nitrogen cycling in grassland soils. Soil Biology & Biochemistry 41, 2272–2282.

Joergensen, R.G., Brookes, P.C., 1990. Ninhydrin-reactive nitrogen measurements of microbial biomass in 0.5 M K_2SO_4 soil extracts. Soil Biology & Biochemistry 22, 1023–1027.

Jones, D., Willett, V., 2006. Experimental evaluation of methods to quantify dissolved organic nitrogen (DON) and dissolved organic carbon (DOC) in soil. Soil Biology & Biochemistry 38, 991–999.

Jones, D.L., 1999. Amino acid biodegradation and its potential effects on organic nitrogen capture by plants. Soil Biology & Biochemistry 31, 613–622.

Jönsson, A.-S., Jönsson, B., 1991. The influence of nonionic and ionic surfactants on hydrophobic and hydrophilic ultrafiltration membranes. Journal of Membrane Science 56, 49–76.

Kanerva, S., Smolander, A., Kitunen, V., Ketola, R.A., Kotiaho, T., 2013. Comparison of extractants and applicability of MALDI-TOF-MS in the analysis of soil proteinaceous material from different types of soil. Organic Geochemistry 56, 1–9.

Keiblinger, K.M., Wilhartitz, I.C., Schneider, T., Roschitzki, B., Schmid, E., Eberl, L., Riedel, K., Zechmeister-Boltenstern, S., 2012. Soil metaproteomics - Comparative evaluation of protein extraction protocols. Soil Biology & Biochemistry 54, 14–24.

Leary, D.H., Hervey, W.J., Deschamps, J.R., Kusterbeck, A.W., Vora, G.J., 2013. Which metaproteome? The impact of protein extraction bias on metaproteomic analyses. Molecular and Cellular Probes 27, 193–199.

Makarov, M.I., Malysheva, T.I., Menyailo, O. V, Soudzilovskaia, N.A., P Van Logtestijn, R.S., C Cornelissen, J.H., 2015. Effect of K_2SO_4 concentration on extractability and isotope signature ($\delta^{13}C$ and $\delta^{15}N$) of soil C and N fractions. European Journal of Soil Science 66, 417–426.

Marchetti, E., Accinelli, C., Epifani, R., 2007. Persistence of Cry toxins and cry genes from genetically modified plants in two agricultural soils. Agronomy for Sustainable Development 27, 231–236.

Mariano, E., Jones, D.L., Hill, P.W., Trivelin, P.C.O., 2016. Mineralisation and sorption of dissolved organic nitrogen compounds in litter and soil from sugarcane fields. Soil Biology & Biochemistry 103, 522–532.

Masciandaro, G., Macci, C., Doni, S., Elena Maserti, B., Calvo-Bado Leo, A., Ceccanti, B., Wellington, E., 2008. Comparison of extraction methods for recovery of extracellular β-glucosidase in two different forest soils. Soil Biology & Biochemistry 40, 2156–2161.

Matsumoto, S., Ae, N., Yamagata, M., 2000. Extraction of mineralizable organic nitrogen from soils by a neutral phosphate buffer solution. Soil Biology & Biochemistry 32, 1293–1299.

Murase, A., Yoneda, M., Ueno, R., Yonebayashi, K., 2003. Isolation of extracellular protein from greenhouse soil. Soil Biology & Biochemistry 35, 733–736.

Nannipieri, P., Ceccanti, B., Crevelli, S., Sequi, P., 1974. Use of 0.1 M pyrophosphate to extract urease from a podzol. Soil Biology & Biochemistry 6, 359–362.

Nielsen, K.M., Calamai, L., Pietramellara, G., 2006. Stabilization of Extracellular DNA and Proteins by Transient Binding to Various Soil Components, in: Nannipieri, P., Smalla, K. (Eds.), Nucleic Acids and Proteins in Soil. Springer-Verlag, Berlin Heidelberg, pp. 141–157.

Norde, W., 2008. My voyage of discovery to proteins in flatland …and beyond. Colloids and Surfaces B: Biointerfaces 61, 1–9.

Peacock, M., Evans, C.D., Fenner, N., Freeman, C., Gough, R., Jones, T.G., Lebron, I., 2014. UV-visible absorbance spectroscopy as a proxy for peatland dissolved organic carbon (DOC) quantity and quality: considerations on wavelength and absorbance degradation. Environmental Science: Processes & Impacts.

Qian, C., Hettich, R.L., 2017. Optimized Extraction Method To Remove Humic Acid Interferences from Soil Samples Prior to Microbial Proteome Measurements.

Journal of Proteome Research 16, 2537–2546.

Quiquampoix, H., Abadie, J., Baron, M.H., Leprince, F., Matumoto-Pintro, P.T., Ratcliffe, R.G., Staunton, S., 1995. Mechanisms and Consequences of Protein Adsorption on Soil Mineral Surfaces, in: Horbett, T.., Brash, J.L. (Eds.), Proteins at Interfaces II. Fundamentals and Applications. American Chemical Society, Washington, DC, pp. 321–333.

R Core Team, 2018. R: A language and environment for statistical computing.

Rhoades, J.D., 1982. Cation exchange capacity, in: Page, A.L., Miller, R.H., Keeney, D.R. (Eds.), Methods of Soil Analysis. Part 2. Chemical and Microbiological Properties. Soil Science Society of America, Madison, Wisconsin, pp. 149–157.

Roberts, P., Jones, D.L., 2008. Critical evaluation of methods for determining total protein in soil solution. Soil Biology & Biochemistry 40, 1485–1495.

Rosier, C.L., Hoye, A.T., Rillig, M.C., 2006. Glomalin-related soil protein: Assessment of current detection and quantification tools. Soil Biology & Biochemistry 38, 2205–2211.

Rousk, J., Jones, D.L., 2010. Loss of low molecular weight dissolved organic carbon (DOC) and nitrogen (DON) in H_2O and 0.5 M K_2SO_4 soil extracts. Soil Biology & Biochemistry 42, 2331–2335.

Rowell, M.J., Ladd, J.N., Paul, A.A., 1973. Enzymically active complexes of proteases and humic acid analogues. Soil Biology & Biochemistry 5, 699–703.

Sarkar, J.M., 1986. Formation of [^{14}C] cellulase-humic complexes and their stability in soil. Soil Biology & Biochemistry 18, 251–254.

Schneider, T., Keiblinger, K.M., Schmid, E., Sterflinger-Gleixner, K., Nther Ellersdorfer, G., Roschitzki, B., Richter, A., Eberl, L., Zechmeister-Boltenstern, S., Riedel, K., 2012. Who is who in litter decomposition? Metaproteomics reveals major microbial players and their biogeochemical functions. ISME Journal 6, 1749–1762.

Schnitzer, M., 1982. Organic Matter Characterization, in: Page, A.L., Miller, R.H., Keeney, D.R. (Eds.), Methods of Soil Analysis. Part 2. Chemical and Microbiological Properties. American Society of Agronomy, Soil Science Society of America, Madison, U.S.A, pp. 581–594.

Schubert, P.F., Finn, R.K., 1981. Alcohol precipitation of proteins: The relationship of denaturation and precipitation for catalase. Biotechnology and Bioengineering 23, 2569–2590.

Serban, A., Nissenbaum, A., 1986. Humic acid association with peroxidase and catalase. Soil Biology & Biochemistry 18, 41–44.

Shaff, J.E., Schultz, B.A., Craft, E.J., Clark, R.T., Kochian, L. V, 2010. GEOCHEM-EZ: a chemical speciation program with greater power and flexibility. Plant and Soil 330, 207–214.

Shih, Y.-C., Prausnitz, J.M., Blanch, H.W., 1992. Some characteristics of protein precipitation by salts. Biotechnology and Bioengineering 40, 1155–1164.

Swenson, T.L., Jenkins, S., Bowen, B.P., Northen, T.R., 2015. Untargeted soil metabolomics methods for analysis of extractable organic matter. Soil Biology & Biochemistry 80, 189–198.

Vepsäläinen, M., Kukkonen, S., Vestberg, M., Sirviö, H., Maarit Niemi, R., 2001. Application of soil enzyme activity test kit in a field experiment. Soil Biology & Biochemistry 33, 1665–1672.

Wallage, Z.E., Holden, J., 2010. Spatial and temporal variability in the relationship between water colour and dissolved organic carbon in blanket peat pore waters. Science of The Total Environment 408, 6235–6242.

Warren, C.R., 2015. Comparison of methods for extraction of organic N monomers from soil microbial biomass. Soil Biology & Biochemistry 81, 67–76.

Whiffen, L.K., Midgley, D.J., McGee, P.A., 2007. Polyphenolic compounds interfere with quantification of protein in soil extracts using the Bradford method. Soil Biology & Biochemistry 39, 691–694.

Wickham, H., 2016. ggplot2: Elegant Graphics for Data Analysis.

Wilkesman, J., Kurz, L., 2009. Protease Analysis by Zymography: A Review on Techniques and Patents. Recent Patents on Biotechnology 3, 175–184.

Wright, S.F., Upadhyaya, A., 1998. A survey of soils for aggregate stability and glomalin, a glycoprotein produced by hyphae of arbuscular mycorrhizal fungi. Plant and Soil 198, 97–107.

Yu, W.H., Li, N., Tong, D.S., Zhou, C.H., Lin, C.X., Xu, C.Y., 2013. Adsorption of proteins and nucleic acids on clay minerals and their interactions: A review. Applied Clay Science 80, 443–452.

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Chapter 4

Is soluble protein mineralisation and protease activity in soil regulated by supply or demand?

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LG, DJ and PH conceived the experiment. LG conducted the experiment and LG and DJ wrote the manuscript. LG and FS conducted the statistical analysis. LG, DJ, PH, EP and EB reviewed the manuscript.

Abstract

Protein represents a major input of organic matter to soil and is an important source of carbon (C) and nitrogen (N) for microorganisms. Therefore, determining which soil properties influence protein mineralisation in soil is key to understanding and modelling soil C and N cycling. However, the effect of different soil properties on protein mineralisation, and especially the interactions between soil properties, are poorly understood. We investigated how topsoil and subsoil properties affect protein mineralisation along a grassland altitudinal (catena) sequence that contained a gradient in soil type and primary productivity. We devised a schematic diagram to test the key edaphic factors that may influence protein mineralisation in soil (e.g. pH, microbial biomass, inorganic and organic N availability, enzyme activity and sorption). We then measured the mineralisation rate of ^{14}C-labelled soluble plant-derived protein and amino acids in soil over a two-month period. Correlation analysis was used to determine the associations between rates of protein mineralisation and soil properties. Contrary to expectation, we found that protein mineralisation rate was nearly as fast as for amino acid turnover. We ascribe this rapid protein turnover to the low levels of protein used here, its soluble nature, a high degree of functional redundancy in the microbial community and microbial enzyme adaptation to their ecological niche. Unlike other key soil N processes (e.g. nitrification, denitrification), protease activity was not regulated by a small range of factors, but rather appeared to be affected by a wide range of interacting factors whose importance was dependent on altitude and soil depth [e.g. above-ground net primary productivity (NPP), soil pH, nitrate, cation exchange capacity (CEC), C:N ratio]. Based on our results, we hypothesise that differences in soil N cycling and the generation of ammonium are more related to the rate of protein supply rather than limitations in protease activity and protein turnover *per se*.

Keywords: Decomposition, Mineralisation, Nutrient cycling, Protease activity, Soil quality indicator

4.1. Introduction

Nitrogen (N) availability represents one of the major factors limiting primary productivity in agroecosystems (Vitousek and Howarth, 1991). Although our understanding of the behaviour and fate of inorganic N in soil is well understood, the factors influencing organic N cycling remain poorly characterised. The main input of organic N to soil is in the form of protein through the addition of plant and microbial residues (Schulten and Schnitzer, 1997; Stevenson and Cole, 1999). As plants and microbes may contain thousands of proteins, each differing in their solubility, charge, size and structure, they represent a diverse group of compounds (Ramírez-Sánchez et al., 2016). Although the relative contribution of these proteins to soil organic matter (SOM) remains unknown, it has been estimated that ca. 40% of total soil N and 9–16% of soil organic C is proteinaceous (Schulten and Schnitzer, 1997; Stevenson and Cole, 1999). Therefore, protein is a significant fraction of SOM and the central reservoir of organic N in soil. Further, studies involving the addition of large amounts of protein to soil have shown that protein depolymerisation to oligopeptides and amino acids by protease enzymes is the rate limiting step of the soil N cycle irrespective of soil type, environmental conditions or management (Hu et al., 2018; Jan et al., 2009; Jones and Kielland, 2012; Mariano et al., 2016; Simpson et al., 2017). The key factors that regulate protease activity and protein mineralisation at low (more realistic) doses need to be elucidated so we can improve our mechanistic knowledge of the soil N cycle and improve predictive models of plant N supply from the soil. This improved mechanistic knowledge can then be used to identify management options to regulate and optimise N available for plants and reduce N losses to the wider environment.

Protein mineralisation rates depend on substrate availability and the net production of proteases by the microbial community. However, the effect of soil properties on these two factors are complex (Vranová et al., 2013). So far, studies have investigated the impact of microbial biomass, organic N compounds, inorganic N concentration, C:N ratio, temperature, water content and pH on protein mineralisation in soil (Allison and Vitousek, 2005; Farrell et al., 2014; Fierer et al., 2003; Geisseler and Horwath, 2008; Giagnoni et al., 2011). However, the magnitude of influence these soil properties have on protein mineralisation processes is variable and the results are often based on treatment studies rather than observational data. For example, a study by Allison and Vitousek (2005) showed inorganic N addition to decrease soil protease activity compared to an increase seen by Geisseler and

Horwath (2008). In addition, past studies have tended to measure the effect of soil properties in isolation through treatments or just in a single soil type (e.g. Geisseler and Horwath, 2008). Soil properties do not act in isolation and thus we need to understand the interactive effects between soil properties to enhance our mechanistic understanding.

Altitude causes natural variations in soil characteristics, plant communities and the quantity and quality of organic inputs entering the soil due to variations in temperature and precipitation (Warren, 2017). Soil gradients also occur with depth. The topsoil has a higher root abundance resulting in increased organic C and N inputs into soil via root turnover and exudation as well as a higher microbial abundance and diversity (Loeppmann et al., 2016; Philippot et al., 2013; Razavi et al., 2016). These gradients provide a range of soil properties to examine how rates of protein mineralisation are affected.

Protein mineralisation occurs in two main steps (Fig. 4.1); the first step is proteolysis catalysed by protease enzymes. This step is considered to be the rate-limiting step of soil N mineralisation (Jan et al., 2009). Firstly, primary productivity determines the input of protein into the soil system through plant litter, rhizodeposition and microbial necromass. Increasing primary productivity will increase the supply of protein from root turnover and to a lesser extent leaf matter (Schulten and Schnitzer, 1997). Protein can then remain free in the soil solution or stabilised on soil particles by adsorption onto clay mineral surfaces and polyphenol-rich organic compounds (Boyd and Mortland, 1990; Burns, 1982). Cation exchange capacity (CEC) provides a proxy for charge density and surface binding potential (Manrique et al., 1991). Soil pH may subsequently regulate the mechanism of protein binding by affecting the charge of the protein and CEC of the sorbing surfaces (Kleber et al., 2007; Quiquampoix et al., 1993). In plants, the isoelectric point (IEP) for proteins ranges from 1.99 to 13.96 and has a triphasic distribution, however, proteins with an acidic IEP (ca. 5.6) are slightly more abundant than proteins with a basic IEP (ca. 8.37; Mohanta et al., 2019). Therefore, proteins present in a soil pH ≤ 7 are likely to be adsorbed onto soil surfaces with a lower pH favouring stronger bond types (Bingham and Cotrufo, 2016). It is still unclear whether proteins are protected from attack by proteases when adsorbed onto soil surfaces so for this study we consider stabilised protein to be unavailable for protein mineralisation (Lutzow et al., 2006). Available protein is hydrolysed into polypeptides and amino acids catalysed by proteases (Fig. 4.1).

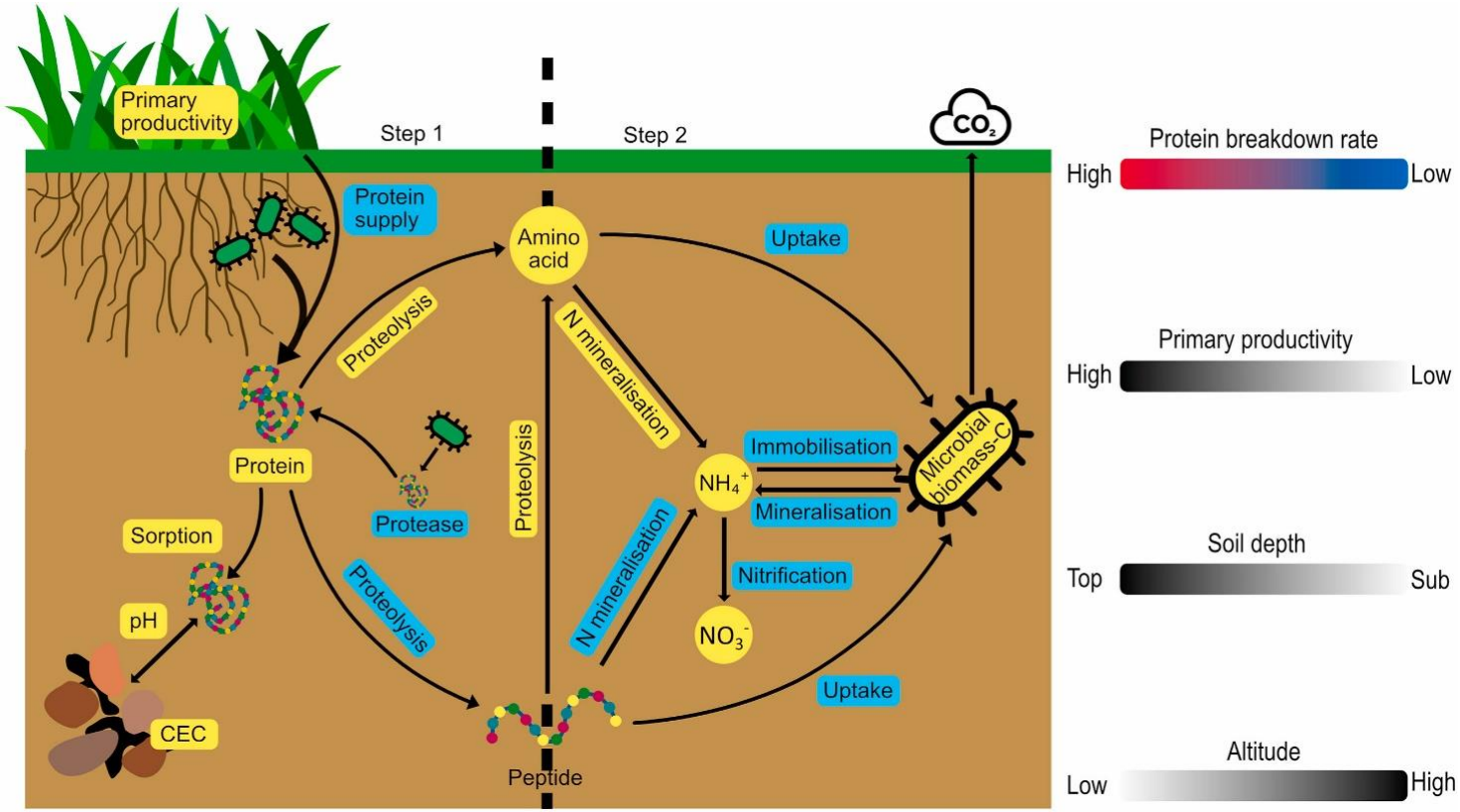

Figure 4.1. Schematic diagram showing the main soil properties and processes regulating the microbially-mediated mineralisation of protein in soil. Step 1 represents the depolymerisation of protein to peptides and amino acids by proteolysis, catalysed by extracellular protease enzymes, and step 2 represents the utilisation of peptides and amino acids by microorganisms and their subsequent immobilisation of C in the biomass or mineralisation to CO_2. Yellow boxes represent the main soil parameters that we measured in this study while the blue boxes represent the main processes that would drive or limit the rate of protein mineralisation associated with the soil parameters we measured. The bars on the side show our hypotheses relating to the speed of protein turnover and either primary productivity, soil depth or altitude. CEC indicates cation exchange capacity.

The second key step is the consumption of oligopeptides and amino acids by microorganisms. Based on the low C:N ratio of peptides and amino acids and their subsequent transamination and deamination reactions after uptake which produced keto acids, ca. 30% of the C in these compounds is typically mineralised to CO_2, leading to ammonium (NH_4^+) excretion back into solution (Hill and Jones, 2019; Roberts et al., 2009). Some of the NH_4^+ excreted is subsequently nitrified to nitrate (NO_3^-) with some NH_4^+ and NO_3^- also lost from the system by leaching or conversion to gaseous forms (e.g. NH_3, NO, N_2O and N_2). NH_4^+ and NO_3^- not lost, can be utilised by plants (Schimel and Bennett, 2004). Together, these processes result in the complete mineralisation of protein by soil microorganisms (i.e. protein → peptides → amino acids → NH_4^+ + CO_2).

The aim of the study was to determine how key regulators described above may affect protein mineralisation rates and, thus, the limiting factors on the soil N cycle. We hypothesise that 1) key regulators (NH_4^+, NO_3^-, protein, amino acid, microbial biomass-C, pH, CEC, N mineralisation, sorption and primary productivity) will predict protein mineralisation rates as these drive or limit degradation processes; 2) The rate of protein mineralisation will decrease along the grassland altitudinal gradient (from low to high altitude) as primary productivity, pH and C and N availability reduce microbial activity, and 3) Protein mineralisation is negatively correlated with depth as protein inputs and microbial biomass C decreases in the subsoil relative to the topsoil (Liu et al., 2016). Our hypotheses are shown schematically in Fig. 4.1.

4.2. Materials and methods

4.2.1. Soil sampling

We evaluated the rate of protein mineralisation at ten sites along a grassland altitudinal catena sequence. We collected soils from a grassland altitudinal gradient to reflect different soil characteristics as a result of differing environmental factors e.g. altitude and temperature. Protein mineralisation rates were measured under constant temperature to remove bias in temperature effects along the gradient. We then measured the key regulators and rate of protein mineralisation. In this study, we define protein mineralisation in soil to be the decomposition of protein until it is respired as CO_2 by microorganisms. Altitude ranged from 5 m to 410 m.a.s.l at Abergwyngregyn, Gwynedd, UK (53°13′ N, 4°00’ W, Table 4.1). Mean annual soil surface temperature

at 10 cm depth ranged from 10.6 °C at Site 1 to 6.9 °C at Site 10 with annual rainfall ranging from 800 mm at Site 1 to 2300 mm at Site 10 (Farrell et al., 2011a). In all cases, replicate batches of soil (ca. 1 kg; *n* = 3) across each site were collected from the topsoil (0–15 cm) and subsoil (15–30 cm). Aboveground biomass was also removed and dried (80 °C, 24 h) for analysis. The soil was homogenised by hand to minimise disturbance. Rocks, earthworms, and large root masses were removed, and soils stored at 4°C for a maximum of two weeks until required. Time sensitive properties e.g. mineralisation rates were started immediately after soil had been processed. The general soil properties are described in Table 4.1. All soil properties are expressed on a volumetric basis (soil depth 0–15 cm) to account for the difference in bulk densities along the altitudinal gradient.

Above-ground primary productivity was measured according to Vile et al. (2006). Briefly, after cutting the grass to ground level at the start of the growing season (March), wire mesh cages with an area of 0.126 m^2 were placed on top of the grass to exclude grazers. Cages were then secured to the ground and left for 2 months at which point the cages were removed, and the grass cut to ground level and recovered. Subsequently, the grass cuttings were dried (80°C, 24 h) and weighed to determine net primary production.

Table 4.1. General site description. Values represent means ± SEM (*n* = 3).

Site	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Classification	Eutric Cambisol	Eutric Cambisol	Eutric Cambisol	Eutric Cambisol	Cambic Podzol	Cambic Podzol	Cambic Podzol	Cambic Podzol	Fibric Histosol	Fibric Histosol
Altitude (m.a.s.l)	5	10	60	80	220	290	340	350	400	410
Land use	Improved grassland	Improved grassland	Improved grassland	Semi-improved grassland	Semi-improved grassland	Semi-improved grassland	Semi-improved grassland	Semi-improved grassland	Acidic grassland	Acidic grassland
Texture	Clay loam	Clay loam	Sandy clay	Sandy clay	Sandy clay	Sandy clay loam	Sandy clay loam	Sandy clay loam	Sandy clay loam	Sandy clay loam

4.2.1.1. Determination of chemical soil properties

Total C and N of soil and above-ground biomass were determined with a TruSpec® CN analyser (Leco Corp., St Joseph, MI). Cation exchange capacity (CEC) was measured according to Rhoades (1982) by flame photometry. Free amino acids (FAA) and hydrolysable protein content were measured in soil extracts (1:5 w/v soil-to-0.5 M K_2SO_4). FAA were determined by fluorescence assays according to the OPAME method of Jones et al. (2002). To determine soil solution protein content, the soil was subjected to acid hydrolysis under N_2 (Bremner, 1950) and the resulting amino acids concentration measured as FAA after neutralisation. Ammonium (NH_4^+) and nitrate (NO_3^-) concentrations were both determined colorimetrically according to Mulvaney (1996) and Miranda et al. (2001) respectively. Soluble phenolic compounds were measured in 1:5 (w/v) soil-to-distilled water extracts using the Folin-Ciocalteu reagent according to Swain and Hillis (1959). Soil pH and electrical conductivity (EC) were measured in 1:5 (w/v) soil:H2O extracts using standard electrodes.

4.2.1.2. Determination of biological soil properties

Soil microbial biomass (C and N) was determined by the chloroform fumigation-extraction method according to Vance et al. (1987) by measuring dissolved organic C (DOC) and total dissolved N (TDN) from fumigated and unfumigated soils using a Multi-N/C Series NPOC-TN analyser (Analytik Jena, Germany). Dissolved organic nitrogen (DON) was calculated as the difference between TDN and dissolved inorganic N. Basal respiration was measured at 20°C over 30 min using an EGM-5 CO_2 Gas Analyser (PP Systems, Amesbury, MA). N mineralisation was measured according to the anaerobic incubation procedure of Waring and Bremner (1964) and (Keeney, 1982). This procedure prevents nitrification and thus provides a good measure of ammonification rate (Mariano et al., 2013; Soon et al., 2007). Briefly, 2 g of fresh soil was placed in 20 cm^3 polypropylene containers and filled with deionised water to the top. Containers were shaken and a control set analysed immediately for NH_4^+ and NO_3^- as above by adding 1.875 g KCl to make a 1 M KCl extractant. The second set was incubated for 7 d at 40°C then analysed as per the control set.

4.2.1.3. Determination of physical soil properties

Gravimetric water content was determined by oven drying soil (105°C, 24 h). Bulk density was determined using 100 cm^3 stainless steel coring rings in the field as described in Rowell (1994).

4.2.2. Leucine aminopeptidase activity in soil

A leucine aminopeptidase assay was performed as a proxy for potential protease activity according to Vepsäläinen et al. (2001). Briefly, samples were extracted with deionised water (1:5 (w/v) soil:H2O) and 100 µl pipetted onto a 96 well plate. Subsequently, 100 µl of substrate (500 µM L-leucine 7-amino-4-methlycoumarin hydrochloride) was added to the sample. Standards were prepared for each sample by adding 100 µl of 7-amino-4-methylcoumarin (7-AMC) at different concentrations (0, 0.5, 1, 5, 10, 15 and 25 µM) to 100 µl of sample for quench correction. After a 3 h incubation at 30 °C, fluorescence was measured at an excitation wavelength of 335 nm and emission wavelength of 460 nm on a Cary Eclipse Fluorimeter (Agilent Corp., Santa Clara, CA). A calibration curve was fitted for each sample. Blank sample and substrate measurements were subtracted from the assay reading.

4.2.3. Protein and amino acid mineralisation in soil

The protein and amino acid mineralisation rates were measured as described in Jan et al. (2009). Uniformly ^{14}C-labelled protein from *Nicotiana tabacum* L. leaves (0.5 ml; 0.064 mg C l^{-1}; 0.0063 mg N l^{-1}; 2.0 kBq ml^{-1}; 3–100 kDa; custom produced by American Radiolabeled Chemicals, St Louis, MO) was secondary purified by ultrafiltration in an Amicon® stirred cell using a 3 kDa Ultracel® cut off membrane (Millipore UK Ltd., Watford, UK) to remove any oligopeptides and added to 50 ml polypropylene tubes with 5 g of field-moist soil (n = 3). To another set of 50 ml polypropylene tubes with 5 g of field-moist soil, a uniformly ^{14}C-labelled amino acid mixture (0.5 ml; 0.012 mg C l^{-1}; 0.0036 mg N l^{-1}; 2.0 kBq ml^{-1}; composed of: 8% Ala, 7% Arg, 8% Asp, 12.5% Glu, 4% Gly, 1.5% His, 6.5% Ile, 12.5% Leu, 6% Lys, 8% Phe, 5% Pro, 4% Ser, 5% Thr, 4% Tyr, 8% Val; PerkinElmer Inc., Waltham, MA) was added (n = 3). The addition of 0.5 ml of ^{14}C-labelled protein/amino acid mixture increased the initial water content of the field moist soil from an average of 0.37 g g^{-1} to 0.49 g g^{-1} (on a fresh weight basis). Protein was added in a slightly larger quantity to the soil than amino acid, in terms of C and N quantity, to more closely

replicate field conditions. As we do not know the actual rates of protein and amino acid input into these soils (and which is likely to vary by site), we chose to add the same trace amount to the soil. Essentially, this addition should not greatly alter the concentration of the native protein and amino acids pools and therefore act as a better tracer. Further, the amounts added are unlikely to induce microbial growth based on the size of the microbial biomass (Fig. 4.2). Peptide mineralisation was not measured in this study because our focus was on protein mineralisation although we recognise that this is a likely intermediate produced during protein breakdown. We did, however, use amino acid mineralisation as a comparator in this study. Previously, we have shown that amino acid and oligopeptide mineralisation rates are relatively similar in the soil used here (Farrell et al., 2011a). To capture the $^{14}CO_2$ evolved from the soil a 1 M NaOH trap (1 ml) was added to the tube and sealed (Jan et al., 2009). The soils were incubated in the dark at 10 °C to reflect average soil temperatures across the gradient in a LT-2 incubator (LEEC Ltd., Nottingham, UK). The NaOH traps were changed periodically over a 60 d period. The amount of $^{14}CO_2$ captured was determined after addition of Optiphase HiSafe3 scintillation fluid to the NaOH traps and ^{14}C determination using a Wallac 1414 scintillation counter with automated quench correction (PerkinElmer Inc.). The amount of ^{14}C label remaining in the soil after 60 d was determined by a two-step extraction. First, soil was extracted in deionised water (1:5 w/v soil-to-extractant ratio; 200 rev min^{-1}; 30 min), the samples centrifuged (18,000 *g*; 10 min) and the ^{14}C activity in the supernatant determined by liquid scintillation counting as described above. Secondly, after removal of the supernatant, the soil was re-extracted with 0.05 M Na-pyrophosphate (pH 7; 1:5 w/v soil-to-extractant ratio; 200 rev min^{-1}; 30 min; Greenfield et al., 2018) the extracts centrifuged (18,000 *g*; 10 min) and ^{14}C activity measured as above (Appendix 2, Table S1).

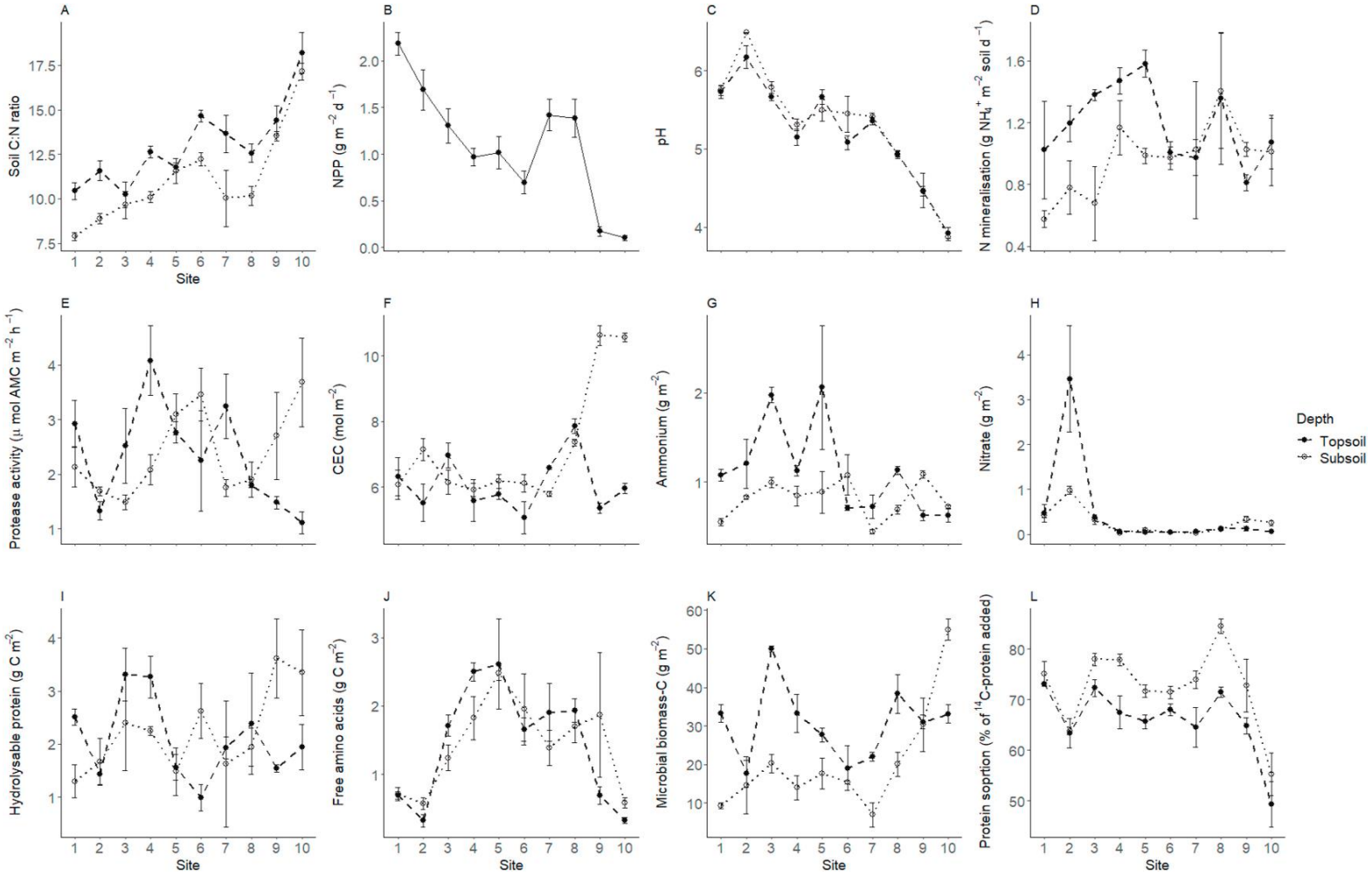

Figure. 4.2. Major characteristics of the grassland altitudinal catena sequence. A) soil C:N ratio, B) net primary productivity (NPP) (g m^{-2} d^{-1}), C) soil pH, D) N mineralisation (g NH_4^+ m^{-2} soil d^{-1}), E) leucine aminopeptidase activity (LAP) (μmol AMC m^{-2} h^{-1}), F) cation exchange capacity (CEC) (mol m^{-2}), G) ammonium (g m^{-2}), H) nitrate (g m^{-2}), I) hydrolysable protein (g C m^{-2}), J) total free amino acids (g C m^{-2}), K) microbial biomass-C (g m^{-2}), and L) protein sorption (% of ^{14}C-labelled protein added). Values represent mean ± SEM (*n* = 3).

4.2.5. Protein and amino acid sorption to soil

The sorption of protein and amino acid to the solid phase was determined by adding ^{14}C-labelled protein and ^{14}C-labelled amino acid (0.5 ml; 2 kBq ml^{-1}) to separate tubes of 1 g of heat-sterilised soil (80°C, 1 h) and incubation for 30 min at 20°C (Greenfield et al., 2018). Subsequently, the soils were shaken with 5 ml of deionised water (30 min; 200 rev min^{-1}), and an aliquot of 1.5 ml transferred to microfuge tubes and centrifuged (18,000 *g*, 5 min) and the supernatant recovered. The amount of ^{14}C recovered in the supernatant was determined as described above and the amount of sorption calculated by difference (Appendix 2, Fig. S1). We acknowledge that heat-sterilisation does not reduce leucine aminopeptidase activity and, thus, protein sorption will measure both protein and its depolymerisation products. However, a previous study found leucine aminopeptidase activity in the 30 min incubation period to be minimal (ca. 2–4 nmol AMC g^{-1} from the low altitudinal and high altitudinal site; Greenfield et al., 2018). In addition, the highest level of $^{14}CO_2$ production in unsterilised soils was ca. 2.7% of the ^{14}C-labelled protein added after 30 min (suggesting that the effect will be small in heat-sterilised soils).

4.2.6. Data and statistical analysis

Amino acid mineralisation was generally biphasic and, thus we described the process by a two-phase double first order kinetic decay model and, subsequently, calculated the half-life and carbon use efficiency (CUE) from the two pools (see Appendix 2 for full description of the calculations and rationale; Figs. S2–S3; Glanville et al., 2016). Protein mineralisation appeared triphasic, however, a kinetic decay model did not fit well because the model does not account for potential factors such as adsorption and desorption of protein to soil surfaces or the induction of soil protease production upon protein addition. Because we could not fit a kinetic decay model to protein mineralisation, we determined the initial rapid linear phase to be up to 3 h and the second slower quasi-linear phase as 39–60 d from Fig. 4.3 and Fig. 4.4. We used these rates in subsequent analysis to assess protein and amino acid mineralisation along the grassland altitudinal gradient. In contrast to the amino acid pool, we acknowledge that the actual levels of isotopic pool dilution are not known for the ^{14}C-labelled protein due to a lack of knowledge about the size, origin, diversity and degree of physical and chemical protection of the native soil protein pool. However, the use of trace levels of protein means their mineralisation rate should be described by the

first order component of the Michaelis-Menten kinetic curve (i.e. turnover rate versus protein concentration). As a similar argument can be made for the ^{14}C-labelled amino acids, we feel that the relative rates of amino acid and protein turnover can thus be compared against each other.

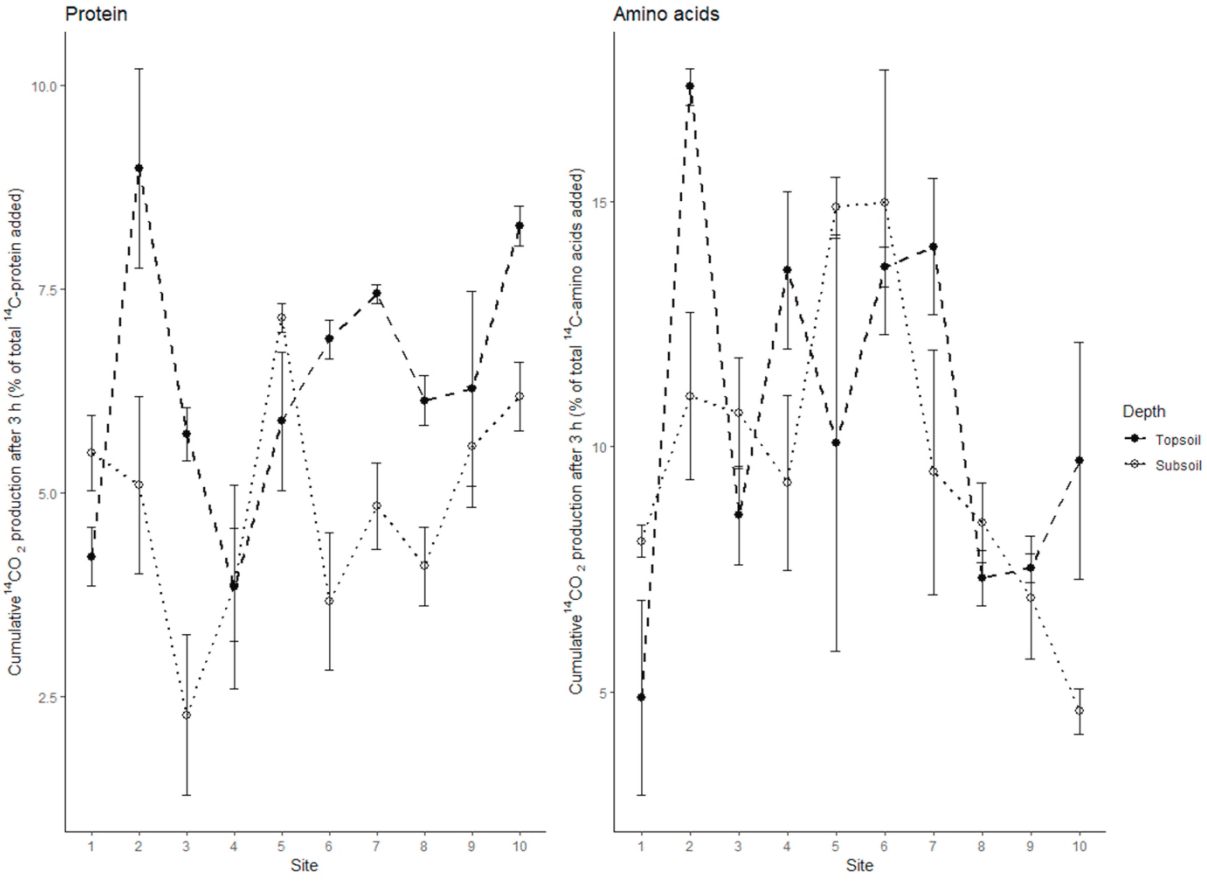

Figure 4.3. Cumulative $^{14}CO_2$ production arising from the mineralisation of ^{14}C-labelled protein (left) and amino acids (right) measured between 0 and 3 h (initial phase) for ten sites along the grassland altitudinal gradient in the topsoil and subsoil (expressed as a % of total ^{14}C-substrate added). Values represent mean ± SEM (*n* = 3).

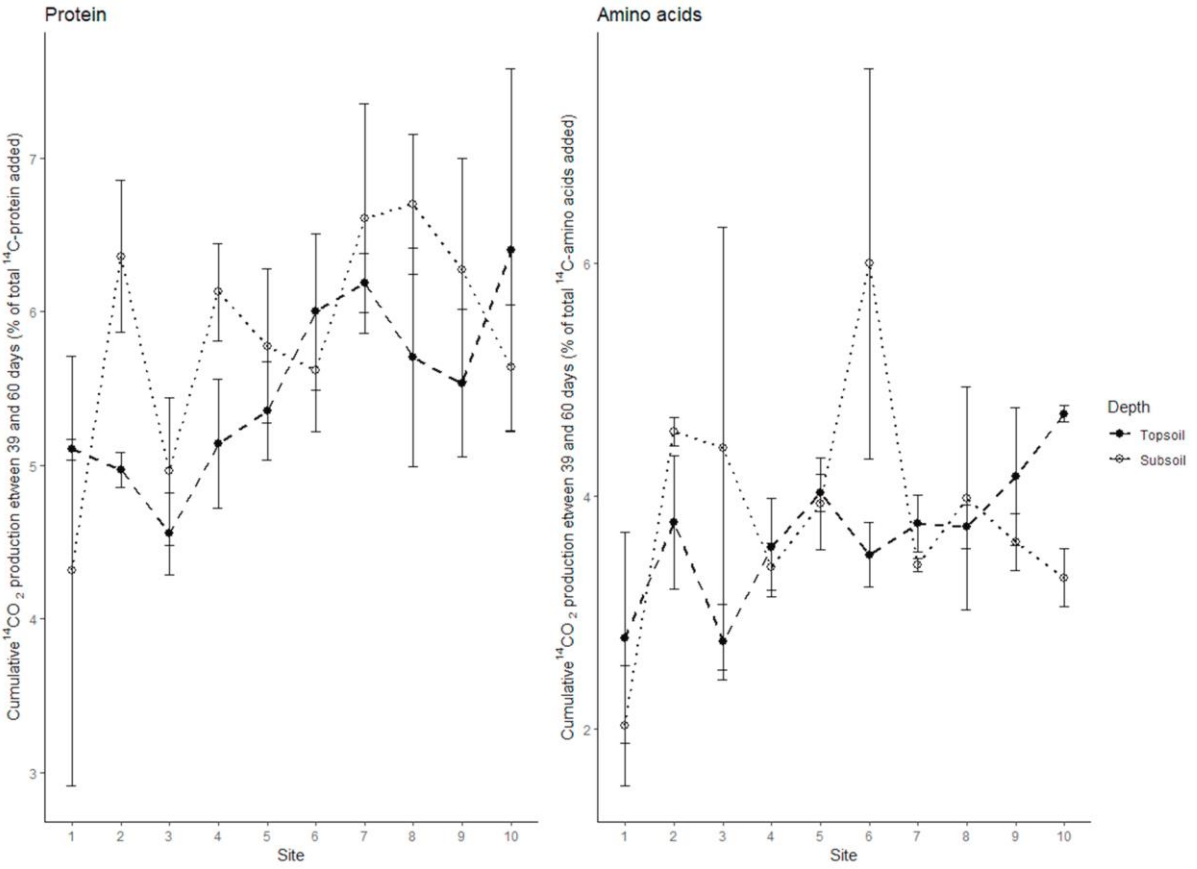

Figure 4.4. Cumulative $^{14}CO_2$ production arising from the mineralisation of ^{14}C-labelled protein (left) and amino acids (right) measured between 39 and 60 d (second, slower phase) for ten sites along the grassland altitudinal gradient in the topsoil and subsoil (expressed as a % of total ^{14}C-substrate added). Values represent mean ± SEM (n = 3).

All treatments were performed in triplicate. All statistical analyses were performed in R 3.5.0 (R Core Team, 2018). Normality of the data was determined by Shapiro-Wilk test ($p > 0.05$) and then visually checked using *qqnorm* plots. Data without a normal distribution was transformed to achieve normality. Homogeneity of variance of the data was determined by Bartlett test ($p > 0.05$) then visually checked using residuals vs. fitted plots. The impact of site and depth on cumulative $^{14}CO_2$ production for both protein and amino acid mineralisation were determined by two-way ANOVA for two time points, 0–3 h (initial phase of substrate mineralisation) and 39–60 d (second phase of substrate mineralisation). A two-way ANOVA was used to test soil parameters for differences with site and depth. A Kruskal-Wallis test was used to determine differences in soil properties between site and depth for data that did not meet the normality assumptions (i.e. the data was not normally distributed).

We explored how soil protein mineralisation rates were related to soil properties using correlation analyses in a way that was consistent with our schematic diagram (Fig. 4.1). Correlations were carried out using the Pearson's product moment correlation using the function *rcorr* in the *Hmisc* package (Harrell and Dupont, 2020). Significant correlations ($p < 0.05$) are presented in a correlation matrix using the function *corrplot* in the package *corrplot* (Wei and Simko, 2017). Multiple comparisons were not considered and *p* values for all correlation coefficients have been presented in Fig. S7.

4.3. Results

4.3.1. Soil properties along the grassland altitudinal gradient

We observed trends in the major characteristics of the grassland altitudinal gradient (Fig. 4.2). Above-ground net primary productivity (NPP), pH and protein sorption both showed a negative trend from the lowest to highest altitude site ($p < 0.0001$; Appendix 2, Table S2). Soil pH showed little difference between the topsoil and subsoil ($p = 0.12$; Appendix 2, Table S2). CEC showed no clear trend in the topsoil but fluctuated along the gradient, whilst, in the subsoil CEC varied from site 1 to site 8 when it nearly doubled to 10 (site: $p < 0.0001$ and depth: $p < 0.0001$; Appendix 2, Table S2). Nitrate spiked at site 2 but otherwise decreased between sites 1 and 10 by seven times in the topsoil and just under half in the subsoil ($p < 0.0001$; Appendix 2, Table S2) though the two depths were not significantly different ($p = 0.936$; Appendix 2, Table S2). Ammonium decreased by 0.46 g m^{-2} along the altitudinal gradient in the topsoil but increased by 0.17 g m^{-2} in the subsoil. However, the trends in ammonium varied within the middle of the gradient (site: $p < 0.0001$ and depth: $p = 0.004$; Appendix 2, Table S2). Protein-C, amino acid-C and microbial biomass-C were highly variable along the gradient; however, this was not significant for protein-C (Appendix 2, Table S2). Only microbial biomass-C showed differences between soil depths ($p < 0.0001$; Appendix 2, Table S2). N mineralisation increased along the first half of the gradient (sites 1–5) and varied between sites ($p = 0.15$; Appendix 2, Table S2). N mineralisation in the topsoil was ca. twice higher than the subsoil between sites 1–5 and then similar between the depths in the second half of the gradient ($p = 0.02$; Appendix 2, Table S2). Overall, leucine aminopeptidase activity varied significantly along the altitudinal gradient ($p < 0.0001$; Appendix 2,

Table S2). However, there was no significant difference in leucine aminopeptidase activity with soil depth (p = 0.41; Appendix 2, Table S2). Other soil properties (plant C:N, bulk density, EC, soil respiration, water content, total C, total N, DOC, DON, soluble phenolics) not used in the correlation analyses are presented in Appendix 2, Fig. S4.

4.3.2. Organic N mineralisation in soil

The overall rates of protein and amino acid mineralisation along the grassland altitudinal gradient in the topsoil and subsoil are presented in Appendix 2, Figs. S5 and S6 respectively. A rapid linear phase of mineralisation was observed up until 3 h after addition for both protein and amino acids (r^2 = 0.91 ± 0.01 and r^2 = 0.85 ± 0.01, respectively) (Fig. 4.3). After 3 h, the rate of mineralisation progressively declined until a second slower quasi-linear phase of mineralisation was observed from day 39 to day 60 when the experiment was terminated.

The initial phase of protein mineralisation (cumulative $^{14}CO_2$ production from ^{14}C-labelled protein after 3 h) doubled from site 1 to site 10 in the topsoil but varied between these sites (Fig. 4.3). There was no trend in the subsoil, but sites varied significantly (p = 0.0001; Table 4.2). Overall, the initial rate was lower in the subsoil compared to the topsoil (p = 0.0001; Table 4.2). The second slower rate (cumulative $^{14}CO_2$ production from ^{14}C-labelled protein between 39 and 60 d) did not show a clear trend along the altitudinal gradient or with depth (p = 0.12 and p = 0.21 respectively; Table 4.2; Fig. 4.4).

Table 4.2. Two-way ANOVA results for cumulative $^{14}CO_2$ production arising from the mineralisation of ^{14}C-labelled protein and ^{14}C-labelled amino acid mixture between 0-3 h and 39-60 d using $p < 0.05$ as the cut off for statistical significance (as indicated by values in bold).

			Site			Soil depth			Site × Soil depth		
Compound	**Time**	***Residuals***	***df***	***F***	***p***	***df***	***F***	***p***	***df***	***F***	***p***
Protein	0-3 h	40	9	5.27	**0.0001**	1	22.6	**0.0001**	9	3.44	**0.003**
	39-60 d	40	9	1.71	0.12	1	1.63	0.21	9	0.80	0.62
Amino acids	0-3 h	39	9	5.96	**0.0001**	1	1.41	0.24	9	2.56	**0.02**
	39-60 d	37	9	2.76	**0.014**	1	0.59	0.45	9	1.10	0.39

Note: *df* = degrees of freedom, *F* = *F* value and *p* = *p* value

The initial phase of amino acid mineralisation doubled in rate along the altitudinal gradient but halved in the subsoil (Fig. 4.3). However, between sites 1 and 10 the initial rate varied significantly ($p < 0.0001$; Table 4.2). The initial rate varied at each depth and was not significant ($p = 0.24$; Table 4.2). The second phase of amino acid mineralisation did not show an obvious trend in rate along the altitudinal gradient (Fig. 4) but the variation between sites was significant ($p = 0.014$, Table 4.2). The differences between the second rate of amino acid mineralisation and soil depth were not significant ($p = 0.45$, Table 4.2). Carbon use efficiency (CUE) was highest at sites 1 and 8–10 (between 0.88 and 0.91) but declined in the middle of the altitudinal gradient (Two-way ANOVA: $F_{(9,39)} = 4.4$, $p = 0.0005$; Appendix 2, Fig. S3). There was little difference in CUE between the topsoil and subsoil (Two-way ANOVA: $F_{(1,39)} = 0.2$, $p = 0.66$; Appendix 2, Fig. S3).

A test to determine the binding of protein to soil surfaces showed that sorption of ^{14}C-labelled protein varied along most of the altitudinal gradient except from site 10 which was ca. 25% lower in the topsoil and subsoil (Two-way ANOVA: $F_{(9,40)} = 16.4$, $p < 0.0001$ and $F_{(1, 40)} = 32.7$, $p < 0.0001$ for site and depth respectively; Appendix 2, Fig. S1). In contrast, sorption of total amino acids showed no trend from site 1 to site 10 or with soil depth (Two-way ANOVA: $F_{(9,38)} = 1.5$, $p = 0.20$ and $F_{(1, 38)} = 4.1$, $p = 0.5$ for site and depth respectively; Appendix 2, Fig. S1). Overall, the sorption of protein was 2.2-fold greater than for amino acids ($p < 0.001$).

4.3.3. Effect of soil properties on protein mineralisation rates

Associations between soil properties and protein mineralisation rates differed between the topsoil and subsoil (Fig. 4.5). In the topsoil, there were no significant correlations between amino acid mineralisation rates and any of the soil properties measured. The initial phase of protein mineralisation (0–3 h) had moderate, positive correlations with ammonium concentration, C:N ratio and N mineralisation. The slower phase of protein mineralisation (39–60 d) had moderate, negative correlations with ammonium and nitrate concentration and strong, negative correlations with above-ground NPP and pH.

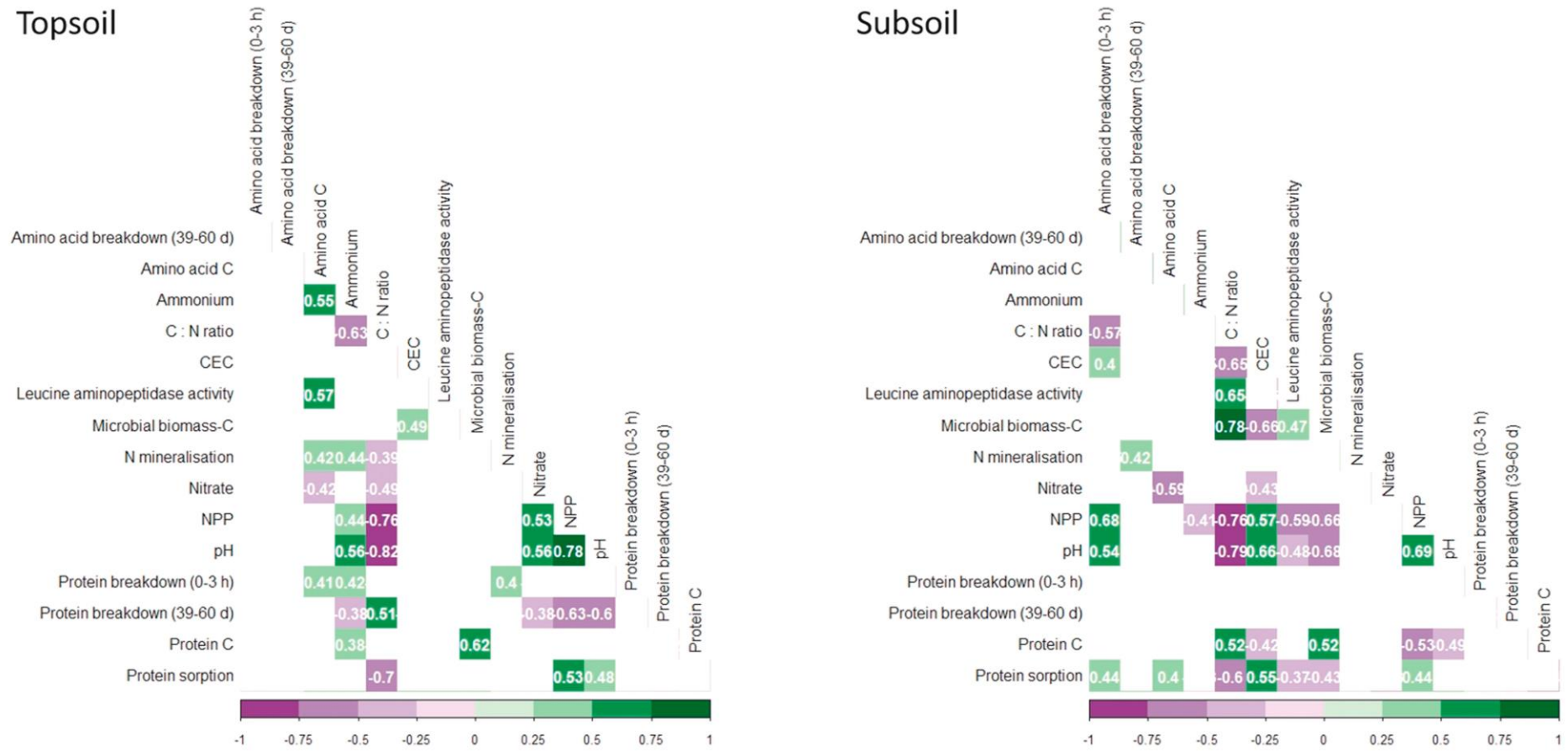

Figure 4.5. Correlation matrix of soil properties and protein mineralisation rates with significance of $p < 0.05$ in the topsoil (left) and subsoil (right). No corrections were made for the *p* values to account for multiple comparisons (see Appendix 2, Fig. S7 for *p* values). Values and colour of the squares represent correlation coefficients.

In the subsoil, there were no significant correlations between protein mineralisation rates and any of the measured soil properties. The initial phase of amino acid mineralisation (0–3 h) had a moderate, negative correlation with soil C:N ratio and moderate positive correlation with CEC, pH and protein sorption. There was a strong, positive correlation with above-ground NPP. The slower phase of amino acid mineralisation (39–60 days) had a moderate, positive correlation with N mineralisation.

4.4. Discussion

4.4.1. Rates of protein mineralisation along a grassland altitudinal gradient

The mineralisation of ^{14}C-labelled protein to $^{14}CO_2$ did not conform well to a classic biphasic first order kinetic model as is typically observed for common low molecular weight solutes in soil (e.g. sugars, organic acids, amino acids; Glanville et al., 2016). This suggests that additional steps occurred during protein mineralisation which were not captured in the kinetic model (e.g. sorption/desorption reactions, up and down-regulation in microbial protease gene expression). While studies have shown that microorganisms can take up small proteins (Whiteside et al., 2009 and references therein), most proteins require some degree of depolymerisation before transportation across cell membranes. The ^{14}C-labelled protein added to the soil consisted of a heterogeneous mixture of proteins ranging from 3 to 100 kDa, therefore, the initial rapid phase may represent the direct uptake of these small proteins followed by a slower phase in which extracellular proteases break down the larger proteins into oligopeptides and amino acids that microorganisms can directly assimilate. It may also reflect the slower mineralisation of proteins bound to the solid phase. After incorporation of the protein-derived-C into the microbial cell, the final mineralisation phase reflects the slow turnover of the microbial biomass during cell maintenance and necromass turnover. Protein mineralisation into oligopeptides and amino acids is typically considered to be the rate limiting step in soil N mineralisation (Jones et al., 2005), yet our study showed relatively similar rates of amino acid and protein turnover when assayed independently. In contrast to these other studies using single animal-derived proteins, our study found no evidence for a lag phase in protein mineralisation, indicating that no *de novo* synthesis of proteases was required to facilitate protein mineralisation (Jan et al., 2009). We ascribe this to the 100 to 1000-fold greater amount of protein used in previous studies in comparison to ours. The unexpectedly large input of protein in these other studies is likely to have induced saturation of the

intrinsic soil protease pool, leading to up-regulation of microbial protease genes and activity in soil, facilitating more rapid use of the resource. This classic substrate-induced respiration response (and associated lag-phase) is well established in soil studies (Blagodataskaya et al., 2010). The amount of protein-C added here (6.4 μg C kg^{-1}) was also well below the critical growth threshold of added C that is needed to induce growth and produce a lag-phase response (200 mg C kg^{-1}; Reischke et al., 2015). It is also possible that the rapid microbial mineralisation of protein observed here reflects the soluble nature of the plant protein used. In comparison to insoluble protein held in SOM, we hypothesise that soluble proteins have a relatively high bioavailability due to their high rates of diffusion in soil solution and potentially less sorption to the solid phase (Quiquampoix et al., 1995). A caveat to our study is that it does not reflect the mineralisation of insoluble proteins which are also abundant in plant cells (e.g. actin, tubulin, membrane proteins) and in SOM.

Our analysis only directly compares the rates of protein and amino acid mineralisation. It did not explicitly consider oligopeptides as an intermediate in the protein breakdown pathway. We note that oligopeptides produced during proteolysis may be taken up directly by the microbial community, thus avoiding the amino acid pool completely. At present, the relative importance of amino acid vs. peptide uptake during protein breakdown remains unknown, however, it is likely that both occur simultaneously as both terminal amino acids and oligopeptides are released during protein breakdown. The comparatively similar rates of protein and amino acid mineralisation observed here suggests that peptidase activity is also not a highly rate limiting process. Further, based on studies across a wide range of soils it is likely that any oligopeptides produced will be rapidly taken up by the soil microbial community, bypassing the need for depolymerisation of oligopeptides (Farrell et al., 2013).

The slower rate of protein mineralisation in the subsoil compared to the topsoil was as we hypothesised. Inputs of C (e.g. from plant roots) into the subsoil are lower and, therefore, microbial biomass-C is less abundant (Loeppmann et al., 2016). Microorganisms utilise the C and N from protein in the soil and, so, a smaller biomass results in lower turnover rates. However, the difference between topsoil and subsoil was not observed in the slower phase of mineralisation between 39 and 60 d (i.e. C immobilised in the biomass). This suggests that topsoil and subsoil microbial communities have similar rates of turnover (Glanville et al., 2016).

Our hypothesis that protein mineralisation rates decreased with altitude is inconsistent with our results. Although protein mineralisation rates differ along the gradient, there was no clear altitudinal trend. Altitude has an indirect influence on soil properties which are driven by other parameters that vary with altitude (Warren, 2017). Parameters include; biological factors e.g. net primary productivity; chemical factors e.g. C and N compounds and concentrations and; physical factors e.g. temperature and soil moisture. We expected that the low altitude grassland sites would have a higher primary productivity with increased plant inputs and higher microbial activity resulting in higher rates of organic N mineralisation. Despite seeing higher primary productivity in the lower altitude sites, they did not correspond to an increase in protein mineralisation rates. It should be noted that we constrained some environmental variables during the experiment (e.g. temperature), so our measurements represent potential protein mineralisation rates rather than actual protein mineralisation rates. Based on the range in temperature across our altitudinal gradient (3.7°C), and assuming a Q_{10} value of 1.7 (Hill et al., 2014), this would only equate to a reduction in microbial enzyme reaction rates of ca. 20% from Site 1 to Site 10, and thus unlikely to greatly alter our conclusions.

Consistent with previous reports, amino acid mineralisation in the soil followed a biphasic pattern. The initial, rapid linear phase of mineralisation up to 3 h corresponds to metabolism of labile C for energy production. The second, slower phase between 39 and 60 d represents the turnover of amino acid-derived-C immobilised in the microbial biomass (Glanville et al., 2016). The initial rapid phase of amino acid mineralisation was twice as fast as protein. If the protein and amino acid pool sizes in soil were the same size, this would suggest that protein mineralisation is a slight bottleneck in the processing of soil organic N. Given the uncertainties in measuring soil protein content (Roberts and Jones, 2008) and thus isotopic pool dilution, it should be noted that this bottleneck may not exist if the protein pool is more than twice the size of the amino acid pool. Overall, we observed few differences between topsoil and subsoil rates of amino acid mineralisation. It is possible that the cut off between topsoil and subsoil at 15 cm was too high to capture differences in soil properties, especially at deeper depths where no roots are present, and the microbial community may be much more C limited. Studies have shown a large variability in the location of the topsoil-subsoil boundary, depending on what soil property is measured (de Sosa et al., 2018; Jones et al., 2018a, Jones et al., 2018b; Loeppmann et al.,

2016a). Future studies may therefore consider separating topsoil from subsoil based on pedogenic horizon rather than depth *sensu stricto*.

As with protein mineralisation, we did not observe a clear decrease in amino acid mineralisation rates along the grassland altitudinal gradient. This is consistent with previous studies measuring amino acid turnover across a global latitudinal gradient (Jones et al., 2009). Microbial CUE of amino acids was high along the entire altitudinal gradient indicating that microorganisms were predominantly using the C for anabolic processes and that the community was C limited at all sites (Geyer et al., 2019). Despite the wide variation in soil type, CUE only varied by ca. 10%, similar to the variability in amino acid mineralisation rates. This low variability in CUE is consistent with previous studies which suggest that the metabolic pathways for amino acid-C use are very similar between soils (Jones et al., 2018a, Jones et al., 2018b).

4.4.2. Effect of soil properties on protein mineralisation

Factors affecting protein mineralisation rates differed between the topsoil and subsoil in our study. Most interestingly, we found no strong associations between the soil properties measured in this study and the rate of protein mineralisation in the subsoil. Similarly, there were no associations between soil properties and the rate of amino acid mineralisation in the topsoil. This suggests that the mechanisms that limit the mineralisation of these two compounds (protein and amino acids) depend on soil depth. Our study indicates that protein mineralisation in the topsoil is associated with the availability of NH_4^+, NO_3^-, amino acids, soil C:N ratio, N mineralisation rate, above-ground NPP and pH, but not in the subsoil. In addition, the main influential drivers of protein mineralisation rate varied in strength with the phase of protein mineralisation (i.e. initial microbial usage phase and the slower microbial turnover phase). Thus, interactions and soil properties that we did not measure also influence protein mineralisation. Therefore, the inability of single soil parameters to determine protein mineralisation consistently leads us to conclude that the regulation of protein mineralisation is both multi-factorial and site-specific. This implies that it will be difficult to accurately parameterise models describing protein turnover and N cycling in soil.

Microorganisms are well adapted to their environment to compete and survive in their ecological niche. For example, a recent study by Puissant et al. (2019) has shown both bacterial and fungal community composition differs in soils at pH 5 and 7 and that the optimal pH for leucine aminopeptidase activity was close to native soil pH

(i.e. functional enzyme adaptation). In addition, a study by Koch et al. (2007) demonstrated that microbial extracellular enzymes involved in C and N mineralisation were adapted to the temperature of their environment. Noll et al. (2019) also found no association between peptidase activity and protein mineralisation rates but showed clear differences between sites (i.e. land use, soil pH and mineralogy) and mineralisation rates. In addition, this was observed by Hu et al. (2020) when measuring the mineralisation of microbial-derived protein. Therefore, microbial community composition and adaptation, shaped by combination of soil and environmental parameters, may exert a stronger influence on mineralisation than specific soil/environmental parameters.

Our experiment was run at the average temperature across the grassland altitudinal gradient thus not encompassing the range of temperatures across the sites. It is likely that substrate availability varies with temperature which will not be captured by our experiment (Kirschbaum, 2006). Furthermore, our *ex situ* assays may not have fully captured the role of rhizosphere microorganisms in protein mineralisation by removal of plant C supply. In addition, our assays do not capture the role of large mesofauna (e.g. earthworms) which are abundant at some locations and whose contribution to SOM turnover is well established (Zeibich et al., 2018). In the topsoil, NH_4^+ and amino acid content and N mineralisation were the main factors which correlated best with the initial rate of protein mineralisation. The positive association of N mineralisation with protein mineralisation rate suggests that protein mineralisation is related to the machinery that drives the process (i.e. protease and microorganism abundance) which in turn is associated to the concentration of intermediate and end products (i.e. amino acids and ammonium). Although we did not measure peptide production and their subsequent use by the microbial community, current evidence from these soils suggest that this process is similarly rapid to amino acid mineralisation (Farrell et al., 2011b). To confirm this would require more mechanistic studies using ^{15}N and ^{13}C isotope pool dilution studies.

With respect to the second, slower phase of protein mineralisation, C:N ratio and soil pH appear to be important influential factors of the rate of protein mineralisation. The association between pH and the rate of protein mineralisation was as we predicted; a more acidic pH is associated with a higher rate of protein mineralisation. The relationship between the soil pH and the isoelectric point (IEP) of a protein determines its availability: below the IEP, proteins unfold on soil mineral

surfaces inhibiting enzyme activity, around the IEP, proteins are adsorbed without effect on their function and above the IEP, less proteins are adsorbed allowing diffusion in soil solution (Quiquampoix et al., 1993). In plants, the IEP ranges from 1.99 to 13.96 and have a triphasic distribution, however, proteins with an acidic IEP (ca. 5.6) are slightly more abundant than proteins with a basic IEP (ca. 8.37; Mohanta et al., 2019). Based on this broad pattern, we would expect the highest protein sorption onto mineral surfaces to occur at the highest altitudinal sites where soil pH is the most acidic. Our results suggest a more neutral pH is associated with higher protein sorption. It is likely that the loose trend in plant protein IEP values is too generalised to predict trends of protein sorption onto clay mineral surfaces. Furthermore, sorption of protein to organic matter follows different patterns than those of mineral surfaces and the mechanisms of sorption are less known due to the vast variety of organic matter in soils (Nannipieri et al., 1996). Alternatively, a different mechanism could explain why a more acidic pH is associated with higher protein mineralisation rates. Soil pH can be considered as a 'master variable' controlling microbial community composition and metabolism as well as protein stabilisation (Aciego Pietri and Brookes, 2009; Jones et al., 2019). Thus, an alternate mechanism like a changing microbial community composition and CUE with soil pH could be a reason for the association between pH and protein mineralisation rates we observed. Further metagenomic and transcriptomic studies are therefore warranted to better explore the relationships between protein mineralisation, microbial community structure and the diversity and expression of proteases produced by this community.

In the subsoil, C:N ratio, CEC, above-ground NPP, pH and protein sorption appeared to be associated with the initial phase of amino acid mineralisation rates. It is interesting that amino acid mineralisation correlated well with above-ground NPP considering we would not expect a direct connection between the above-ground biomass and the subsoil, and particularly as no correlation was seen between NPP and mineralisation rates in the topsoil. Whilst in the slower phase of amino acid mineralisation, only N mineralisation was found to be associated with amino acid mineralisation rates from the soil properties measured in this study. No other correlations were observed with N mineralisation suggesting that properties influencing this process have been missed from this study.

4.4.3. Is protein supply rather than protein turnover the key factor regulating N turnover in soil?

Our study was predicated on the assumption that protein mineralisation in soil would be limited by a range of edaphic factors. Further, we assumed based on previous studies that these factors would influence amino acid turnover in soil to a much lesser extent (i.e. the bottleneck in N cycling was the transformation of protein into amino acids). All the evidence presented here suggests that when added at low concentrations to label the native pool, the turnover rate of soluble protein is rapid and relatively similar to that of amino acids. This strongly implies that N supply in soil is not related to protein depolymerisation rate *per se*, but rather to the rate of protein supply from plant and microbial turnover. As the rates of microbial biomass turnover were similar between our soils, we therefore assume that NPP and subsequent root/shoot turnover are the primary regulator of N supply, rather than protease activity. We do note, however, that above-ground (shoot) and below-ground (roots and associated symbionts) productivity may not always be linked and here we only measured the former (Poeplau, 2016). To some extent this is supported by the very low rates of protein-N accumulation in soil when considered over their pedogenic lifespan of our soils (ca. <5 mg N m^{-2} y^{-1}), especially in comparison to annual rates of above-ground vegetation turnover estimated across our gradient (ca. 1–27 g N m^{-2} y^{-1}). Therefore, we conclude that future studies of organic N turnover should place more emphasis on measuring the actual rates and types of protein entering soil and their use by the microbial community, preferably using isotope tracing and pool dilution techniques (Charteris, 2019; Noll et al., 2019; Reay et al., 2019), rather than relying on proxies such as exoenzyme activities. In addition, in light of the evidence that C inputs from root and arbuscular mycorrhizal turnover can be very large in grasslands (Van Ginkel et al., 1997), the focus should be on net belowground productivity.

4.5. Conclusions

Our results suggest that rates of soluble protein and amino acid mineralisation in soils are similar and that protease is not a major factor limiting the turnover. This is consistent with the finding that phosphatase activity does not limit the use of soluble organic P by the microbial community (Fransson and Jones, 2007). It is also clear that protease activity is affected by a range of edaphic properties, but that none of these have an overriding influence on protein degradation. Rather amino acid and protein

turnover seem to be affected by a range of interacting factors whose importance is dependent on location, substrate type and soil depth. The finding that single soil parameters proved to be poor predictors of protein mineralisation contrasts strongly with other key steps in the soil N cycle (e.g. NO_3^- and N_2O production) which can be modelled using only a small number of soil variables (e.g. pH, organic-C, moisture status). It is possible that this discrepancy can be explained by the large degree of functional redundancy in the microbial community and adaptation of microorganisms and associated proteases to their ecological niche. Based on our results, we hypothesise that differences in soil N cycling and the generation of NH_4^+ supply are more related to the rate of protein supply rather than protein turnover *per se*.

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4.7. References

Aciego Pietri, J.C., Brookes, P.C., 2009. Substrate inputs and pH as factors controlling microbial biomass, activity and community structure in an arable soil. Soil Biology & Biochemistry 41, 1396–1405.

Allison, S.D., Vitousek, P.M., 2005. Responses of extracellular enzymes to simple and complex nutrient inputs. Soil Biology & Biochemistry 37, 937–944.

Bingham, A.H., Cotrufo, M.F., 2016. Organic nitrogen storage in mineral soil: Implications for policy and management. Science of the Total Environment 551–552, 116–126.

Blagodataskaya, E., Blagodatsky, S., Dorodnikov, M., Kuzyakov, Y., 2010. Elevated atmospheric CO_2 increases microbial growth rates in soil: results of three CO_2

enrichment experiments. Global Change Biology 16, 836–848.
Boyd, S.A., Mortland, M.M., 1990. Enzyme interactions with clays and clay-organic matter complexes, In: Bollag, J.., Stotzky, G. (Eds.), Soil Biochemistry: Volume 6. Marcel Dekker, Inc., New York, USA, p. 584.
Bremner, J., 1950. The amino-acid composition of the protein material in soil. Biochemical Journal 47, 538–542.
Burns, R.G., 1982. Enzyme activity in soil: Location and a possible role in microbial ecology. Soil Biology & Biochemistry 14, 423–427.
Charteris, A.F., 2019. Compound-specific amino acid ^{15}N stable isotope probing of nitrogen assimilation by the soil microbial biomass using gas chromatography-combustion-isotope ratio mass spectrometry, In: ^{15}N tracing of microbial assimilation, partitioning and transport of fertilisers in grassland soils. Springer Theses (Recognizing Outstanding Ph.D. Research), Springer, Cham, pp. 57–78.
de Sosa, L.L., Glanville, H.C., Marshall, M.R., Schnepf, A., Cooper, D.M., Hill, P.W., Binley, A., Jones, D.L., 2018. Stoichiometric constraints on the microbial processing of carbon with soil depth along a riparian hillslope. Biology & Fertility of Soils 54, 949–963.
Farrell, M., Hill, P.W., Farrar, J., Bardgett, R.D., Jones, D.L., 2011a. Seasonal variation in soluble soil carbon and nitrogen across a grassland productivity gradient. Soil Biology & Biochemistry 43, 835–844.
Farrell, M., Hill, P.W., Farrar, J., DeLuca, T.H., Roberts, P., Kielland, K., Dahlgren, R., Murphy, D. V., Hobbs, P.J., Bardgett, R.D., Jones, D.l., 2013. oligopeptides represent a preferred source of organic n uptake: A global phenomenon? Ecosystems 16, 133–145.
Farrell, M., Hill, P.W., Wanniarachchi, S.D., Farrar, J., Bardgett, R.D., Jones, D.L., 2011b. Rapid peptide metabolism: A major component of soil nitrogen cycling? Global Biogeochemical Cycles 25, 3014.
Farrell, M., Prendergast-Miller, M., Jones, D.L., Hill, P.W., Condron, L.M., 2014. Soil microbial organic nitrogen uptake is regulated by carbon availability. Soil Biology & Biochemistry 77, 261–267.
Fierer, N., Allen, A.S., Schimel, J.P., Holden, P.A., 2003. Controls on microbial CO_2 production: a comparison of surface and subsurface soil horizons. Global Change Biology 9, 1322–1332.
Fransson, A.M., Jones, D.L., 2007. Phosphatase activity does not limit the microbial

use of low molecular weight organic-P substrates in soil. Soil Biology & Biochemistry 39, 1213–1217.

Geisseler, D., Horwath, W.R., 2008. Regulation of extracellular protease activity in soil in response to different sources and concentrations of nitrogen and carbon. Soil Biology & Biochemistry 40, 3040–3048.

Geyer, K.M., Dijkstra, P., Sinsabaugh, R., Frey, S.D., 2019. Clarifying the interpretation of carbon use efficiency in soil through methods comparison. Soil Biology & Biochemistry 128, 79–88.

Giagnoni, L., Magherini, F., Landi, L., Taghavi, S., Modesti, A., Bini, L., Nannipieri, P., Van der Lelie, D., Renella, G., 2011. Extraction of microbial proteome from soil: potential and limitations assessed through a model study. European Journal of Soil Science 62, 74–81.

Glanville, H.C., Hill, P.W., Schnepf, A., Oburger, E., Jones, D.L., 2016. Combined use of empirical data and mathematical modelling to better estimate the microbial turnover of isotopically labelled carbon substrates in soil. Soil Biology & Biochemistry 94, 154–168.

Greenfield, L.M., Hill, P.W., Paterson, E., Baggs, E.M., Jones, D.L., 2018. Methodological bias associated with soluble protein recovery from soil. Scientific Reports 8, 11186.

Harrell, F.E.J., Dupont, C., 2020. Hmisc: Harrell Miscellaneous. R package version 4.4-0. Avaliable from https://CRAN.R-project.org/package=Hmisc.

Hill, P.W., Garnett, M.H., Farrar, J., Iqbal, Z., Khalid, M., Soleman, N., Jones, D.L., 2014. Living roots magnify the response of soil organic carbon decomposition to temperature in temperate grassland. Global Change Biology 21, 1368–1375.

Hill, P.W., Jones, D.L., 2019. Plant-microbe competition: does injection of isotopes of C and N into the rhizosphere effectively characterise plant use of soil N? New Phytologist 221, 796–806.

Hu, Y., Zheng, Q., Noll, L., Zhang, S., Wanek, W., 2020. Direct measurement of the in situ decomposition of microbial-derived soil organic matter. Soil Biology & Biochemistry 141, 107660.

Hu, Y., Zheng, Q., Zhang, S., Noll, L., Wanek, W., 2018. Significant release and microbial utilization of amino sugars and D-amino acid enantiomers from microbial cell wall decomposition in soils. Soil Biology & Biochemistry 123, 115–125.

Jan, M.T., Roberts, P., Tonheim, S.K., Jones, D.L., 2009. Protein breakdown represents a major bottleneck in nitrogen cycling in grassland soils. Soil Biology & Biochemistry 41, 2272–2282.

Jones, D., Owen, A.G., Farrar, J.F., 2002. Simple method to enable the high resolution determination of total free amino acids in soil solutions and soil extracts. Soil Biology & Biochemistry 34, 1893–1902.

Jones, D.L., Cooledge, E.C., Hoyle, F.C., Griffiths, R.I., Murphy, D. V., 2019. pH and exchangeable aluminum are major regulators of microbial energy flow and carbon use efficiency in soil microbial communities. Soil Biology & Biochemistry 138, 107584.

Jones, D.L., Healey, J.R., Willett, V.B., Farrar, J.F., Hodge, A., 2005. Dissolved organic nitrogen uptake by plants—an important N uptake pathway? Soil Biology & Biochemistry 37, 413–423.

Jones, D. L., Hill, P.W., Smith, A.R., Farrell, M., Ge, T., Banning, N.C., Murphy, D. V., 2018. Role of substrate supply on microbial carbon use efficiency and its role in interpreting soil microbial community-level physiological profiles (CLPP). Soil Biology & Biochemistry 123, 1–6.

Jones, D.L., Kielland, K., 2012. Amino acid, peptide and protein mineralization dynamics in a taiga forest soil. Soil Biology & Biochemistry 55, 60–69.

Jones, D.L., Kielland, K., Sinclair, F.L., Dahlgren, R.A., Newsham, K.K., Farrar, J.F., Murphy, D. V., 2009. Soil organic nitrogen mineralization across a global latitudinal gradient. Global Biogeochemical Cycles 23, 1016.

Jones, D.L., Magthab, E.A., Gleeson, D.B., Hill, P.W., Sánchez-Rodríguez, A.R., Roberts, P., Ge, T., Murphy, D.V., 2018. Microbial competition for nitrogen and carbon is as intense in the subsoil as in the topsoil. Soil Biology & Biochemistry 117, 72–82.

Keeney, D.R., 1982. Nitrogen availability indices, in: Page, A.L., Miller, R.H., Keeny, D.R. (Eds.), Methods of Soil Analysis: Chemical and Microbiological Properties. Soil Science Society of America, Madison, WI, USA, pp. 711–733.

Kirschbaum, M.U.F., 2006. The temperature dependence of organic-matter decomposition - Still a topic of debate. Soil Biology & Biochemistry 38, 2510–2518.

Koch, O., Tscherko, D., Kandeler, E., 2007. Temperature sensitivity of microbial respiration, nitrogen mineralization, and potential soil enzyme activities in

organic alpine soils. Global Biogeochemical Cycles 21, 4017.

Liu, Q., Qiao, N., Xu, X., Xin, X., Han, J.Y., Tian, Y., Ouyang, H., Kuzyakov, Y., 2016. Nitrogen acquisition by plants and microorganisms in a temperate grassland. Scientific Reports 6, 22642.

Loeppmann, S., Blagodatskaya, E., Pausch, J., Kuzyakov, Y., 2016. Enzyme properties down the soil profile - A matter of substrate quality in rhizosphere and detritusphere. Soil Biology & Biochemistry 103, 274–283.

Lutzow, M. v., Kogel-Knabner, I., Ekschmitt, K., Matzner, E., Guggenberger, G., Marschner, B., Flessa, H., 2006. Stabilization of organic matter in temperate soils: mechanisms and their relevance under different soil conditions - a review. European Journal of Soil Science 57, 426–445.

Manrique, L.A., Jones, C.A., Dyke, P.T., 1991. Predicting cation-exchange capacity from soil physical and chemical properties. Soil Science Society of America Journal 55, 787–794.

Mariano, E., Jones, D.L., Hill, P.W., Trivelin, P.C.O., 2016. Mineralisation and sorption of dissolved organic nitrogen compounds in litter and soil from sugarcane fields. Soil Biology & Biochemistry 103, 522–532.

Mariano, E., Trivelin, P.C.O., Leite, J.M., Megda, M.X.V., Otto, R., Franco, H.C.J., 2013. Métodos de incubação para avaliar o nitrogênio mineralizável em solos cultivados com cana-de-açúcar. Revista Brasileira de Ciencia Do Solo 37, 450–461.

Miranda, K.M., Espey, M.G., Wink, D.A., 2001. A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. Biology and Chemistry 5, 62–71.

Mohanta, T.K., Khan, A., Hashem, A., Abdallah, E.F., Al-Harrasi, A., 2019. The molecular mass and isoelectric point of plant proteomes. BMC Genomics 20, 631.

Mulvaney, R.L., 1996. Nitrogen - Inorganic Forms, in: Methods of Soil Analysis. Part 3 - Chemical Methods. Soil Science Society of America, Madison, WI, USA, pp. 1123–1200.

Nannipieri, P., Sequi, P., Fusi, P., 1996. Humus and enzyme activity, in: Piccolo, A. (Ed.), Humic Substances in Terrestrial Ecosystems. Elsevier, Amsterdam, The Netherlands, pp. 293–328.

Noll, L., Zhang, S., Zheng, Q., Hu, Y., Wanek, W., 2019. Wide-spread limitation of

soil organic nitrogen transformations by substrate availability and not by extracellular enzyme content. Soil Biology & Biochemistry 133, 37–49.

Philippot, L., Raaijmakers, J.M., Lemanceau, P., van der Putten, W.H., 2013. Going back to the roots: the microbial ecology of the rhizosphere. Nature Reviews Microbiology 11, 789–799.

Poeplau, C., 2016. Estimating root: shoot ratio and soil carbon inputs in temperate grasslands with the RothC model. Plant and Soil 407, 293–305.

Puissant, J., Jones, B., Goodall, T., Mang, D., Blaud, A., Gweon, H.S., Malik, A., Jones, D.L., Clark, I.M., Hirsch, P.R., Griffiths, R., 2019. The pH optimum of soil exoenzymes adapt to long term changes in soil pH. Soil Biology & Biochemistry 138, 1076012.

Quiquampoix, H., Abadie, J., Baron, M.H., Leprince, F., Matumoto-Pintro, P.T., Ratcliffe, R.G., Staunton, S., 1995. Mechanisms and consequences of protein adsorption on soil mineral surfaces, in: Horbett, T.., Brash, J.L. (Eds.), Proteins at Interfaces II. Fundamentals and Applications. American Chemical Society, Washington, DC, pp. 321–333.

Quiquampoix, H., Staunton, S., Baron, M.-H., Ratcliffe, R.G., 1993. Interpretation of the pH dependence of protein adsorption on clay mineral surfaces and its relevance to the understanding of extracellular enzyme activity in soil. Colloids and Surfaces A: Physicochemical and Engineering Aspects 75, 85–93.

R Core Team, 2018. R: A language and environment for statistical computing.

Ramírez-Sánchez, O., Pérez-Rodríguez, P., Delaye, L., Tiessen, A., 2016. Plant proteins are smaller because they are encoded by fewer exons than animal proteins. Genomics, Proteomics and Bioinformatics 14, 357–370.

Razavi, B.S., Zarebanadkouki, M., Blagodatskaya, E., Kuzyakov, Y., 2016. Rhizosphere shape of lentil and maize: Spatial distribution of enzyme activities. Soil Biology & Biochemistry 96, 229–237.

Reay, M.K., Charteris, A.F., Jones, D.L., Evershed, R.P., 2019. ^{15}N-amino sugar stable isotope probing (^{15}N-SIP) to trace the assimilation of fertiliser-N by soil bacterial and fungal communities. Soil Biology & Biochemistry 138, 107599.

Reischke, S., Kumar, M.G.K., Bååth, E., 2015. Threshold concentration of glucose for bacterial growth in soil. Soil Biology & Biochemistry 80, 218–223.

Rhoades, J.D., 1982. Cation exchange capacity, in: Page, A.L., Miller, R.H., Keeney, D.R. (Eds.), Methods of Soil Analysis. Part 2. Chemical and Microbiological

Properties. Soil Science Society of America, Madison, WI, pp. 149–157.
Roberts, P., Jones, D.L., 2008. Critical evaluation of methods for determining total protein in soil solution. Soil Biology & Biochemistry 40, 1485-1495.
Roberts, P., Stockdale, R., Khalid, M., Iqbal, Z., Jones, D.L., 2009. Carbon-to-nitrogen ratio is a poor predictor of low molecular weight organic nitrogen mineralization in soil. Soil Biology & Biochemistry 41, 1750–1752.
Rowell, D.L., 1994. Soil Science: Methods and Applications. Longman Group UK Limited, Harlow, UK.
Schimel, J.P., Bennett, J., 2004. Nitrogen mineralization: Challenges of a changing paradigm. Ecology 85, 591–602.
Schulten, H.-R., Schnitzer, M., 1997. The chemistry of soil organic nitrogen: a review. Biology & Fertility of Soils 26, 1–15.
Simpson, J., Warren, C., Adams, P., 2017. Potential protease activity and organic nitrogen concentration are rapid tests and accurate indicators of N-availability in Tasmanian *Eucalyptus nitens* plantations. Soil Biology & Biochemistry 115, 152–160.
Soon, Y.K., Haq, A., Arshad, M.A., 2007. Sensitivity of nitrogen mineralization indicators to crop and soil management. Communications in Soil Science and Plant Analysis 38, 2029–2043.
Stevenson, F.J., Cole, M.A., 1999. The Carbon Cycle, in: Stevenson, F.J., Cole, M.A. (Eds.), Cycles of Soil : Carbon, Nitrogen, Phosphorus, Sulfur, Micronutrients. Wiley, IL, USA, pp. 1–78.
Swain, T., Hillis, W.E., 1959. The phenolic constituents of *Prunus domestica.* I.—The quantitative analysis of phenolic constituents. Journal of the Science of Food and Agriculture 10, 63–68.
Van Ginkel, J.H., Gorissen, A., Van Veen, J.A., 1997. Carbon and nitrogen allocation in *Lolium perenne* in response to elevated atmospheric CO_2 with emphasis on soil carbon dynamics. Plant and Soil 188, 299–308.
Vance, E.D., Brookes, P.C., Jenkinson, D.S., 1987. An extraction method for measuring soil microbial biomass C. Soil Biology & Biochemistry 19, 703–707.
Vepsäläinen, M., Kukkonen, S., Vestberg, M., Sirviö, H., Maarit Niemi, R., 2001. Application of soil enzyme activity test kit in a field experiment. Soil Biology & Biochemistry 33, 1665–1672.
Vile, D., Shipley, B., Garnier, E., 2006. Ecosystem productivity can be predicted from

potential relative growth rate and species abundance. Ecology Letters 9, 1061–1067.

Vitousek, P.M., Howarth, R.W., 1991. Nitrogen limitation on land and in the sea: How can it occur? Biogeochemistry 13, 87–115.

Vranová, V., Rejsek, K., Formanek, P., 2013. Proteolytic activity in soil: A review. Applied Soil Ecology 70, 23–32.

Waring, S.A., Bremner, J.M., 1964. Ammonium production in soil under waterlogged conditions as an index of nitrogen availability. Nature 201, 951–952.

Warren, C.R., 2017. Variation in small organic N compounds and amino acid enantiomers along an altitudinal gradient. Soil Biology & Biochemistry 115, 197–212.

Wei, T., Simko, V., 2017. R package “corrplot”: Visualization of a Correlation Matrix (Version 0.84). Available from https://github.com/taiyun/corrplot.

Whiteside, M.D., Treseder, K.K., Atsatt, P.R., 2009. The brighter side of soils: Quantum dots track organic nitrogen through fungi and plants. Ecology 90, 100–108.

Zeibich, L., Schmidt, O., Drake, H.L., 2018. Protein- and RNA-enhanced fermentation by gut microbiota of the earthworm *Lumbricus terrestris*. Applied and Environmental Microbiology 84, e00657-18.

Chapter 5

Do plants use root-derived proteases to promote the uptake of soil organic nitrogen?

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LG, DJ and PH conceived the experiment. LG conducted the experiment and LG and DJ wrote the manuscript. LG, DJ, PH, EP and EB reviewed the manuscript.

Abstract

The capacity of plant roots to directly acquire organic nitrogen (N) in the form of oligopeptides and amino acids from soil is well established. However, plants have poor access to protein, the central reservoir of soil organic N. Our question is: do plants actively secrete proteases to enhance the breakdown of soil protein or are they functionally reliant on soil microorganisms to undertake this role? Growing maize and wheat under sterile hydroponic conditions with and without inorganic N, we measured protease activity on the root surface (root-bound proteases) or exogenously in the solution (free proteases). We compared root protease activities to the rhizosphere microbial community to estimate the ecological significance of root-derived proteases. We found little evidence for the secretion of free proteases, with almost all protease activity associated with the root surface. Root protease activity was not stimulated under N deficiency. Our findings suggest that cereal roots contribute one-fifth of rhizosphere protease activity. Our results indicate that plant N uptake is only functionally significant when soil protein is in direct contact with root surfaces. The lack of protease upregulation under N deficiency suggests that root protease activity is unrelated to enhanced soil N capture.

Keywords: Aminopeptidase, Peptidase, Plant nutrition, Proteinase, Root exudation

5.1. Introduction

The rhizosphere represents a zone of intense competition for nutrient resources between plant roots and soil microorganisms (Jones et al. 2009). This competition is particularly intense for low molecular weight forms of organic N such as amino acids, oligopeptides and urea which can be taken up and assimilated by both plants and microorganisms (Kuzyakov and Xu 2013; Moreau et al. 2019). Conventionally, it is thought that high molecular weight N held in soil organic matter is largely unavailable to plants and that this resource needs to be hydrolysed to induce solubilisation and promote plant availability (Schulten and Schnitzer 1997). This hydrolysis step has been shown to be a major bottleneck in N cycling in many ecosystems (Jan et al. 2009). Of the organic N held in soil organic matter, ca. 40% is typically present in the form of protein which enters soil mainly from plant and microbial necromass. Microorganisms release extracellular protease and deaminase enzymes into the soil to break down this protein into oligopeptides, amino acids and NH_4^+. The soluble products can then a) be taken up and assimilated by the microbial community and any

excess NH_4^+ excreted back into the soil, or b) taken up directly by plant roots and associated mycorrhizas (Schimel and Bennett 2004). However, some studies have reported that plant roots can also release extracellular proteases into the soil (Adamczyk et al. 2010). Although plant roots contain a wide range of intracellular proteases (Tornkvist et al. 2019), the production of extracellular proteases by plant roots has been hypothesised to have at least four distinct functions: 1) enhancing availability of N for nutrition, 2) defence against plant pathogenic organisms, 3) root cell expansion, and 4) regulation of proteins and peptides in response to developmental and environmental cues (i.e. signal transduction; van der Hoorn 2008; Kohli et al. 2012). In addition, roots may unwittingly release proteases into soil during apoptotic cell death (e.g. from border cells or epidermal and cortical cell death) or following lysis caused by mesofaunal damage or physical abrasion (e.g. root hairs) (Wen et al. 2007; Sun et al. 2015; Song et al. 2016). Theoretically, the use of root proteases to promote organic N release may reduce competition with microorganisms, given that only a small proportion of the root surface is colonised by microorganisms (Foster 1986). In addition, it may allow the spatially targeted release of exoenzymes at sites where the N demand is greatest (e.g. root tips). This would be similar to the well-established mechanism of phosphatase release from roots experiencing P limitation (Ciereszko et al. 2011).

Evidence that plant root proteases can increase the supply of N from the soil remains conflicting. For example, Godlewski and Adamczyk (2007) reported that 15 different agricultural and wild plant species have the ability to release proteases. Also, their studies on *Triticum aestivum* (Adamczyk et al. 2008) and *Allium porrum* (Adamczyk et al. 2009; Adamczyk 2014) indicate that these proteases may increases levels of free amino acids in the soil. Paungfoo-Lonhienne et al. (2008) have also observed that plants can secrete root proteases but that they also have the potential to take up exogenously supplied proteins intact via endocytosis. In contrast, Chang and Bandurski (1964) and Vágnerová and Macura (1974) both reported negligible root protease activity in cereals, while Eick and Stöhr (2009) showed no change in membrane-bound protease activity under N deficient conditions. Similarly, Synková et al. (2016) and Paungfoo-Lonhienne et al. (2008) have shown that *Nicotiana tabacum*, *Hakea actites* and *Arabidopsis thaliana* plants grow very poorly when supplied with just protein. Lastly, an upregulation of protease activity may occur under different nutritional stresses (e.g. P deficiency) suggesting that the response is not N-specific

(Tran and Plaxton 2008). These differences in opinion could be attributed to the different methods used to measure protease activity and plant growth conditions (German et al. 2011). This is particularly the case when sampling the root secretome due to i) the release of intracellular proteases from roots damaged during handling, ii) contamination from seed exudates known to be rich in proteases, iii) and difficulties in achieving or maintaining sterile conditions, particularly the elimination of root endophytes (Sánchez-López et al. 2018; Oburger and Jones 2018).

This study focuses on aminopeptidases (E.C.3.4.11) which catalyse the cleavage of N-terminus amino acids from peptide and protein substrates. They are involved in fundamental plant cellular processes (e.g. mitosis, meiosis, oxidative regulation) and in various aspects of plant development via degradation of storage protein (e.g. germination, senescence) (Oszywa et al. 2013; Kania and Gillner 2015; Budic et al. 2016). Plants typically encode many aminopeptidases (e.g. *Arabidopsis thaliana* encodes at least 28) which can have broad specificity (Ogiwara et al. 2005; Walling 2006). Scranton et al. (2012) found that leucine aminopeptidase can moonlight as a molecular chaperone to aid plant defence. In addition, aminopeptidases are induced under both drought and metal stress in the plant roots (Wang et al. 2011; Boulila-Zoghlami et al. 2011). Importantly, aminopeptidases have also been implicated in autophagy under N deficiency (Xia et al. 2012; Xu et al. 2019), suggesting that they are a good candidate to investigate for their role in protein-N mobilisation a rhizosphere context.

Investigations of the role of plant proteases in N acquisition have generally focused on proteases secreted from roots (Vágnerová and Macura 1974; Godlewski and Adamczyk 2007). Proteomic studies of the apoplast and cell wall, however, have revealed the presence of a wide range of proteases, most of which have unknown roles (Rodríguez-Celma et al. 2016; Calderan-Rodrigues et al. 2019). Therefore, with a focus on aminopeptidases, our aim was to determine: a) whether plants release free proteases from their roots or if the proteases remain root surface-bound, b) if proteins and/or their breakdown products are taken up by the plant, c) if root protease activity is up- or down-regulated in the presence of inorganic N and, d) how root protease activity compares to rhizosphere protease activity. We hypothesise that plants will both secrete proteases from their roots but also retain surface-bound protease activity to maximise protein-N capture from soils. We also expect protease activity to be induced in the absence of an inorganic N supply (Godlewski and Adamczyk 2007). Finally, we

hypothesise that protease activity from rhizosphere soil will be proportionally higher than for roots as it is more energetically favourable for the soil microbial community to use the products of protein hydrolysis rather than inorganic N (Abaas et al. 2012).

5.2. Materials and methods

5.2.1 Growth of plants

Maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.) were chosen as the study species as both plants are cereals with wide agricultural use but have different N use efficiencies (Liang et al. 2013). Seeds were surface sterilised by shaking with 70% ethanol for 5 min and then with 10% sodium hypochlorite containing one drop of Tween 20 for 5 min. The seeds were then rinsed four times in sterile, deionised water. The seeds were germinated and grown for up to two weeks in sterile Phytatrays® (Sigma-Aldrich, Poole, UK) on autoclaved agar with either inorganic N or zero N nutrient solution added. Seedlings were grown at 20°C, 12 h photoperiod at 500 µmol photons m^{-2} s^{-1} PAR.

5.2.2. Nutrient solution

Seedlings were supplied with either a zero N nutrient solution or inorganic N nutrient solution in the agar. The zero N nutrient solution consisted of 1.5 mM $MgSO_4$, 2 mM K_2SO_4, 4 mM $CaCl_2$, 1.87 mM NaH_2PO_4, 0.13 mM Na_2HPO_4, 0.14 mM H_3BO_3, 0.02 mM $MnSO_4$, 0.002 mM $ZnSO_4$, 0.003 mM $CuSO_4$, 0.0002 mM Na_2MoO_4, 0.089 mM Fe(III)-citrate in 0.1 mM of MES buffer (pH 5.6) (Hewitt 1966). The inorganic N solution consisted of 4 mM $NaNO_3$ and 4 mM NH_4Cl in addition to the zero N nutrient solution.

5.2.3. Extracellular root protease: proteases in solution

After one-week, sterile seedlings (n = 8 for each treatment per plant) of similar height and root length were transferred from the Phytatrays® into a pre-autoclaved hydroponic growth system. The plants were firstly placed into a 1.5 ml Eppendorf tube with the bottom removed. This was then placed into the top of a 50 cm^3 polypropylene centrifuge tube containing nutrient solution and then into a larger sterile box. Nutrient solution was injected into each centrifuge tube via silicone tubing connected to a 0.22-µm filter located outside the box. The nutrient solution in the centrifuge tube was continually aerated by passing 0.22-µm filtered air into the solution via silicone tubing located outside the box. An air outlet from the centrifuge tube was via silicon tubing

with a hydrophobic 0.22-µm filter (Appendix 3, Fig. S1). Weekly, nutrient solutions were removed from the hydroponic system through a 0.22-µm filter and protease activity measured. Fresh nutrient solution was then injected into each centrifuge tube through a 0.22-µm filter. Nutrient solutions were changed weekly to ensure nutrients were never limited and provide a weekly time series of protease activity over the seedling's growth. A negative control consisted of nutrient solution with no plant present. All work was carried out in a sterile, laminar flow cabinet. After four weeks of growth, under the constant conditions outlined previously, the experiment was stopped. The roots and shoots were separated, the fresh weight recorded, then oven dried at 80°C for 24 h after which the dry weight was recorded.

To ensure that the system was sterile, an open Petri-dish with nutrient agar was placed at the bottom of the hydroponic system. At the end of the experiment, nutrient solution was plated onto nutrient agar. If no microbial growth was observed after one week at 37°C, the system was considered sterile.

5.2.4. Protease assay

Leucine aminopeptidase activity was used as an exemplar to measure potential protease activity according to Vepsäläinen et al. (2001). The nutrient solution was pipetted (100 µl) into a 96 well plate. Substrate (100 µl of 500 µM L-leucine 7-amido-4-methylcoumarin hydrochloride dissolved in sterile water and passed through a 0.22-µm filter to ensure no microbial contamination) was added to the sample (pH 5.7). Standards were prepared for each sample by adding 100 µl of 7-amido-4-methylcoumarin (AMC) at different concentrations (0, 0.5, 1, 5, 10, 15, 25 and 50 µM) to 100 µl of sample for quench correction. After a 3 h incubation at 20°C, fluorescence was measured at an excitation wavelength of 335 nm and emission wavelength 460 nm on a Cary Eclipse Fluorescence Spectrophotometer (Agilent Corp., Santa Clara, CA). A calibration curve was then fitted for each sample. Blank sample and substrate measurements were subtracted from the assay reading.

5.2.5. Extracellular root protease: proteases in the root

To determine surface bound root protease activity, we carried out a protease assay in situ. After two weeks of growth, plants ($n = 4$) were transferred into a sterile 50 cm^3 centrifuge tube where the protease assay was carried out as described above except the assay solution consisted of 5 ml of sterile nutrient solution and 5 ml of 500

μM L-leucine 7-amido-4-methlycoumarin hydrochloride. Plants were incubated at 20°C for 3 h in the sterile laminar flow cabinet. The plants were removed and 200 μl of assay solution were pipetted into a 96-well plate for fluorescence measurement. At the end of each experiment, roots and shoots were separated and the fresh weight recorded, then oven dried at 80°C for 24 h and the dry weight recorded (Appendix 3, Fig. S2).

5.2.6. ^{14}C-protein uptake experiment

To determine whether plants use protein and/or its derivatives as a sole N source we carried out a ^{14}C-protein uptake experiment. After two weeks of growth, plants (n = 4) were removed from the nutrient agar and placed in 10 mL sterile zero N nutrient solution in a 50 cm^3 sterile centrifuge tube in a laminar flow cabinet. Each plant was placed in a sterile plastic air-tight box. Uniformly ^{14}C-labelled protein from *Nicotiana tabacum* L. leaves (1 ml; 0.064 mg C l^{-1}; 0.0063 mg N l^{-1}; 3.3 kBq ml^{-1}; >3 kDa; custom produced by American Radiolabeled Chemicals, St Louis, MO) was secondary purified by ultrafiltration in an Amicon® stirred cell using a 3 kDa Ultracel® cut off membrane (Millipore UK Ltd., Watford, UK) to remove any oligopeptides and pipetted into the nutrient solution. To capture the $^{14}CO_2$ evolved from plant respiration a 1 M NaOH trap (1 ml) was added to the box. After 24 h the plants were removed, and the roots washed in 0.1 M $CaCl_2$. The roots and shoot were separated, weighed and dried at 80°C for 24 h. To measure the ^{14}C in the root and shoot biomass, the dried samples were oxidised on a Harvey OX400 Biological Oxidiser (Harvey Instruments Corp., Hillsdale, NJ, USA) and $^{14}CO_2$ captured in Oxysolve C-400 Scintillant (Zinsser Analytic, Frankfurt, Germany) and ^{14}C determination using a Wallac 1414 scintillation counter with automated quench correction (PerkinElmer Inc., Waltham, MA). The amount of $^{14}CO_2$ captured was determined after addition of Optiphase HiSafe3 scintillation fluid to the NaOH traps and ^{14}C determination using a Wallac 1414 scintillation counter with automated quench correction (PerkinElmer Inc.). We acknowledge that we do not know the forms of ^{14}C that were taken up into the plant (i.e. intact protein or hydrolysis products such as peptides or amino acids), but we assume it is as an organic N compound.

5.2.7. Rhizosphere protease activity

To compare root protease activity to rhizosphere soil protease activity, we collected an agricultural topsoil (0–15 cm) from Abergwyngregyn, UK (53°14′29” N, 4°01′15” W). The soil was characterised as a Eutric Cambisol (pH 6.8; 27.8 g C kg^{-1}; 3.4 g N kg^{-1}). Soil was sieved (<2 mm) and added to boxes (8 cm × 10.5 cm × 4 cm) to achieve a dry bulk density of 1 g cm^{-3}. Maize and wheat seeds were germinated and densely planted in the soil (1 seed per 1 cm^3) to maximise the rhizosphere effect and grown at 20°C, 12 h photoperiod at 500 µmol photons m^{-2} s^{-1} PAR. Seedlings were watered daily. After 2 weeks, the rooting was dense and, therefore, all soil was considered to be rhizosphere soil. Soil was sampled and a soil slurry created by adding 0.2 g to 20 ml sterile, 0.1 mM MES buffer (pH 5.6) and shaking for 30 min at 250 rev min^{-1}. Protease activity was also measured at the native soil pH (6.8) in a soil slurry with sterile, deionised water (1:100 soil:water ratio). Protease activity did not significantly differ between the two assay pHs (unpaired t test: $p = 0.21$). Rhizosphere protease activity was compared to extracellular root protease activity under inorganic N treatment for each species. We determined the volume of root to be 0.00785 cm^3 for maize and 0.00502 cm^3 for wheat with 1 cm root length and 1 mm and 0.8 mm diameter for maize and wheat, respectively (Eq. 1).

$$\text{Volume of root } (cm^3) = \pi r^2 h \quad (1)$$

We assumed the root density to be 1 g cm^{-3} and, thus, the fresh root weight to be 0.00785 g and 0.00502 g for maize and wheat respectively. Assuming, 90% water, the dry root weight is 0.000785 cm^3 and 0.000502 g cm^{-3} (Eq. 2).

$$\text{Dry root weight} = 0.1\frac{1.0\ g\ cm^{-3}}{Volume\ of\ root\ (cm^3)} \quad (2)$$

We determined the rhizosphere extent to be 2 mm from the root surface. Therefore, the volume of soil surrounding 1 cm of root would be 0.126 cm^3 (Eq. 1). The soil dry bulk density is 1 g cm^{-3}, thus, the soil weight would be 0.126 g. We then determined the final soil weight surrounded by the root to be 0.118 g and 0.121 g of soil for maize and wheat respectively (Eq. 3).

Final soil weight (g) = total soil weight (g) − dry weight of root (g) (3)

Rhizosphere protease activity was then compared to extracellular root protease activity (μmol AMC cm^{-1} root h^{-1}).

5.2.8. Statistical analysis

All experiments were performed in quadruplicate. All statistical analyses were performed on R version 3.5.0 (R Core Team 2018). Normality of the data was determined by Shapiro-Wilk test ($p > 0.05$) then visually checked using *qqnorm* plots. Homogeneity of variance of the data was determined by Bartlett test ($p > 0.05$) then visually checked using residuals vs. fitted plots. One-way ANOVAs were used to determine if there was a significant difference ($p < 0.05$) between N treatment for extracellular protease activity and ^{14}C-labelled protein uptake for each species. Unpaired t-tests were used to determine if there was a significant difference ($p < 0.05$) between rhizosphere and extracellular root protease activity.

5.3. Results

5.3.1. Root protease activity

We found no evidence of protease activity in the nutrient solution that the seedlings were grown in (no significant difference from the control, unpaired t-test: $p = 0.84$; data not presented). However, we did observe measurable protease activity in the *in situ* protease assay. Extracellular root protease activity ranged from 2 to 5 μmol AMC mg^{-1} root h^{-1} in maize roots and 5–6 μmol AMC mg^{-1} root h^{-1} in wheat roots (Fig. 5.1). We assume all protease activity measured *in situ* to be extracellular root protease at or in the root surface because we found no evidence when protease activity was measured in the solution only. Protease activity was not significantly different between N treatments, but under the N-addition treatments, protease activity was two times higher for maize and ca. 14% higher for wheat ($F_{(1,6)} = 6.4$, $p = 0.53$ and $F_{(1,6)} = 0.13$, $p = 0.73$, respectively).

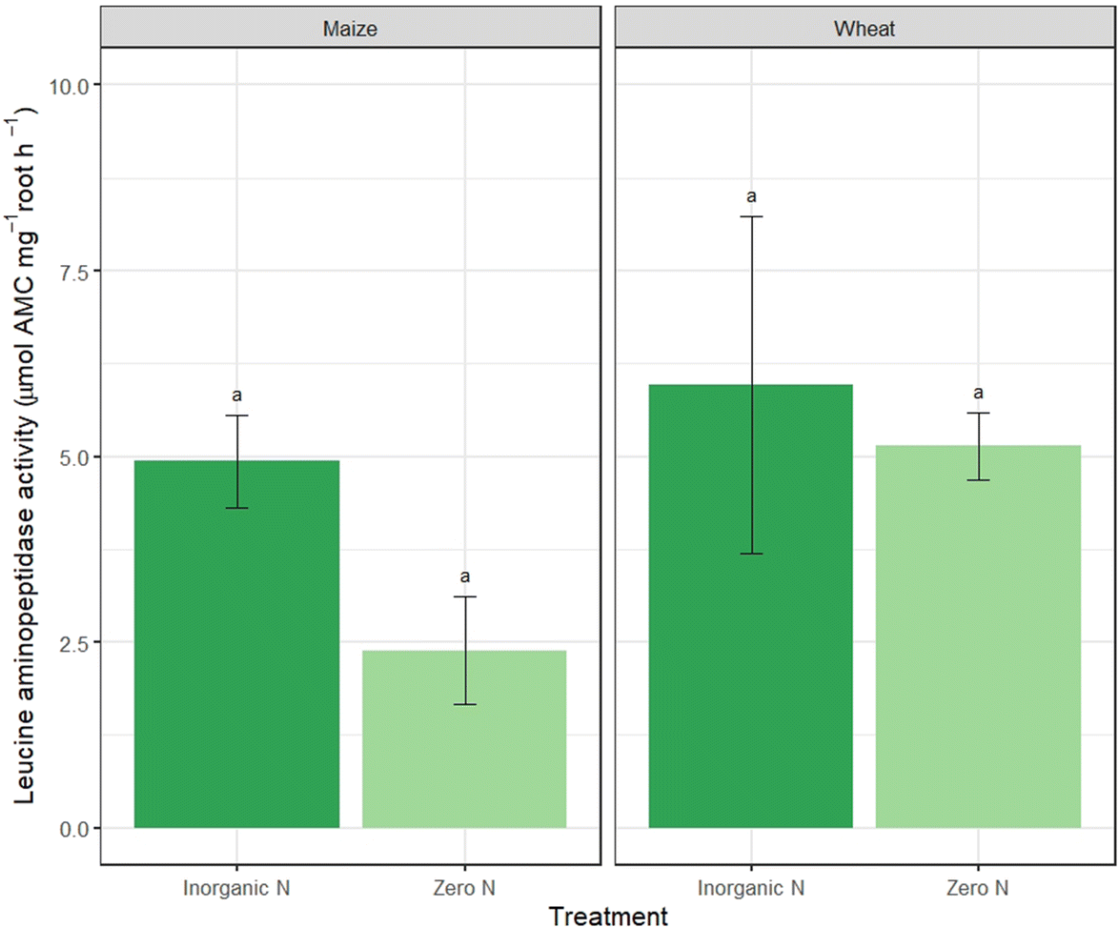

Figure 5.1. Extracellular root leucine aminopeptidase activity (µmol AMC mg^{-1} root h^{-1} of maize and wheat under inorganic N and zero N treatments measured using the *in situ* assay. Different letters represent significant difference between N treatments for each plant ($p < 0.05$). Values represent mean ± SEM ($n = 4$).

5.3.2. ^{14}C-protein uptake

We measured plant uptake of ^{14}C derived from labelled protein to determine whether the breakdown products from proteolysis were utilised by the plant. Mineralisation of ^{14}C-protein to $^{14}CO_2$ was similar between N treatments for both maize and wheat ($p = 0.06$ and 0.54 respectively) (Fig. 5.2). Root uptake of ^{14}C was ca. twice as high under the inorganic N than zero N treatment in maize ($p = 0.03$) (Fig. 5.2). However, wheat root uptake of ^{14}C-protein was similar between treatments ($p = 0.43$). Uptake of ^{14}C-protein into the plant shoot was ca. three times higher under inorganic

N than zero N for maize and ca. twice as high for wheat ($p = 0.04$ and 0.02 respectively) (Fig. 5.2).

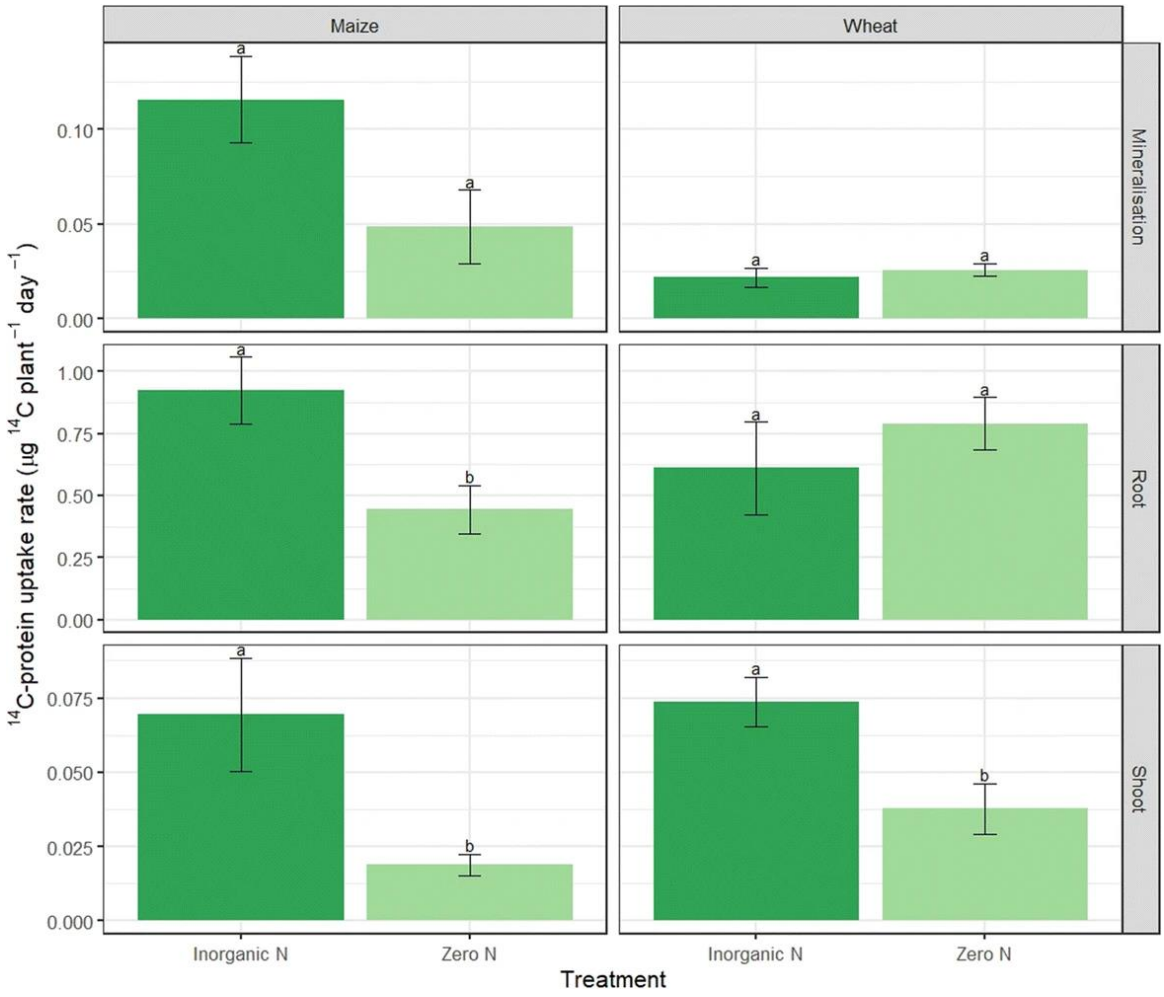

Figure 5.2. ^{14}C-labelled protein respired, root and shoot uptake rate (µg ^{14}C plant^{-1} day^{-1}) of maize and wheat under inorganic N and zero N treatments. Different letters represent significant difference between N treatments for each plant ($p < 0.05$). Values represent mean ± SEM ($n = 4$).

5.3. Rhizosphere and root protease activity

We compared root protease activity to rhizosphere protease activity to determine the potential ecological significance of plant root protease activity. Extracellular root protease activity contributed 15% and 19% of rhizosphere protease activity (Fig. 5.3) (t-test: $p = 0.006$ and $p < 0.0001$ for maize and wheat respectively).

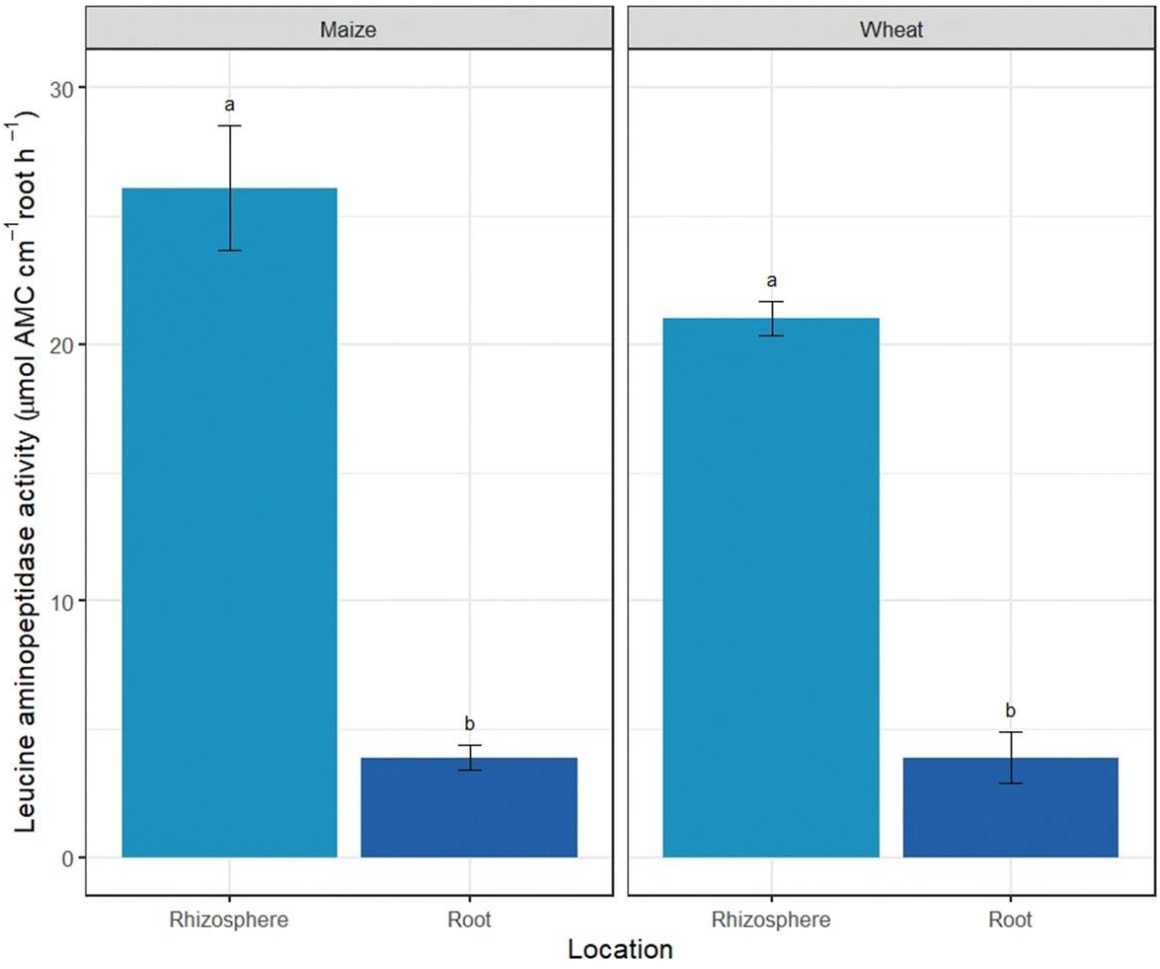

Figure 5.3. Comparison of leucine aminopeptidase activity in the rhizosphere and extracellular root (µmol AMC cm^{-1} root h^{-1}) of maize and wheat. Different letters represent significant difference between N treatments for each plant ($p < 0.05$). Values represent mean ± SEM ($n = 4$).

5.4. Discussion

5.4.1. Free versus surface bound root protease activity

Here we evaluated the possible importance of four different mechanisms for the use of protein-derived N by plant roots, and their likely importance in plant N nutrition: A) Proteases are released from the root into the external medium where they diffuse away and encounter proteins on soil surfaces and/or free in solution and the products released diffuse back to the root where they can be taken up (Adamczyk et al. 2010); B) Proteins come in direct contact with the root surface enabling cleavage by outward facing cell wall bound proteases and uptake of soluble products; C) Proteins diffuse through pores in the cell wall, entering the apoplast where plasma membrane or inward-facing cell wall bound proteases break them into soluble

products (Chang and Bandurski 1964); and D) Small proteins are taken up by the root cell via endocytosis (Carpita et al. 1979) (Fig. 5.4). In this study we found no evidence to show that root proteases are released into the external medium in significant quantities (mechanism A), however, we did find strong evidence for root-bound protease activity (mechanisms B and C). In this study, it was not possible to determine the direct contribution of mechanism D as this can only be confirmed when mechanisms A and B are absent using our methods. Our findings are therefore consistent with studies of plant proteomes which have revealed a high diversity and proportion of proteases among cell wall proteins (ca. 15% of the total; Albene et al. 2014; Canut et al. 2016). These proteases have been shown to be important regulators of plant growth and development, however, their potential role in N nutrition remains unclear (van der Hoorn 2008). Their known functions include: i) breakdown of cell wall proteins to facilitate cell wall re-organisation (e.g. at the root-symbiont interface), ii) removal of oxidised/damaged proteins (Takeda et al. 2009), iii) the production of active peptides important for plant defence responses (immune signalling; Plattner and Verkhratsky 2015; Hou et al. 2018), iv) the synthesis of anti-microbial peptides (Schaller et al. 2018), v) regulators of programmed cell death (phytaspases; Chichkova et al. 2010), vi) cell wall loosening to enable mucilage release (Rautengarten et al. 2008), and vii) potential salvage of C and N resources in senescing tissues (Polge et al. 2009). To date, all the evidence suggests that these events are highly spatially and temporally co-ordinated in response to specific environmental stimuli and developmental cues (van der Hoorn 2008; Plattner and Verkhratsky 2015). The activity of these proteases also appears to target specific protein substrates, consistent with the view that they are not generalist proteases involved in the breakdown of soil-derived protein. Although there is a lack of evidence for their direct involvement in N nutrition, it is clear that many could have an indirect role on N nutrition; for example, through improved N recycling and N use efficiency in the plant, reducing microbial growth and competition for exogenous N, enhancing soil-root contact, and promoting symbioses that promote N acquisition (e.g. N fixation, mycorrhizas). Of critical significance is that many of these proteases are upregulated in response to environmental stress (e.g. Jorda and Vera 2000; Golldack et al. 2003), a feature that was not seen in our experiments when N was withheld from the plants. This suggests that the degradation of exogenous proteins at the root surface is either a constitutively expressed trait, or more likely is just an indirect consequence of foreign

proteins adhering to the root surface or entering the apoplast where proteolysis occurs. A similar argument has been made for the indirect capture of amino acids and peptides from soil as transporters for these solutes are also not up-regulated in cereals under N deficiency (Jones and Darrah 1994). In this latter situation, the active uptake of amino acids and oligopeptides at the epidermal surface and apoplast is likely associated with the recapture of solutes lost in root exudation by passive diffusion (Jones et al. 2009) and not uptake of organic N from soil (Kuzyakov and Xu 2013).

While cell wall proteases may indirectly lead to some cleavage of proteins, further action of cell wall endo/exopeptidases may still be required to transform larger peptides into oligopeptides capable of active transport into the cell. To date, there is no evidence suggesting these enzymes are regulated by plant N status with most implicated in the recycling of damaged proteins (e.g. TPP(II) cell wall exopeptidase; Book et al. 2005; Polge et al. 2009). Again, this indicates that while the root possesses a full complement of enzymatic machinery required for proteolysis and the uptake of soluble products, this may have no direct involvement in N acquisition. One caveat we note is that our study only focused on fluorescent substrates targeted by aminopeptidases. Further studies are warranted on other types of fluorescent substrates which can target alterative proteases.

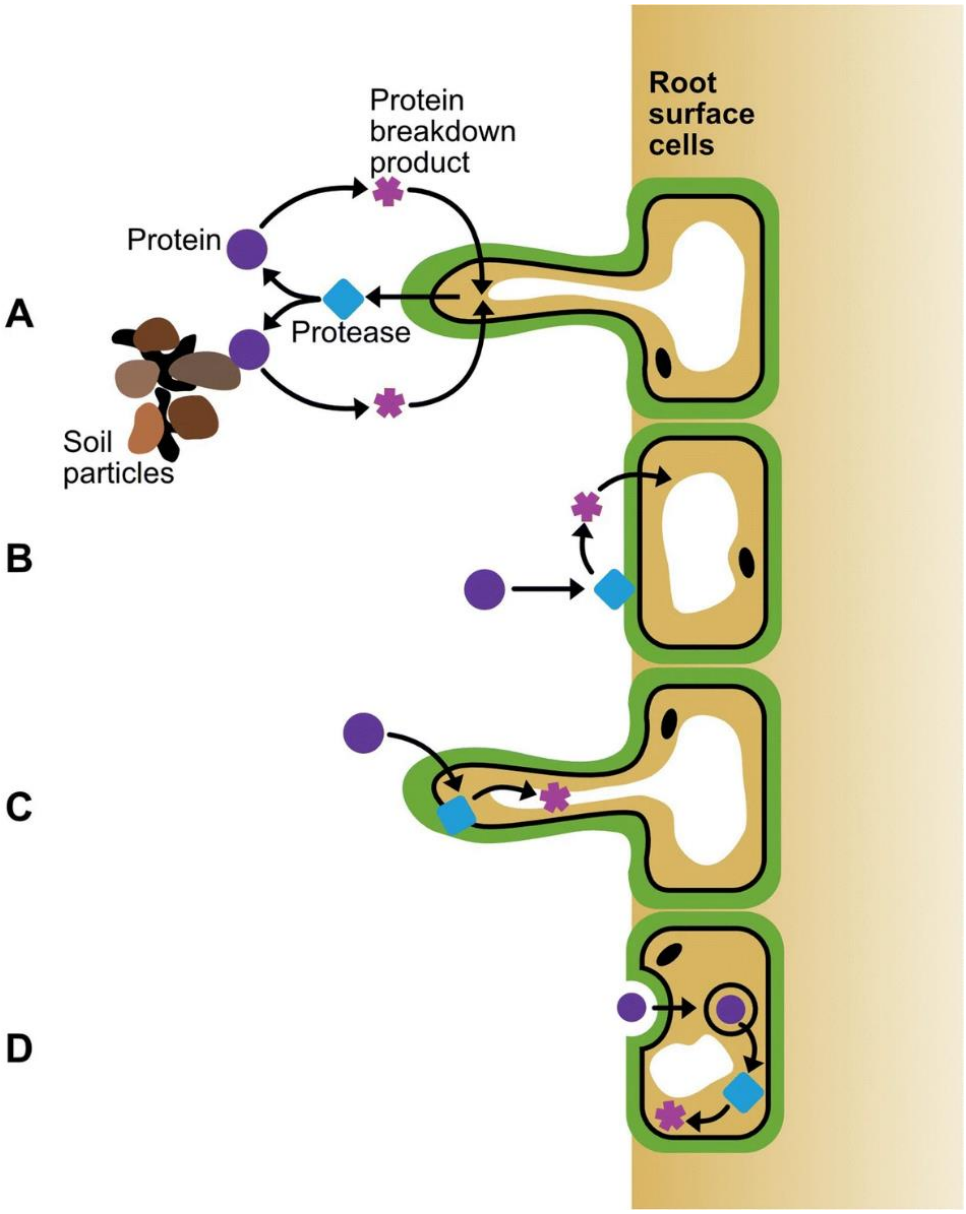

Figure 5.4. Schematic diagram for the mechanisms of root protease activity in order to obtain N for nutrition.

5.4.2. Are root proteases quantitatively important in nitrogen uptake from soil?

Most studies on the direct uptake of exogenous proteins by roots have been undertaken in the absence of soil and at very high soluble protein concentrations, conditions that might be viewed as ecologically unrealistic (White et al. 2015). In addition, even when purified protein forms are used these do not represent soil proteins and can still contain substantial amounts of oligopeptide impurities. In our study, we secondary-purified our plant-derived protein to remove oligopeptides, however, this was still added directly to the nutrient medium. In these situations, proteins tend to be attracted to the charged root surface where clumping can occur (White et al. 2015). However, in soil it is more likely that soluble proteins will preferentially sorb to soil particles and/or denature and precipitate, hampering their movement and bioavailability (Fiorito et al. 2008). This implies that soil-borne protein needs to be in close proximity to the root surface for root-mediated, protein-derived N uptake to occur. This is consistent with our results and others showing that roots contain both inward and outward facing cell wall proteases (Figueiredo et al. 2018; Hou et al. 2018), indicating that they can cleave large proteins outside the cell wall (mechanism B) and either cleave or directly take up smaller ones diffusing through the cell wall (mechanism C and D; Fig. 5.4).

The ^{14}C-labelled proteins used in this study contained a range of molecular weights (3–100 kDa) and therefore sizes. It is likely that this also affects their potential for uptake. Conventionally, the cell wall rather than the plasma membrane is thought to represent the main barrier to protein uptake. This is due to the charged nature of the wall which induces protein binding and retention (Albene et al. 2014), but also due to the small pores (4–5 nm diameter) in the wall which prevents the inward movement of larger proteins (>30 kDa; Palocci et al. 2017). This is consistent with the inward movement and intact uptake of the highly stable, green fluorescent protein (~27 kDa) from solution by Arabidopsis roots (mechanism D; Paungfoo-Lonhienne et al. 2008). However, Read and Bacic (1996) suggest that, albeit less frequent, 6–9 nm diameter pores may also exist, which would allow the ingress and potential uptake of much larger proteins (65–100 kDa), although the significance of this pathway remains unknown. We hypothesize that at least some of our ^{14}C-labelled proteins would have been capable of passing through the cell wall and being available for root uptake. Unfortunately, the molecular weight distribution of proteins in soil solution remains

virtually unknown. Based on the root uptake of a wide range of synthetic nanoparticles (up to 50 nm diameter) it also implies that this is not a protein specific pathway (Lv et al. 2019). Consequently, although evidence exists for low molecular weight protein uptake, it may not necessarily mean that it is quantitatively important in N nutrition.

A study, that investigated whether Arabidopsis could use protein as a N source, found that growth was higher in plants grown on a combination of organic and inorganic N sources rather than protein alone (protein and inorganic N > inorganic N > protein) (Paungfoo-Lonhienne et al. 2008). It is therefore possible that plant N limitation could inhibit protease synthesis. However, we would also expect that if outward facing protease activity was a preferred plant strategy under N limitation that it would preferentially allocate N resources to this function. By analogy, in the case of root C starvation, it is well established that a large proportion of intracellular protein can be degraded to provide C skeletons for respiration without a loss of basic metabolism (Brouquisse et al. 1991). It is also possible that the presence of proteins in the rhizosphere could induce extracellular protease production which the absence of proteins in our experiments would have prevented. However, this mechanism has only been observed in fungi so far (e.g. Hanson and Marzluf 1975; Boer and Peralta 2000). In addition, when ^{14}C-labelled protein was added, the uptake of ^{14}C-derived from protein into the shoot was also higher under the inorganic N treatment. This suggests that proteases are not induced under N deficiency. We hypothesise that the supply of inorganic N drives faster growth which in turn leads to greater cell wall reorganisation, more plasma membrane vesicle fusion events (facilitating protein internalisation) and greater cell wall protease activity.

5.4.3. Root versus rhizosphere protease activity

Rhizosphere protease activity was higher than extracellular root protease activity for both maize and wheat. We expected rhizosphere protease activity to be high because the rhizosphere is a hotspot for microbial activity (Kuzyakov and Blagodatskaya 2015). Soil microorganisms are largely C limited and they produce proteases to liberate both C and N from proteinaceous compounds, with a large proportion of the protein-C subsequently used in catabolic processes (Gonzales and Robert-Baudouy 1996; Jan et al. 2009). Furthermore, they do not favour the uptake of NO_3^- as this is energetically unfavourable (Abaas et al. 2012). This contrasts with crop plants who often favour NO_3^- as a source of N due to its fast diffusion in soil and who

are rarely C limited (Iqbal et al. 2020). Previous reports for protease and other enzymes (e.g. Badalucco et al. 1996; Gramss et al. 1999; Brzostek et al. 2013) have shown that roots contribute little to overall rhizosphere hydrolytic activity. In contrast, our study shows up to one-fifth of rhizosphere protease activity is of root origin. In future, it is important to consider the potential contribution of plant root proteases in rhizosphere activity.

5.5. Conclusions

Although plants have the potential to contribute to rhizosphere protease activity and possess the capacity to take up and metabolise protein breakdown products, current evidence suggests that this plays a minor role in N nutrition. Our study found no evidence for the root-release of proteases into the soil solution. In contrast, we present strong evidence for root-bound protease activity and breakdown of soluble proteins. However, our results suggest that the use of exogenous protein may be an indirect by-product of other processes occurring in the root. In particular, the lack of up-regulation in protease activity under N deficiency and low intrinsic rates of protease activity in comparison to soil microbial-derived protease activity suggests it plays a minor role in overall plant N acquisition.

5.6. Acknowledgements

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5.7. References

Abaas, E., Hill, P.W., Roberts, P., Murphy, D.V., Jones, D.L., 2012. Microbial activity differentially regulates the vertical mobility of nitrogen compounds in soil. Soil Biology & Biochemistry 53, 120–123.

Adamczyk, B., 2014. Characterization of proteases secreted by leek roots. Russian Journal of Plant Physiology 61, 714–717.

Adamczyk, B., Godlewski, M., Smolander, A., Kitunen, V., 2009. Degradation of proteins by enzymes exuded by *Allium porrum* roots–A potentially important strategy for acquiring organic nitrogen by plants. Plant Physiology and Biochemistry 47, 919–925.

Adamczyk, B., Godlewski, M., Zimny, J., Zimny, A., 2008. Wheat (*Triticum aestivum*) seedlings secrete proteases from the roots and, after protein addition, grow well on medium without inorganic nitrogen. Plant Biology 10, 718–724.

Adamczyk, B., Smolander, A., Kitunen, V., Godlewski, M., 2010. Proteins as nitrogen source for plants: a short story about exudation of proteases by plant roots. Plant Signaling & Behavior 5, 817–819.

Albene, C., Canut, H., Hoffman, L., Jamet, E., 2014. Plant Cell Wall Proteins: A Large Body of Data, but What about Runaways? Proteomes 2, 224–242.

Badalucco, L., Kuikman, P.J., Nannipleri, P., 1996. Protease and deaminase activities in wheat rhizosphere and their relation to bacterial and protozoan populations. Biology & Fertility of Soils 23, 99–104.

Boer, C.G., Peralta, R.M., 2000. Production of extracellular protease by Aspergillus tamarii. Journal of Basic Microbiology 40, 75–81.

Book, A.J., Yang, P., Scalf, M., Smith, L.M., Vierstra, R.D., 2005. Tripeptidyl peptidase II. An oligomeric protease complex from *Arabidopsis*. Plant Physiology 138, 1046–1057.

Brzostek, E.R., Greco, A., Drake, J.E., Finzi, A.C., 2013. Root carbon inputs to the rhizosphere stimulate extracellular enzyme activity and increase nitrogen availability in temperate forest soils. Biogeochemistry 115, 65–76.

Calderan-Rodrigues, M.J., Fonseca, J.G., de Moraes, F.E., Setem, L.V., Begossi, A.C., Labate, C.A., 2019. Plant cell wall proteomics: A focus on monocot species, *Brachypodium distachyon*, *Saccharum* spp. and *Oryza sativa*. International Journal of Molecular Sciences.

Canut, H., Albene, C., Jamet, E., 2016. Post-translational modifications of plant cell wall proteins and peptides: A survey from a proteomics point of view. Biochimica et Biophysica Acta - Proteins and Proteomics 1864, 983–990.

Carpita, N., Sabularse, D., Montezinos, D., Delmer, D.P., 1979. Determination of the Pore Size of Cell Walls of Living Plant Cells. Science 205, 1144–1147.

Chang, C.W., Bandurski, R.S., 1964. Exocellular Enzymes of Corn Roots. Plant Physiology 39, 60–64.

Chichkova, N. V., Shaw, J., Galiullina, R.A., Drury, G.E., Tuzhikov, A.I., Kim, S.H., Kalkum, M., Hong, T.B., Gorshkova, E.N., Torrance, L., Vartapetian, A.B., Taliansky, M., 2010. Phytaspase, a relocalisable cell death promoting plant protease with caspase specificity. EMBO Journal 29, 1149–1161.

Ciereszko, I., Szczygła, A., Zebrowska, E., 2011. Phosphate deficiency affects acid phosphatase activity and growth of two wheat varieties. Journal of Plant Nutrition 34, 815–829.

Eick, M., Stöhr, C., 2009. Proteolysis at the plasma membrane of tobacco roots: Biochemical evidence and possible roles. Plant Physiology and Biochemistry 47, 1003–1008.

Figueiredo, J., Sousa Silva, M., Figueiredo, A., 2018. Subtilisin-like proteases in plant defence: the past, the present and beyond. Molecular Plant Pathology 19, 1017–1028.

Fiorito, T.M., Icoz, I., Stotzky, G., 2008. Adsorption and binding of the transgenic plant proteins, human serum albumin, β-glucuronidase, and Cry3Bb1, on montmorillonite and kaolinite: Microbial utilization and enzymatic activity of free and clay-bound proteins. Applied Clay Science 39, 142–150.

Foster, R.C., 1986. The ultrastructure of the rhizoplane an rhizosphere. Annual Review of Phytopathology 24, 211–234.

German, D.P., Weintraub, M.N., Grandy, A.S., Lauber, C.L., Rinkes, Z.L., Allison, S.D., 2011. Optimization of hydrolytic and oxidative enzyme methods for ecosystem studies. Soil Biology & Biochemistry 43, 1387–1397.

Godlewski, M., Adamczyk, B., 2007. The ability of plants to secrete proteases by roots. Plant Physiology and Biochemistry 45, 657–664.

Golldack, D., Vera, P., Dietz, K.-J., 2003. Expression of subtilisin-like serine proteases in *Arabidopsis thaliana* is cell-specific and responds to jasmonic acid and heavy metals with developmental differences. Physiologia Plantarum 118, 64–73.

Gramss, G., Voigt, K.D., Kirsche, B., 1999. Oxidoreductase enzymes liberated by plant roots and their effects on soil humic material. Chemosphere 38, 1481–1494.

Hanson, M.A., Marzluf, G.A., 1975. Control of the synthesis of a single enzyme by multiple regulatory circuits in *Neurospora crassa*. Proceedings of the National Academy of Sciences of the United States of America 72, 1240–1244.

Hewitt, E.J., 1966. Sand and water culture methods used in the study of plant nutrition, Revised 2n. ed. Commonwealth Agricultural Bureaux, Farnham Royal (Bucks), UK.

Hou, S., Jamieson, P., He, P., 2018. The cloak, dagger, and shield: proteases in plant–pathogen interactions. Biochemical Journal 475, 2491–2509.

Iqbal, A., Dong, Q., Wang, X., Gui, H., Zhang, H., Pang, N., Zhang, X., Song, M., 2020. Nitrogen preference and genetic variation of cotton genotypes for nitrogen use efficiency. Journal of the Science of Food and Agriculture 100, 2761–2773.

Jan, M.T., Roberts, P., Tonheim, S.K., Jones, D.L., 2009. Protein breakdown represents a major bottleneck in nitrogen cycling in grassland soils. Soil Biology & Biochemistry 41, 2272–2282.

Jones, D.L., Darrah, P.R., 1994. Amino-acid influx at the soil-root interface of *Zea mays* L. and its implications in the rhizosphere. Plant and Soil 163, 1–12.

Jones, D.L., Nguyen, C., Finlay, R.D., 2009. Carbon flow in the rhizosphere: Carbon trading at the soil-root interface. Plant and Soil 321, 5–33.

Jorda, L., Vera, P., 2000. Local and systemic induction of two defense-related subtilisin-like protease promoters in transgenic *Arabidopsis* plants. Luciferin induction of PR gene expression. Plant Physiology 124, 1049–1057.

Kohli, A., Narciso, J.O., Miro, B., Raorane, M., 2012. Root proteases: reinforced links between nitrogen uptake and mobilization and drought tolerance. Physiologia Plantarum 145, 165–179.

Kuzyakov, Y., Blagodatskaya, E., 2015. Microbial hotspots and hot moments in soil: Concept & review 83, 184–199.

Kuzyakov, Y., Xu, X., 2013. Competition between roots and microorganisms for nitrogen: mechanisms and ecological relevance. New Phytologist 198, 656–669.

Liang, B., Zhao, W., Yang, X., Zhou, J., 2013. Fate of nitrogen-15 as influenced by soil and nutrient management history in a 19-year wheat-maize experiment. Field Crops Research 144, 126–134.

Lv, J., Christie, P., Zhang, S., 2019. Uptake, translocation, and transformation of metal-based nanoparticles in plants: recent advances and methodological challenges. Environmental Science: Nano 6, 41–59.

Moreau, D., Bardgett, R.D., Finlay, R.D., Jones, D.L., Philippot, L., 2019. A plant perspective on nitrogen cycling in the rhizosphere. Functional Ecology 33, 540–552.

Oburger, E., Jones, D.L., 2018. Sampling root exudates – Mission impossible? Rhizosphere 6, 116–133.

Palocci, C., Valletta, A., Chronopoulou, L., Donati, L., Bramosanti, M., Brasili, E., Baldan, B., Pasqua, G., 2017. Endocytic pathways involved in PLGA nanoparticle uptake by grapevine cells and role of cell wall and membrane in size selection. Plant Cell Reports 36, 1917–1928.

Paungfoo-Lonhienne, C., Lonhienne, T.G.A., Rentsch, D., Robinson, N., Christie, M., Webb, R.I., Gamage, H.K., Carroll, B.J., Schenk, M., Schmidt, S., 2008. Plants can use protein as a nitrogen source without assistance from other organisms. PNAS 105, 4524–4529.

Plattner, H., Verkhratsky, A., 2015. The ancient roots of calcium signalling evolutionary tree. Cell Calcium.

Polge, C., Jaquinod, M., Holzer, F., Bourguignon, J., Walling, L., Brouquisse, R., 2009. Evidence for the existence in *Arabidopsis thaliana* of the proteasome proteolytic pathway: Activation in response to cadmium. Journal of Biological Chemistry 284, 35412–35424.

R Core Team, 2018. R: A language and environment for statistical computing.

Rautengarten, C., Usadel, B., Neumetzler, L., Hartmann, J., Büssis, D., Altmann, T., 2008. A subtilisin-like serine protease essential for mucilage release from *Arabidopsis* seed coats. The Plant Journal 54, 466–480.

Read, S.M., Bacic, A., 1996. Cell Wall Porosity and Its Determination, in: Plant Cell Wall Analysis. Springer, Berlin, Germany, pp. 63–80.

Rodríguez-Celma, J., Ceballos-Laita, L., Grusak, M.A., Abadía, J., López-Millán, A.F., 2016. Plant fluid proteomics: Delving into the xylem sap, phloem sap and apoplastic fluid proteomes. Biochimica et Biophysica Acta - Proteins and Proteomics 1864, 991-1002.

Sánchez-López, A., Pintelon, I., Stevens, V., Imperato, V., Timmermans, J.-P., González-Chávez, C., Carrillo-González, R., Van Hamme, J., Vangronsveld, J., Thijs, S., 2018. Seed Endophyte Microbiome of Crotalaria pumila Unpeeled: Identification of Plant-Beneficial Methylobacteria. International Journal of Molecular Sciences 19, 291.

Schaller, A., Stintzi, A., Rivas, S., Serrano, I., Chichkova, N. V., Vartapetian, A.B., Martínez, D., Guiamét, J.J., Sueldo, D.J., van der Hoorn, R.A.L., Ramírez, V., Vera, P., 2018. From structure to function - a family portrait of plant subtilases.

New Phytologist 218, 901–915.

Schimel, J.P., Bennett, J., 2004. Nitrogen Mineralization: Challenges of a Changing Paradigm. Ecology 85, 591–602.

Schulten, H.-R., Schnitzer, M., 1997. The chemistry of soil organic nitrogen: a review. Biology & Fertility of Soils 26, 1–15.

Scranton, M.A., Yee, A., Park, S.Y., Walling, L.L., 2012. Plant leucine aminopeptidases moonlight as molecular chaperones to alleviate stress-induced damage. Journal of Biological Chemistry 287, 18408–18417.

Song, Y., Ling, N., Ma, J., Wang, J., Zhu, C., Raza, W., Shen, Y., Huang, Q., Shen, Q., 2016. Grafting Resulted in a Distinct Proteomic Profile of Watermelon Root Exudates Relative to the Un-Grafted Watermelon and the Rootstock Plant. Journal of Plant Growth Regulation 35, 778–791.

Sun, L., Song, J., Peng, C., Xu, C., Yuan, X., Shi, J., 2015. Mechanistic study of programmed cell death of root border cells of cucumber (*Cucumber sativus* L.) induced by copper. Plant Physiology and Biochemistry 97, 412–419.

Synková, H., Hýsková, V., Garčeková, K., Křížová, S., Ryšlavá, H., 2016. Protein as a sole source of nitrogen for in vitro grown tobacco plantlets. Biologia Plantarum 60, 635–644.

Takeda, N., Sato, S., Asamizu, E., Tabata, S., Parniske, M., 2009. Apoplastic plant subtilases support arbuscular mycorrhiza development in *Lotus japonicus*. The Plant Journal 58, 766–777.

Tornkvist, A., Liu, C., Moschou, P., 2019. Proteolysis and Nitrogen: emerging insights. Journal of Experimental Botany 70, 2009–2019.

Tran, H.T., Plaxton, W.C., 2008. Proteomic analysis of alterations in the secretome of *Arabidopsis thaliana* suspension cells subjected to nutritional phosphate deficiency. Proteomics 8, 4317–4326.

Vágnerová, K., Macura, J., 1974. Determination of protease activity of plant roots. Folia Microbiologica 19, 322–328.

van der Hoorn, R.A.L., 2008. Plant Proteases: From Phenotypes to Molecular Mechanisms. Annual Review of Plant Biology 59, 191–223.

Vepsäläinen, M., Kukkonen, S., Vestberg, M., Sirviö, H., Maarit Niemi, R., 2001. Application of soil enzyme activity test kit in a field experiment. Soil Biology & Biochemistry 33, 1665–1672.

Wen, F., Vanetten, H.D., Tsaprailis, G., Hawes, M.C., 2007. Extracellular proteins in

pea root tip and border cell exudates. Plant Physiology 143, 773–783.
White, J.F., Chen, Q., Torres, S., Mattera, R., Irizarry, I., Tadych, M., Bergen, M., 2015. Collaboration between grass seedlings and rhizobacteria to scavenge organic nitrogen in soils. AoB Plants 7, plu093.

Chapter 6

Protein addition to soil increases rhizosphere extent and promotes protease activity

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Abstract

Protein typically represents the largest input of organic nitrogen (N) into soil. Proteases subsequently make this protein available for use by both plants and microorganisms, however, the factors that regulate protein breakdown in the rhizosphere remain limited. Root exudation of carbon (C) and N into soil promotes microbial growth and thus enzyme production, which is further enhanced by root morphological traits such as root hairs. However, it is not clear how inputs of protein from external sources (e.g. necromass) effect enzyme activity in the rhizosphere. Understanding the interaction between protein addition and root morphology will further our understanding of plant and microbial strategies for enhancing N acquisition. Using soil zymography, we investigated the spatial distribution of leucine aminopeptidase activity in the rhizosphere of *Hordeum vulgare* L. with and without root hairs subject to localised protein addition. Seedlings of barley were grown for two weeks in rhizoboxes and soluble protein was applied 48 h before analysis of leucine aminopeptidase activity. *In situ* zymography was used to quantitatively visualise leucine aminopeptidase activity while *ex situ* sampling was used to determine its enzyme kinetics. In the zymograms, we found that mean and maximal leucine aminopeptidase activity was highest in the barley genotype with root hairs and in the presence of soil protein hotspots. This suggests that microorganisms and plant roots in the rhizosphere of genotypes with root hairs have a greater advantage in accessing protein hotspots in the soil. Leucine aminopeptidase activity did not follow the same trends when analysed by *in situ* zymography and *ex situ* sampling. Therefore, we recommend the use of *in situ* zymography to detect the spatial distribution of enzymatic hotspots and rhizosphere extent followed by *ex situ* sampling for assessing enzyme kinetics in the hotspot areas detected by *in situ* sampling. However, sampling biases must be considered to ensure enzyme activities are being interpreted as the true rhizosphere.

Keywords: Nitrogen mineralisation, Enzyme activity, Soil zymography, Soil organic matter

6.1. Introduction

Protein is an important source of carbon (C) and nitrogen (N) for soil microorganisms and N is a key macronutrient for plant growth. Furthermore, protein is estimated to contribute ca. 40% of total soil N and 9-16% of soil organic C (Schulten and Schnitzer, 1997; Stevenson and Cole, 1999). However, soil protein is generally considered a stable fraction of soil organic matter (SOM) due to its ability to form many bonds and complexes with components in the soil matrix e.g. polyphenols and clay mineral surfaces (Rillig et al., 2007). Yet, many soil systems have regular inputs of protein available for degradation and use by microorganisms. These include plant litter, root biomass and decaying micro- and macrofauna as well as functional proteins released into the soil by plants and microorganisms to carry out specific functions (Rillig et al., 2007). Based on estimates of below-ground turnover in cropping systems, we estimate that the annual input of protein into soil from roots alone is 0.4-0.8 t ha^{-1} y^{-1} (Steingrobe et al., 2001). Considering the age of most agricultural soils and amount of protein held in SOM, this suggests that only a small amount of protein enters the stable SOM pool each year. Extracellular protease enzymes produced by microorganisms and plant roots break down proteins into oligopeptides and amino acids. However, whether plant root-derived proteases have any functional significance in N nutrition remains unclear (Greenfield et al., 2020a).

Microbial activity is typically greater in the rhizosphere (relative to the bulk soil) due to the high rates of root C exudation of a variety of compounds (e.g. carbohydrates, amino acids, enzymes, proteins and phenols) (Jones et al., 2009; Koo et al., 2005). Studies have found C and N exudation to enhance enzyme activities in the rhizosphere (Brzostek et al., 2013; Kandeler et al., 1994). Furthermore, protein mineralisation and protease activity have been found to be regulated by supply of substrate rather than limitations in protein turnover (Greenfield et al., 2020b). Thus, we hypothesise that the influx of soluble protein into the soil system is likely to stimulate protease activity in the rhizosphere. The rate of protein mineralisation is faster in soil-plant systems compared to bulk soil and particularly in the rhizosphere (Jan et al., 2009; Loeppmann et al., 2016). Due to the short turnover time of proteinaceous N, estimated to range from hours to days (Greenfield et al., 2020b; Hill et al., 2012; Jan et al., 2009), an excess of easily available C and N is likely to be depleted by the microbial community within a few days (Kuzyakov and Xu, 2013). However, few studies have investigated the spatial responses to external protein

addition on enzyme activity in the rhizosphere (e.g. necromass hotspots) that can create 'hot moments' (Hill et al., 2019; Kuzyakov and Blagodatskaya, 2015).

Root morphology plays a major role in determining the quality, quantity and distribution of exudates along the surface of the root. One key root morphological trait which is known to increase C substrate availability for microorganisms is the presence of root hairs (Holz et al., 2018; Jones et al., 2009). They are also responsible for the majority of N uptake in roots (Waisel et al., 2002). Root hairs increase the rhizosphere extent of rhizosphere enzyme activity by increasing the surface area and volume of soil exploited by the root (Holz et al., 2020; Ma et al., 2018). Interestingly, it has been found that hairless genotypes compensate for the reduced surface area and volume by increasing enzyme activity close to the root surface in response to phosphorous limitation (Holz et al., 2020). However, plants with root hairs are likely to be able to access N from larger soil volumes due to the larger rhizosphere extent. Understanding the interaction between protein addition and root morphology will further extend our knowledge of plant and microbial strategies for N acquisition.

We applied soil zymography, a two-dimensional imaging technique, and leucine aminopeptidase kinetics to determine the effect of soluble protein addition on the spatial distributions of leucine aminopeptidase activity in the rhizosphere of barley (*Hordeum vulgare* L.) with and without root hairs. We hypothesised that 1) protein addition would increase leucine aminopeptidase activity in the rhizosphere due to an increase in substrate, 2) root hairs would accelerate leucine aminopeptidase activity in the rhizosphere by providing a higher surface area and more root exudates, and 3) root hairs would increase the rhizosphere extent of leucine aminopeptidase activity due to the larger surface area of the root.

6.2. Materials & methods

6.2.1. Soil and plant preparation

The soil was collected from the top 20 cm of a grassland (*Lolium perenne* L.) sandy clay loam, classified as a Eutric Cambisol, located in Abergwyngregyn, Wales (53°13′ N, 4°00' W). Prior to use, the soil was 2 mm sieved to remove stones and plant residues. General soil properties are presented in Table 6.1. Soil pH(H_2O) and electrical conductivity (EC) were measured in a 1:5 (w/v) soil:distilled water suspension. Organic matter (OM) was determined by loss-on-ignition (Ball, 1964).

Dissolved organic C (DOC) and total dissolved N (TDN) were measured using 1:5 (w/v) soil:0.5 M K_2SO_4 extracts on a Multi-N/C Series NPOC-TN analyser (Analytik Jena, Germany). Dissolved organic nitrogen (DON) was calculated as the difference between TDN and dissolved inorganic N. Ammonium (NH_4^+) and nitrate (NO_3^-) concentrations were both determined colorimetrically according to the salicylic acid procedure of Mulvaney (1996) and VCl_3 procedure of Miranda et al. (2001) respectively. Plant-available P was measured using the 0.5 M acetic acid of Vaz et al. (1994). Rhizoboxes with inner dimensions of 12.5 × 12.5 × 2.5 cm were filled with soil to a final density of 1.2 g cm^{-3}.

Table 6.1. General properties of the soil used in the experiments. Values are expressed on a dry weight basis and represent mean ± SE (*n = 3*).

Soil property	Units	Value
pH(H_2O)		6.50 ± 0.11
EC	(µS cm^{-1})	34.4 ± 3.54
Organic matter	(mg kg^{-1})	556 ± 1.50
DOC	(mg kg^{-1})	41.0 ± 5.21
DON	(mg kg^{-1})	7.28 ± 2.41
NH_4^+	(mg kg^{-1})	7.98 ± 4.14
NO_3^-	(mg kg^{-1})	1.98 ± 0.60
Available P	(mg kg^{-1})	3.54 ± 1.67

Two genotypes of barley (*Hordeum vulgare* L.), the wild type and a hairless mutant, were germinated on moist filter paper for 72 h. More detailed information on the two genotypes of barley and microscope images of the roots can be found in Brown et al. (2012). One seedling was planted at a depth of 5 mm into each rhizobox with 3 biological replications in separate boxes. The rhizoboxes were kept in a climate-controlled chamber at 20 ± 1 °C, 55 % relative humidity and 12 h photoperiod with a photosynthetically active radiation intensity of 500 µmol photons m^{-2} s^{-1}. During the growth period, the rhizoboxes were kept inclined at an angle of 45° so that the roots grew along the lower wall of the rhizobox. The rhizoboxes were irrigated with distilled water to maintain a water content at 60% of the water holding capacity (i.e. 60% field capacity). No additional nutrients were added to the soil.

To determine the effect of protein hotspots on the plant-soil interface, bovine serum albumin (BSA) was added to soil in 0.5 cm tall protein horizontal bands (Appendix 4; Fig. S1). Briefly, 100 ml of 6 mg ml^{-1} BSA was added to each 0.5 x 0.5 cm area along a protein band. This concentration of protein was used to ensure saturation of the soil with protein. Three control bands were also added by pipetting 100 ml of sterile, distilled water into 0.5 x 0.5 cm regions along the band. Rhizoboxes were then incubated for 48 h, to allow sufficient time for microorganisms to respond to the protein addition, under the same conditions as growth before imaging.

6.2.2. Soil zymography

After two weeks of growth and 48 h after soluble protein addition, direct zymography (Sannullah et al. 2016) was applied to visualise the activity of leucine aminopeptidase (E.C. 3.4.11.1). Leucine aminopeptidase was used as a proxy to measure protease activity. It catalyses the cleavage of N-terminus amino acids from peptide and protein substrates and is involved in fundamental plant rhizosphere processes and plant development e.g. degradation of storage protein (Kania and Gillner, 2015). Thin polyamide membrane filters (Tao Yuan, China) with a size of 10 × 10 cm and a pore size of 0.45 mm were saturated with 10 mM L-leucine-7-amido-4-methyl-coumarin hydrochloride (dissolved in 0.05 M Trizma buffer, pH 7). We chose pH 7 because the pH optimum of leucine aminopeptidase is ca. 7 (Puissant et al., 2019). The rhizoboxes were opened on the lower, rooted side and the saturated membranes were placed directly on to the root-soil surface. After 1 h incubation, the membranes were carefully removed from the rhizobox and any attached soil particles gently removed using a small soft brush. The membranes were placed under ultraviolet (UV) light in a dark room. The distance between the camera (EOS M50, Canon), the sample, and the UV light were fixed, and a photograph of the membrane taken. The camera settings were f/5.6, ISO 800, 40 mm zoom and exposure 1/40 seconds.

Images were calibrated by saturating 4 cm^2 membranes in 60 ml AMC with the following concentrations: 0, 0.05, 0.1, 0.2, 0.5, 1, 2, 3, 4, 5, 10 mM. The amount of AMC on an area basis was calculated from the volume of solution taken up by the membrane and its size (Spohn and Kuzyakov, 2014). The membranes used for calibration were imaged under UV light in the same way as described for the rhizoboxes. A calibration curve was fitted using a power function equation $y = ax^b$, by

plotting concentrations (pmol mm^{-2}) versus grey values obtained in Matlab (MATLAB, The MathWorks) using a script published in Razavi et al. (2019).

6.2.3. Image processing and analysis

Images were processed and analysed in ImageJ 1.x (Schindelin et al., 2012). Images were transformed to 32-bit grayscale images as matrices and corrected for light variations and camera noise (Razavi et al., 2016). The grey value blank from the 0 mM AMC standard was used as a referencing signal and subtracted from the zymograms. Then a power function ($y = ax^b$) of the calibration was used to relate the grey values to leucine aminopeptidase activity.

Rhizosphere extent was measured as the distance of a region with at least 30% higher enzyme activity than the bulk soil from the point the enzyme activity started increasing to the point it ceased to increase using a threshold by a default algorithm in ImageJ (Tajima and Kato, 2011). Ten locations (lines across the root) in each band were selected and measurements taken. The diameter of the root was measured at the same locations as rhizosphere extent from root masks thresholded by a triangle algorithm in ImageJ (Tajima and Kato, 2011). Root diameter was then subtracted from the rhizosphere distance and divided by two to obtain the rhizosphere extent from the root surface (mm). Leucine aminopeptidase activity in the bulk soil was defined in the region with the absence of elevated activities activity. The mean and maximum leucine aminopeptidase activity across the rhizosphere extent was measured.

6.2.4. Enzyme kinetics

Rhizosphere enzyme kinetics were measured according to Marx et al. (2001) with some modifications. After two weeks of growth, soil was collected from the rhizosphere of each of the three protein and control bands and combined to make a composite sample for each treatment (protein or control) to give 0.2 g soil for each biological replicate (*n* = 3). Rhizosphere soil was collected carefully with a needle to avoid mixing with bulk soil from as close to the root surface as possible. A soil slurry was created by adding 20 ml of sterile deionised water to the soil. The soil slurry was homogenised by shaking at 250 rev min^{-1} for 30 min. 50 µl of soil suspension, 100 µl a range of substrate concentrations from low to high (0, 5, 10, 20, 40, 80, 100, 200 µM) and 50 µL of Trizma buffer (pH 7) was added to a 96-well microplate.

Fluorescence was measured in microplates at an excitation wavelength of 355 nm and an emission wavelength of 460 nm, and a slit width of 20 nm, with a Cary Eclipse Fluorescence Spectrophotometer (Agilent Corp., Santa Clara, CA). Enzyme activities were measured 30 min, 1 h and 2 h after adding soil solution, buffer and substrate solution. Microplates were incubated at 20°C between measurements. The difference between activities at 2 h and 1 h was used to determine AMC release in nmol per g dry soil per hour (nmol g^{-1} dry $soil^{-1}$). The leucine aminopeptidase assays were performed in three analytical replicates. The Michaelis-Menten constant K_m and V_{max} were determined using the Michaelis-Menten equation:

$$v = \frac{Vmax[S]}{Km+[S]} \quad (1)$$

where V is the reaction rate (as a function of substrate concentration), [S] is the substrate concentration, K_m is the substrate concentration at half-maximal rate and V_{max} is the maximum reaction rate.

6.2.5. Statistical analysis

All statistical analyses were performed in R 3.5.0 (R Core Team, 2018). Treatments were performed in triplicate (n = 3). Normality of the data was determined by Shapiro-Wilk test (p > 0.05) then visually checked using *qqnorm* plots. Data without a normal distribution was square root transformed to achieve normality. Homogeneity of variance of the data was visually determined using residuals vs. fitted plots. Two-way ANOVAs followed by the Duncan Test (p < 0.05) were used to determine if there was a significant difference between root morphology and protein addition.

6.3. Results

Examples of leucine aminopeptidase zymograms clearly demonstrated the spatiotemporal distribution of enzyme activity in the rhizosphere of the two types of barley (+/- root hairs) (Fig. 6.1). Faint protein bands can be seen on the zymogram of barley without root hairs, but no bands are visible on the zymogram with root hairs.

The rhizosphere extent of the leucine aminopeptidase was significantly greater when soluble protein was applied but did not differ between barley genotypes with or without root hairs (Table 6.2; Fig. 6.2). Mean and maximum leucine aminopeptidase activity was 1.5 times greater in the protein-rich areas with root hairs compared to the control and over three-times more in soil of the barley genotype

without root hairs (Table 6.2, Fig. 6.2). The interaction between root hair and protein treatments was significant for rhizosphere extent only (Table 6.2).

Enzyme kinetics (V_{max} and K_m) showed no significant differences between roots with and without root hairs and protein addition (Table 6.2; Fig. 6.3). V_{max} was slightly higher in the barley genotype without root hairs whilst K_m was highest in the control treatment of the barley genotype with no root hairs.

Table 6.2. Two-way ANOVA results for each of the measured variables using $p < 0.05$ as the cut-off for statistical significance (as indicated by values in bold).

Variable	**Residuals**	**Root hairs**			**Protein**			**Root hairs x protein**		
		df	*F*	*p*	*df*	*F*	*p*	*df*	*F*	*p*
Rhizosphere extent	356	1	14.7	**<0.001**	1	47.0	**<0.001**	1	5.0	**0.03**
Mean LAP activity	356	1	419	**<0.001**	1	41.5	**<0.001**	1	3.38	0.07
Max LAP activity	356	1	370	**<0.001**	1	45.9	**<0.001**	1	0.73	0.39
V_{max}	8	1	1.26	0.29	1	0.05	0.83	1	0.004	0.95
K_m	8	1	0.50	0.50	1	1.41	0.27	1	3.24	0.11

Note: LAP = leucine aminopeptidase activity, *df* = degrees of freedom, *F* = *F* value and *p* = *p* value.

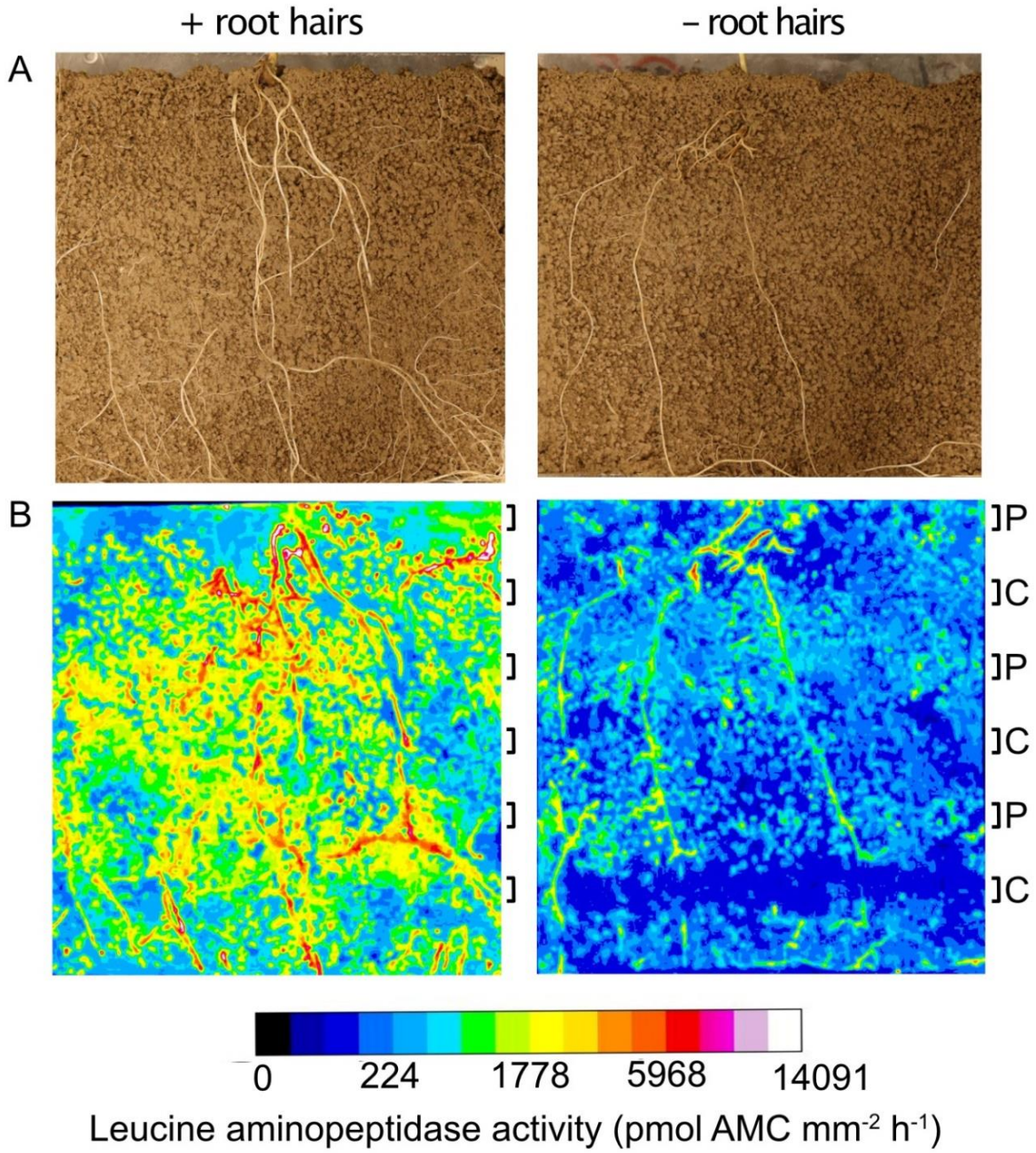

Figure 6.1. Examples of barley roots grown in rhizoboxes (A) and the spatial distribution of leucine aminopeptidase activity in the soil and rhizosphere of barley roots (B) with and without root hairs. Side colour bar is proportional to the enzyme activity (pmol mm^{-2} h^{-1}) and protein (P) and control (C) band positions are indicated on the right-hand side of the zymograms.

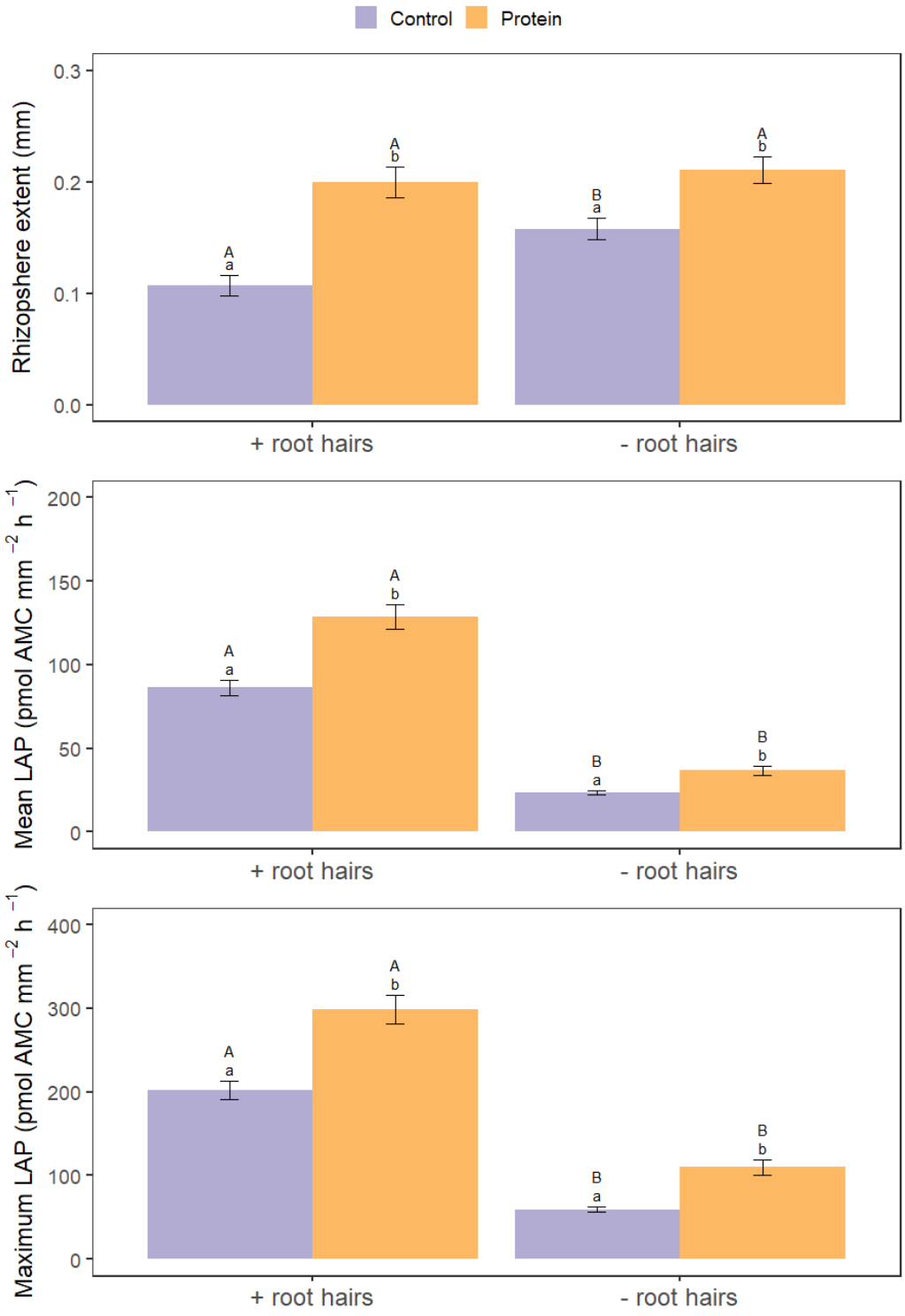

Figure 6.2. Rhizosphere extent of leucine aminopeptidase activity (distance from the root surface, mm), mean leucine aminopeptidase activity (LAP) across the rhizosphere and maximum leucine aminopeptidase activity of the rhizosphere (pmol AMC mm^{-2} h^{-1}) for barley genotypes with and without roots hairs and with protein or control (sterile water) addition. Values represent mean ± SE (n = 90). Different lowercase letters indicate a significant difference between protein and control treatments and different uppercase letters indicate a significant difference between root hair genotype ($p < 0.05$).

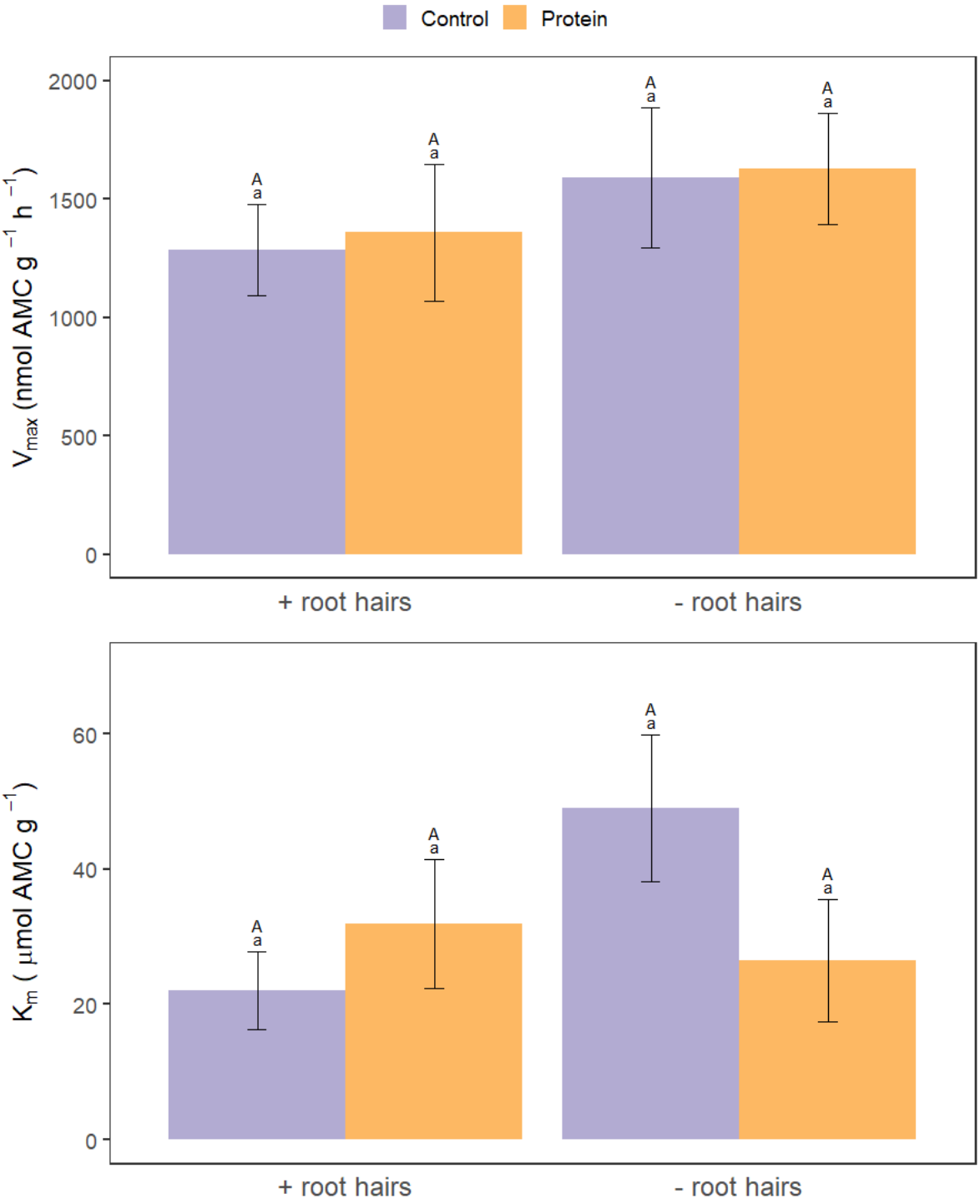

Figure 6.3. The kinetic parameters V_{max} (nmol AMC g^{-1} h^{-1}) and K_m (µmol AMC g^{-1}) of leucine aminopeptidase activity for barley genotypes with and without roots hairs and with protein or control (sterile water) addition. Values represent mean ± SE (n = 3). Different lowercase letters indicate a significant difference between protein and control treatments and different uppercase letters indicate a significant difference between root hair genotype (p < 0.05).

6.4. Discussion

6.4.1. Effect of root hairs and protein addition on protease activity

As we hypothesized, mean and maximum rhizosphere leucine aminopeptidase activity were highest for the barley genotype with root hairs. It is likely that root hairs increase the availability of substrates in the rhizosphere through their high surface area per unit root length and thus greater rates of passive C exudation (i.e. which in turn increases microbial enzyme activity) (Holz et al., 2018; Jones et al., 2009). Thus, the increase in protease activity due to root hairs induces greater rhizosphere priming thereby enhancing the release of N from soil organic matter (SOM) required to fuel microbial growth (Dijkstra et al., 2013). The rhizosphere extent of leucine aminopeptidase activity in the rhizosphere was largest when soluble protein was applied, and this enhancement was seen for both barley genotypes irrespective of the presence of root hairs. We hypothesised that root hairs would increase the area of high protease activity in the rhizosphere compared to roots without hairs via an increased surface area of the root for microbial colonisation (Gilroy and Jones, 2000; Haling et al., 2013). Similarly, an increased rhizosphere extent was observed when measuring phosphatase activity of barley with and without root hairs (Holz et al., 2020) as well as plants with differing length hairs (Ma et al., 2018). A possible reason for the similar rhizosphere extents of leucine aminopeptidase activity could be a result of soluble protein addition having a more dominant effect on rhizosphere extent than presence of root hairs. In addition, root hairs had a greater effect on the enzyme activity per root surface area rather than overall rhizosphere extent. Overall, the influence of the root on protease activity seems to be limited to a narrow zone <0.2 mm from the root surface. This is an efficient strategy for plants to compete with microorganisms in the rhizosphere for N (Kuzyakov and Xu, 2013). Enzyme activities are less reported than for many other rhizosphere properties (e.g. pH) which extend much further from the root surface. This could be due to slow diffusion of proteases relative to other solutes (e.g. H^+) and that they are primarily produced by the most active microorganisms in the rhizosphere which are likely to be located on the rhizoplane.

As expected, soluble protein addition increased leucine aminopeptidase activity in the rhizosphere of the barley plants. Many studies have shown organic fertiliser to increase soil protease activity due to the removal of substrate limitation (Liu

et al., 2019; Ma et al., 2020; Marinari et al., 2000; Melero et al., 2006; Niemi et al., 2008). Fewer studies have assessed protein amendments specifically, but those that have, have also found protein addition to increase protease activity (e.g. Geisseler and Horwath, 2009). The increase in protease activity has been shown to be faster and larger when amended with soluble protein e.g. casein compared to insoluble protein e.g. gluten and zein (Geisseler and Horwath, 2009). This is probably due to the greater ability of soluble proteins to diffuse through soil solution and thus come into contact with free exoenzymes and membrane-bound proteases. Protein addition also resulted in the largest rhizosphere extent in both the barley genotypes irrespective of the presence of root hairs by <50%. Our finding is supported by a study on the effect of root exudate compounds on artificial rhizosphere extent which found that alanine substantially increased the rhizosphere extent compared to water (Zhang et al., 2019).

Leucine aminopeptidase activity was higher in the rhizosphere with root hairs and protein addition compared to the other treatment combinations. This suggests that microorganisms and plant roots in the rhizosphere of barley genotype with root hairs have a greater advantage in utilising protein hotspots in the soil. The ability to obtain more N is particularly important in agricultural soils where crops tend to be more N limited (Rütting et al., 2018).

6.4.2. In situ versus ex situ sampling

Rhizosphere leucine aminopeptidase activity did not follow the same trends when analysed by *in situ* zymography and *ex situ* destructive sampling. This suggests these two methods are not measuring the same part of the rhizosphere or causing unreliable results due to the sampling method. The rhizosphere extent of leucine aminopeptidase activity we measured in our experiment by zymography extended <0.2 mm beyond the root surface. Yet, our *ex situ* destructive sampling method involved collecting soil from distances up to 2 mm from the root surface. Our ability to constrain the sampling to smaller distances from the root surface proved impossible due to (a) logistical difficulties in recovering rhizosphere soil, (b) aggregation of the soil, and (c) the requirement for enough soil to perform the enzymatic assays. This shows that our *ex situ* measurements of leucine aminopeptidase activity included soil from around ten times further away from the root surface than the rhizosphere of the barley plants extended. It is also probable that the *in situ* measurements of leucine aminopeptidase activity were of the rhizoplane (produced by root and root related microorganisms)

rather than the rhizosphere as the highest activity occurred when the substrate-impregnated membrane was placed over the root. It should also be noted that 20% of the enzyme reactions that occur in the volume of the rhizosphere are in direct contact with the zymograph membrane and, thus, 80% of rhizosphere enzyme activity is not measured by *in situ* zymography (Guber et al., 2018). Oburger and Jones (2018) reviewed sampling techniques used to measure root exudation from the rhizosphere and concluded that current techniques have myriad of problems that cause biases when determining exudation dynamics. It is likely that a large bias from destructively sampling and sample area is occurring in our *ex situ* sampling causing a lack of congruence with the *in situ* measurements. Destructive sampling can cause damage to roots and fungal hyphae (Oburger and Jones, 2018), whilst the sampling area is likely larger than the true rhizosphere area i.e. not all soil adhered to the root surface and thus sampling soil more related to bulk soil properties (Neumann et al., 2009). Furthermore, it is likely that destructive sampling and removal of plant C inputs may induce changes in microbial metabolism (i.e. less metabolically active) and the types of organisms which remain active (Oburger and Jones, 2009). Therefore, we recommend *in situ* spatial sampling techniques for a more representative measurement of the spatial distribution of rhizosphere enzyme activity combined with *ex situ* assays to determine enzyme kinetics. Together these will provide an insight into the catalytic mechanism of the enzyme. However, caution is needed when interpreting rhizosphere enzyme activity from *ex situ* destructive sampling as it may greatly underestimate enzyme activity at the root surface.

6.5. Conclusions

We found the combination of root hairs and protein addition to produce the highest leucine aminopeptidase activity creating an advantage for plants with root hairs to access protein hotspots in soil. The ability to obtain more N from organic sources is particularly important in agricultural soils where crops tend to be more N limited and rely on inorganic N fertilisers. In addition, our results have shown clear evidence on the disparity between *in situ* and *ex situ* rhizosphere sampling methods for protease activity. Therefore, we recommend the use of combination of *in situ* and *ex situ* sampling techniques when assessing rhizosphere enzyme activity, but consideration must be taken to determine biases in both sampling techniques.

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6.7. References

Ball, D.F., 1964. Loss-on-ignition as an estimate of organic matter and organic carbon in non-calcareous soils. European Journal of Soil Science 15, 84–92.

Brown, L.K., George, T.S., Thompson, J.A., Wright, G., Lyon, J., Dupuy, L., Hubbard, S.F., White, P.J., 2012. What are the implications of variation in root hair length on tolerance to phosphorus deficiency in combination with water stress in barley (*Hordeum vulgare*)? Annals of Botany 110, 319–328.

Brzostek, E.R., Greco, A., Drake, J.E., Finzi, A.C., 2013. Root carbon inputs to the rhizosphere stimulate extracellular enzyme activity and increase nitrogen availability in temperate forest soils. Biogeochemistry 115, 65–76.

Dijkstra, F.A., Carrillo, Y., Pendall, E., Morgan, J.A., 2013. Rhizosphere priming: a nutrient perspective. Frontiers in Microbiology 4, 216.

Geisseler, D., Horwath, W.R., 2009. Relationship between carbon and nitrogen availability and extracellular enzyme activities in soil. Pedobiologia 53, 87–98.

Gilroy, S., Jones, D.L., 2000. Through form to function: Root hair development and nutrient uptake. Trends in Plant Science 5, 56–60.

Greenfield, L.M., Hill, P.W., Paterson, E., Baggs, E.M., Jones, D.L., 2020a. Do plants use root-derived proteases to promote the uptake of soil organic nitrogen? Plant and Soil.

Greenfield, L.M., Hill, P.W., Seaton, F.M., Paterson, E., Baggs, E.M., Jones, D.L., 2020b. Is soluble protein mineralisation and protease activity in soil regulated by supply or demand? 150, 108007.

Guber, A., Kravchenko, A., Razavi, B.S., Uteau, D., Peth, S., Blagodatskaya, E., Kuzyakov, Y., 2018. Quantitative soil zymography: Mechanisms, processes of substrate and enzyme diffusion in porous media. Soil Biology & Biochemistry 127, 156–167.

Haling, R.E., Brown, L.K., Bengough, A.G., Young, I.M., Hallett, P.D., White, P.J., George, T.S., 2013. Root hairs improve root penetration, root-soil contact, and phosphorus acquisition in soils of different strength. Journal of Experimental Botany 64, 3711–3721.

Hill, E.J., Jones, D.L., Paterson, E., Hill, P.W., 2019. Hotspots and hot moments of amino acid N in soil: Real-time insights using continuous microdialysis sampling. Soil Biology & Biochemistry 131, 40–43.

Hill, P.W., Farrell, M., Jones, D.L., 2012. Bigger may be better in soil N cycling: Does rapid acquisition of small l-peptides by soil microbes dominate fluxes of protein-derived N in soil? Soil Biology & Biochemistry 48, 106–112.

Holz, M., Zarebanadkouki, M., Carminati, A., Becker, J.N., Spohn, M., 2020. The effect of root hairs on rhizosphere phosphatase activity. Journal of Plant Nutrition and Soil Science 183, 382–388.

Holz, M., Zarebanadkouki, M., Kuzyakov, Y., Pausch, J., Carminati, A., 2018. Root hairs increase rhizosphere extension and carbon input to soil. Annals of Botany 121, 61–69.

Jan, M.T., Roberts, P., Tonheim, S.K., Jones, D.L., 2009. Protein breakdown represents a major bottleneck in nitrogen cycling in grassland soils. Soil Biology & Biochemistry 41, 2272–2282.

Jones, D.L., Nguyen, C., Finlay, R.D., 2009. Carbon flow in the rhizosphere: Carbon trading at the soil-root interface. Plant and Soil 321, 5–33.

Kandeler, E., Eder, G., Sobotik, M., 1994. Microbial biomass, N mineralization, and the activities of various enzymes in relation to nitrate leaching and root distribution in a slurry-amended grassland. Biology & Fertility of Soils 18, 7–12.

Kania, J., Gillner, D., 2015. Aminopeptidases isolated from plants of great economic value - role and characteristics. Chemik 69, 466–468.

Koo, B.-. J., Adriano, D.C., Bolan, N.S., Barton, C.D., 2005. Root exudates and microorganisms, in: Encyclopedia of Soils in the Environment. Elsevier, pp. 421–428.

Kuzyakov, Y., Blagodatskaya, E., 2015. Microbial hotspots and hot moments in soil:

Concept & review 83, 184–199.
Kuzyakov, Y., Xu, X., 2013. Competition between roots and microorganisms for nitrogen: mechanisms and ecological relevance. New Phytologist 198, 656–669.
Liu, Y., Hou, H., Ji, J., Lv, Z., Liu, X., Liu, G., Li, Z., 2019. Long-term fertiliser (organic and inorganic) input effects on soil microbiological characteristics in hydromorphic paddy soils in China. Soil Research 57, 459.
Loeppmann, S., Semenov, M., Blagodatskaya, E., Kuzyakov, Y., 2016. Substrate quality affects microbial- and enzyme activities in rooted soil. Journal of Plant Nutrition and Soil Science 179, 39–47.
Ma, Q., Wen, Y., Wang, D., Sun, X., Hill, P.W., Macdonald, A., Chadwick, D.R., Wu, L., Jones, D.L., 2020. Farmyard manure applications stimulate soil carbon and nitrogen cycling by boosting microbial biomass rather than changing its community composition. Soil Biology & Biochemistry 144, 107760.
Ma, X., Zarebanadkouki, M., Kuzyakov, Y., Blagodatskaya, E., Pausch, J., Razavi, B.S., 2018. Spatial patterns of enzyme activities in the rhizosphere: Effects of root hairs and root radius. Soil Biology & Biochemistry 118, 69–78.
Marinari, S., Masciandaro, G., Ceccanti, B., Grego, S., 2000. Influence of organic and mineral fertilisers on soil biological and physical properties. Bioresource Technology 72, 9–17.
Marx, M.-C., Wood, M., Jarvis, S.., 2001. A microplate fluorimetric assay for the study of enzyme diversity in soils. Soil Biology & Biochemistry 33, 1633–1640.
Melero, S., Porras, J.C.R., Herencia, J.F., Madejon, E., 2006. Chemical and biochemical properties in a silty loam soil under conventional and organic management. Soil and Tillage Research 90, 162–170.
Miranda, K.M., Espey, M.G., Wink, D.A., 2001. A Rapid, Simple Spectrophotometric Method for Simultaneous Detection of Nitrate and Nitrite. Biology and Chemistry 5, 62–71.
Mulvaney, R.L., 1996. Nitrogen - Inorganic Forms, in: Methods of Soil Analysis. Part 3 - Chemical Methods. Soil Science Society of America, Madison, WI, USA, pp. 1123–1200.
Neumann, G., George, T.S., Plassard, C., 2009. Strategies and methods for studying the rhizosphere-the plant science toolbox. Plant and Soil 321, 431–456.
Niemi, R., Vepsäläinen, M., Wallenius, K., Erkomaa, K., Kukkonen, S., Palojärvi, A., Vestberg, M., 2008. Conventional versus organic cropping and peat

amendment: Impacts on soil microbiota and their activities. European Journal of Soil Biology 44, 419–428.

Oburger, E., Jones, D.L., 2018. Sampling root exudates – Mission impossible? Rhizosphere 6, 116–133.

Oburger, E., Jones, D.L., 2009. Substrate mineralization studies in the laboratory show different microbial C partitioning dynamics than in the field. Soil Biology & Biochemistry 41, 1951–1956.

Puissant, J., Jones, B., Goodall, T., Mang, D., Blaud, A., Gweon, H.S., Malik, A., Jones, D.L., Clark, I.M., Hirsch, P.R., Griffiths, R., 2019. The pH optimum of soil exoenzymes adapt to long term changes in soil pH. Soil Biology & Biochemistry 138, 1076012.

R Core Team, 2018. R: A language and environment for statistical computing.

Razavi, B.S., Zarebanadkouki, M., Blagodatskaya, E., Kuzyakov, Y., 2016. Rhizosphere shape of lentil and maize: Spatial distribution of enzyme activities. Soil Biology & Biochemistry 96, 229–237.

Razavi, B.S., Zhang, X., Bilyera, N., Guber, A., Zarebanadkouki, M., 2019. Soil zymography: Simple and reliable? Review of current knowledge and optimization of the method. Rhizosphere 11, 100161.

Rillig, M.C., Caldwell, B.A., Wösten, H.A.B.B., Sollins, P., 2007. Role of proteins in soil carbon and nitrogen storage: Controls on persistence. Biogeochemistry 85, 25–44.

Rütting, T., Aronsson, H., Delin, S., 2018. Efficient use of nitrogen in agriculture. Nutrient Cycling in Agroecosystems 110, 1–5.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.Y., White, D.J., Hartenstein, V., Eliceiri, K., Tomancak, P., Cardona, A., 2012. Fiji: An open-source platform for biological-image analysis. Nature Methods 9, 676–682.

Schulten, H.-R., Schnitzer, M., 1997. The chemistry of soil organic nitrogen: a review. Biology & Fertility of Soils 26, 1–15.

Steingrobe, B., Schmid, H., Gutser, R., Claassen, N., 2001. Root production and root mortality of winter wheat grown on sandy and loamy soils in different farming systems. Biology and Fertility of Soils 33, 331–339.

Stevenson, F.J., Cole, M.A., 1999. The Carbon Cycle, in: Stevenson, F.J., Cole, M.A. (Eds.), Cycles of Soil : Carbon, Nitrogen, Phosphorus, Sulfur,

Micronutrients. Wiley, IL, USA, pp. 1–78.

Tajima, R., Kato, Y., 2011. Comparison of threshold algorithms for automatic image processing of rice roots using freeware ImageJ. Field Crops Research 121, 460–463.

Vaz, M.D.., Edwards, A.C., Shan, C.A., Cresser, M.S., 1994. Changes in the chemistry of soil solution and acetic-acid extractable P following different types of freeze/thaw episodes. European Journal of Soil Science 45, 353–359.

Zhang, X., Dippold, M.A., Kuzyakov, Y., Razavi, B.S., 2019. Spatial pattern of enzyme activities depends on root exudate composition. Soil Biology & Biochemistry 133, 83–93.

Chapter 7

Critical review and meta-analysis of methods used to assess soil protease activity

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This manuscript has been not yet been submitted.

LG conducted the systematic review. LG, DJ and JP discussed the meta-analysis and LG conducted the analysis. LG wrote the manuscript and LG, DJ, and JP reviewed the manuscript.

Abstract

Proteases play a crucial role in the soil nitrogen (N) cycle by converting protein to oligopeptides and amino acids. They are often viewed as a bottleneck in terrestrial N cycling; therefore, it is vital that we have robust methods for evaluating protease activity in soil. In response to this, several laboratory-based protease methods have been developed and subsequently modified. However, the validity of these different approaches remains largely unknown. In addition, the lack of standardised protocols makes is difficult to compare protease activity across studies. In this systematic review, we critically evaluate the most common colorimetric and fluorimetric methods used to measure soil protease activity involving 680 independent studies and 1,491 individual assays. To investigate the key regulators of soil protease activity, we collected associated metadata on environmental (mean annual temperature and soil pH) and methodological (assay temperature and pH) factors. Protease activity for colorimetric substrates were concentrated at ca. 1000 nmol product g^{-1} h^{-1}, whilst the fluorimetric substrate was lower at ca. 100 nmol product g-1 h^{-1}. However, we found soil protease activity varied widely around these peaks due to study biases rather than environmental or methodological factors. We present the following recommendations for measuring soil protease activity: 1) report assay conditions and soil characteristics, particularly pH and temperature, 2) conduct the assay at either field or optimised pH and temperature and, 3) check that measurements lie between 0-1000 nmol product g^{-1} h^{-1}. Following these recommendations will provide a robust method for measuring enzyme activity and a self-check for reliable results. Our study also sets a precedent for future meta-analyses on other soil enzymes to determine the impact of methodological approach.

Keywords: Enzyme assay, Mineralisation, Organic nitrogen, Peptidase, Proteinase

7.1. Introduction

Protease activity is an important process in the soil N cycle and is often considered to be the rate-limiting step of N mineralisation (Jan et al., 2009; Weintraub and Schimel, 2005). Proteases catalyse the hydrolysis of proteins and polypeptides into oligopeptides and amino acids. In the soil, extracellular proteases are released by microorganisms, plants, animal excrements and leached from agro-industrial fertilisers, though microorganisms are considered the dominant producer (Greenfield et al., 2020; Vranová et al., 2013). Protease activity, alongside other enzymes, are increasingly being used as soil quality indicators (Schloter et al., 2018; Trasar-Cepeda et al., 2008). Therefore, standardised soil sample pre-treatment, assay protocol and measurement units are vital to ensure comparability across studies.

Methods used to assay soil protease activity can be split into two main categories: fluorometric and colorimetric analysis. Both methods are based on the addition of a substrate bonded to a fluorophore or chromophore which is added to the soil solution or soil slurry and the breakdown products are then measured directly or indirectly. Fluorometric assays are more sensitive than their colorimetric counterparts with a limit of detection around 50 pmol of substrate (Deng et al., 2013; Dick et al., 2018). However, both are susceptible to interference from other soil components, which must be accounted for (Deng et al., 2013). Both techniques offer substrates that can be measured using ‘bench-top’ or ‘microplate’ based protocols with the latter allowing for a larger number of low volume samples to be processed but can incur more measurement error e.g. in pipetting (Bell et al., 2013; Deng et al., 2013). The proteases targeted by an assay method tend to fall into two distinct categories related to the substrate used: 1) specific proteases, and 2) non-specific proteases. For example, the leucine-7-AMC substrate is hydrolysed by leucine aminopeptidase (EC 3.4.11.1) which preferentially catalyses the hydrolysis of leucine at the N-terminus of polypeptides and proteins. In contrast, the substrate, casein, is cleaved in many places by casein-hydrolysing peptidases like trypsin (EC 3.4.21.4). Indirect methods of analysis tend to measure non-specific proteases e.g. casein measured colorimetrically. Despite being one of the oldest techniques (Ladd and Butler, 1972), colorimetric analysis with casein remains a popular method due to its broader analysis of soil proteases. However, little work has been done to determine whether different substrates provide a similar estimate of soil protease activity and organic N processing rates in soil.

Another key difference in protease assay methods is whether the assay is conducted under ‘laboratory-optimised conditions’ or ‘field-relevant conditions’. The former normally involves optimisation of the pH, temperature and substrate conditions to maximise catalytic activity (Tabatabai, 1994). In the field, soil pH varies greatly according to soil type, land use and pollution events while temperature is determined by season, altitude and climate (Slessarev et al., 2016). The optimum pH of protease activity is around 7 and a temperature around 40-60˚C although this can vary depending on the soil, location and microbial origin of the protease (Ladd and Butler, 1972; Puissant et al., 2019; Vazquez et al., 2005). Therefore, the choice of field-relevant or laboratory-optimised conditions is likely to have a marked effect on the measured soil protease activity and thus the interpretation of the results obtained.

The wide range of assay methods and substrates used leads to questions over how comparable studies are. There have been many objective reviews over the years that conclude the need for standardisation of methods when measuring soil enzyme activity (e.g. Dick, 2011; Fornasier et al., 2011; German et al., 2011; Nannipieri et al., 2017). Despite these reviews, standardisation and transparency of reporting key methodological and environment variables that affect enzyme activity is lacking. Furthermore, there are no commercially available reference materials for quality assurance purposes (e.g. standard soil proteins or soil proteases) and no standard reference values for protease activity for use as a soil health metric. This has led to the publication of questionable results, exacerbated by pitfalls in methodologies and activity calculations (German et al., 2011).

The aim of this study was to 1) review current colorimetric and fluorimetric methods used to measure protease activity in soil, 2) determine the impact of using field-relevant versus laboratory-optimised pH and temperature conditions in protease assays, 3) evaluate the numerical range of soil protease activity at a global scale 4) identify potential factors that may help explain the natural variation in protease activity, and 5) provide guidance for future measurements and reporting of soil protease activity.

7.2. Methods

7.2.1. Systematic review and data exportation

We conducted a systematic review in March 2020 to obtain studies that had measured soil protease activity. We used Web of Science as our primary database and used the search string “TS = (soil* NEAR/10 (protease* OR peptidase* OR aminopeptidase* OR proteinase* OR proteolytic OR assay? OR enzyme? OR "enzyme activity"))” (see Appendix 5, Table S1 for search term strategy). Following this, we used ScienceDirect to search full texts for common assay substrates (“soil” followed by “7-Amino-4-Methylcoumarin”, “N-benzoyl L-arginine amide”, “casein”, “gelatin”, “*p*-nitroaniline”, “haemoglobin” and “benzyloxycarbonyl phenylalanyl leucine”, “azocoll” and “azocasein”). We also searched common synonyms and acronyms for these substrates and filtered results for journals in the relevant discipline (Appendix 5, Table S2). Studies were selected using predetermined criteria (Appendix 5, Table S3) and in total, 680 studies met the criteria for inclusion (Appendix 5, Fig. S1. PRISMA diagram). Once these studies were selected, we exported data on assay conditions into an Excel spreadsheet. In addition, we exported data on methodological and environmental factors: substrate used, assay pH, assay temperature, mean soil protease activity and its standard deviation, soil pH, sample location and mean annual temperature (MAT). Mean soil protease activity data was exported using predetermined criteria (Appendix 5, Table S4) and studies that did not meet criteria for soil protease activity data were not used in analysis for aims 3 and 4 of this study. When more than one assay was included in a study (e.g. studies that measured different soil types or under different assay conditions) they were counted as an independent protease activity measurement. Therefore, from the 680 individual studies collected there were 1,491 individual assays. Protease activity was converted into nmol product g^{-1} h^{-1} where applicable and studies were grouped according to the substrate used: AMC, casein, BAA, Z-phe-leu and pNA. We acknowledge that AMC, Z-phe-leu and pNA come in multiple forms, but for the purposes of this study we grouped the variations of each of these substrates (Appendix 5, Table S5). We have not analysed or reported on the substrates azcoll, azocasein, haemoglobin, gelatin and native (no substrate) because these were represented by <10 studies in the dataset.

7.2.2. Data analysis

All data analysis was conducted in R 3.5.0 (R Core Team, 2018) and all graphs were drawn using the package *ggplot2* (Wickham, 2016). Protease activity values were converted to nmol product g^{-1} h^{-1} in order to compare within substrates (product = AMC, tyrosine, NH_4^+, leucine and pNA for AMC, casein, BAA, Z-phe-leu and pNA substrates). Outliers were removed by estimating the maximum activity possible for the assay based on the amount of substrate added (i.e. theoretically impossible values where the reported protease activity exceeded the amount of substrate added were not deemed scientifically credible and were thus omitted). From this method of outlier removal, we excluded 103 assays from 53 studies from analysis for aims 3 and 4 (i.e. 7.8% of the total studies; Appendix 5, Fig. S2). Mean annual temperature (MAT) data was extracted using packages *sp* and *raster* in R according to the GPS coordinates for assays that reported no MAT (Bivand et al., 2013; Fick and Hijmans, 2017; Hijmans, 2020; Pebesma and Bivand, 2005). The pH optima of leucine aminopeptidase measured by Puissant et al. (2019) at pH 7 was used to determine the difference between assay pH reported and pH optima of the enzyme. Although we acknowledge that leucine aminopeptidase does not represent the pH optimum of all protease enzymes targeted by the substrates analysed in this study, there is little information on the soil pH optimum of the other proteases targeted by casein, BAA and Z-phe-leu substrates. Work by Ladd and Butler (1972) suggests the pH optimum is between 6.8-8.8 for Z-phe-leu, BAA and casein. Other studies including Niemi and Vepsäläinen (2005) and Sinsabaugh et al. (2008) have measured a pH optimum of approximately 7 for leucine aminopeptidase.

A linear regression model was used to determine the extent to which environmental (soil pH and MAT) and methodological factors (assay pH and assay temperature) explain the variation in protease activity across studies. A linear model using the function *lm* was built to test the effect of environmental and methodological factors on mean soil protease activity collected from the studies. Protease activity measurement of each assay was only included in linear regression analysis if there was a value for mean soil protease activity, MAT, soil pH, assay pH and assay temperature. The linear regression models were tested for normality using the Shapiro-Wilks test and then visually assessed using a *qqnorm* plot of the residuals. In order to meet normality assumptions of the model soil protease activity was $\log_{10}$ transformed.

7.3. Results

7.3.1. Soil protease activity methods

Fluorimetric-based protease assays

Fluorimetric assays for quantifying soil enzyme activity were first introduced by Pancholy and Lynd (1972) to measure soil lipase activity. However, our analysis shows that their use for measuring soil protease activity did not become commonplace until the 2000s (Fig. 7.1). Since their introduction, the use of fluorescent substrates has become increasingly popular. Overall, our analysis suggests that they account for 40% of the total soil protease studies, while in the last 5 years they account for nearly 60% of the total. Most fluorimetric assays use the fluorescent compound 4-methlumbelliferone (MUB), however, protease assays use 7-amino-4-methlycoumarin (AMC or MUC) which has an amide group attached to one of the benzene rings instead of a hydroxide group, allowing for an amino acid to bond to the amide group via an amide bond (Table 7.1). Aminopeptidase enzymes hydrolyse the amide bond producing the amino acid and AMC. The latter, upon excitation by UV light at 330-380 nm, emits fluorescence at 440-480 nm that is read by a fluorometer. Due to their specificity, aminopeptidase assays do not provide an overall measurement of soil protease activity but a proxy to indicate rates of activity. Despite being expensive per gram of substrate, due to the low quantities needed (mg per assay) it is a cost-effective method (ca. £0.34 sample^{-1}; Table 7.1). Alkalinisation e.g. addition of NaOH is used in some protocols to increase fluorescence of acidic solutions. However, German et al. (2011) found fluorescence was only difficult to quantify at pH values below 4.5. A geographical analysis of the use of fluorescent substrates revealed a large research bias towards their use in the Northern Hemisphere, particularly Europe and the USA (Fig. 7.2).

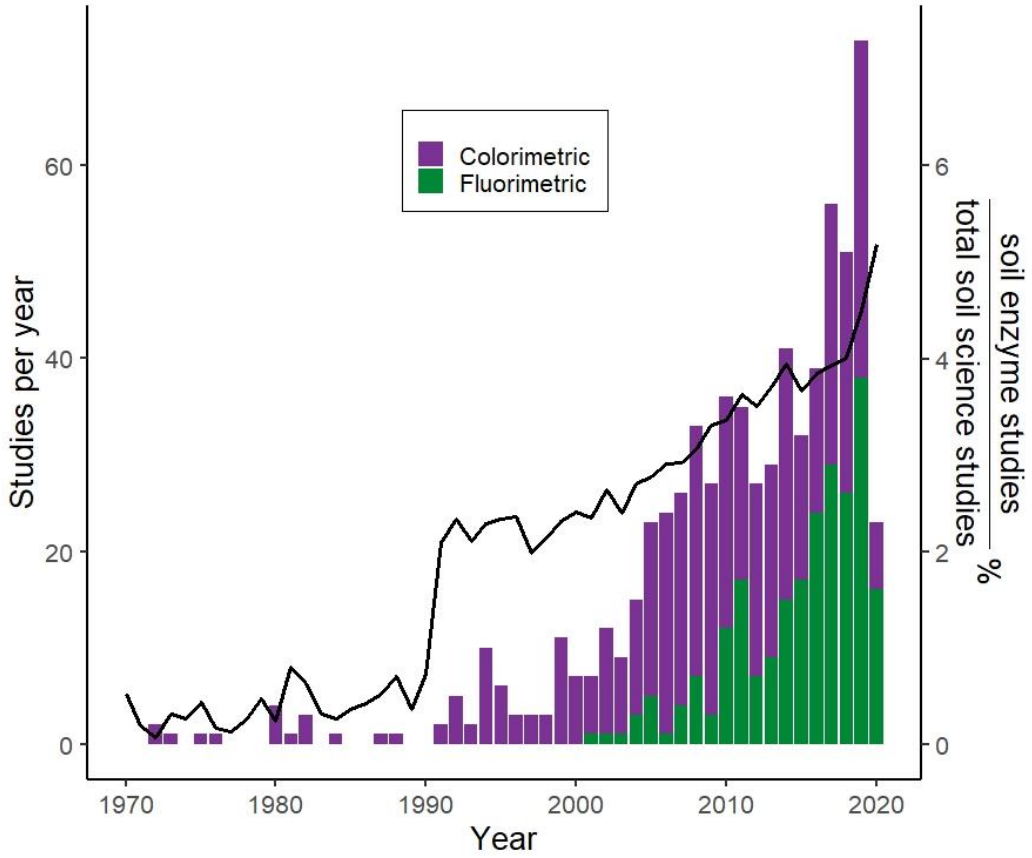

Figure 7.1. Number of studies that have measured soil protease activity using colorimetric and fluorimetric techniques between 1970 and 2020 and that were considered within this systematic review. The black line represents the number of soil enzyme studies published each year as a percentage of total soil science studies published each year.

Colorimetric-based protease assays

Casein is a milk-derived phosphoprotein substrate with a very high molecular weight, which can be broken down by a range of proteases (e.g. endo- and exoproteases) to produce peptide chains and amino acids (Table 7.1; Dewan et al., 1974; Landi et al., 2011). The Folin-Ciocalteu reagent is the most common chromophore reagent used to determine the quantity of breakdown products (used in a ca. 40% of colorimetric studies collected in this meta-analysis) and reacts with tyrosine residues produced as a breakdown product by protease enzymes to form a blue chromophore. It was originally used as a protein assay by Lowry et al. (1951). As it reacts with tyrosine, the amount of tyrosine produced over a certain time can be measured and compared to a tyrosine standard. However, Folin-Ciocalteu reagent also reacts with many other compounds in soil and due to the complex nature of soil this means that several other compounds could be measured as well (e.g. humic substances, buffers, chelating agents and lipids) (Peterson, 1979).

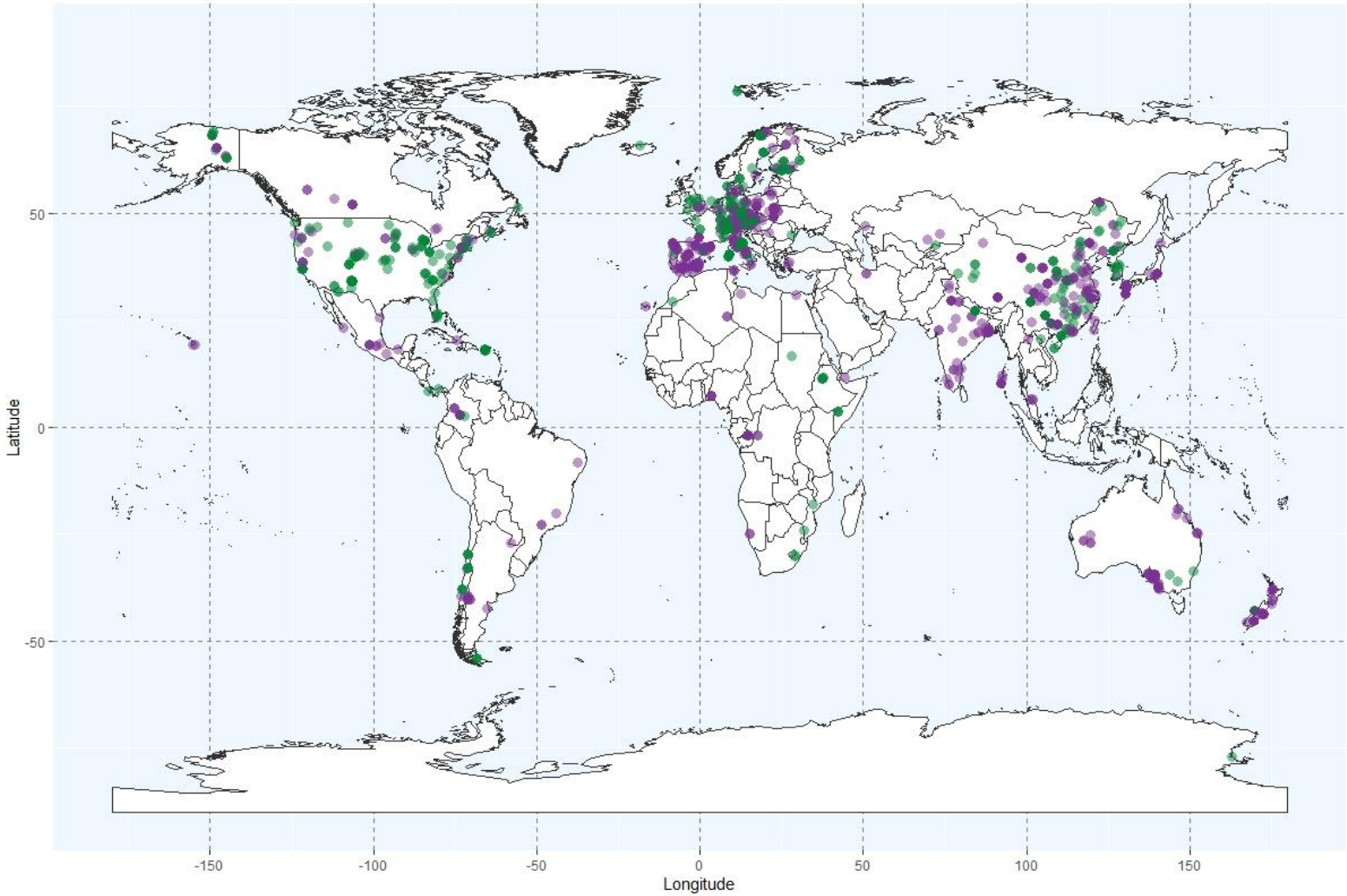

Figure 7.2. Geographical location where soil protease activity has been measured either colorimetrically (n = 393, purple symbols) or fluorometrically (n = 179, green symbols).

BAA is a typical substrate for a trypsin-like enzyme producing NH_4^+ (Table 7.1) (Landi et al., 2011). NH_4^+ can then be measured colorimetrically. Z-phe-leu is a low molecular weight substrate that is hydrolysed by carboxypeptidases to produce leucine which is also measured colorimetrically (Ladd and Butler, 1972). Both substrates are typically measured with ninhydrin reagent. Hydrinantin (reduced ninhydrin) is added directly to the reaction to prevent the precipitation of certain salts affecting accuracy. The carbonyl group on the ninhydrin reacts with nucleophilic groups on amino acids (e.g. NH_2-R) to form a ninhydrin chromophore of deep blue/purple colour (Moore and Stein, 1954). An amino acid standard e.g. leucine is used to determine the quantity of amino acids in the solution. However, as it also reacts with NH_4^+ which is immobilised by microorganisms in soil, toluene is often used to inhibit microbial activity (used in ca. 7% of colorimetric studies following the Watanabe and Hayano, 1995 method). Reiskind et al. (2011) found toluene to only reduce immobilisation in organic tundra soils with no effect in the mineral tundra soils also tested. Thus, studies using ninhydrin reagent are likely to also be strongly impacted by microbial immobilisation of substrate released by protease action. No studies accounted for this in a quantitative way. Z-phe-leu is a low molecular weight substrate also typically measured using the ninhydrin reagent (Ladd and Butler, 1972). It is hydrolysed by carboxypeptidases to produce leucine which is then measured colorimetrically. Due to using the same reagent, Z-phe-leu can also react with NH_4^+ and thus could be measuring microbial mineralisation as well as protease activity.

Lastly, p-nitroaniline (pNA) is a chromophore commonly used to measure aminopeptidase activity in soil (Sinsabaugh et al., 1993). The assay works similarly to AMC whereby the chromophore is bonded to an amino acid (e.g. glycine and leucine) and when this bond is hydrolysed by an aminopeptidase it turns purple (absorbance measured at ca. 410 nm) (Table 7.1). Like AMC assays, due to their specificity, aminopeptidase assays do not provide an overall measurement of soil protease activity but a proxy to indicate rates of activity. This assay can be carried out via bench- and microplate-scale making it versatile (Deng et al., 2013). Colorimetric-based assays have remained a popular method despite the rise in fluorimetric assay use, accounting for 60% of the total soil protease studies (Fig. 7.1).

Table 7.1. Summary of substrates used to measure soil protease activity and their basic properties. Leucine-7-amino-4-methlycoumarin and glycine *p*-nitroaniline have been used as examples for AMC and pNA respectively as these are the most common forms for the substrate group (Appendix 5; Table S5).

Substrate	Method	Protease measured	Hydrolysis mechanism	EC number	Product measured	Cost per sample (£)*	Main studies cited for protocol
Leucine-7-amino-4-methylcoumarin (AMC)	Fluorimetric	Leucine aminopeptidase	Exopeptidase (N-terminus)	3.4.11.	AMC	0.34	Marx et al. 2001 Saiya-Cork et al. 2002 Bell et al. 2013 Vepsäläinns et al. 2001 Stursova et al. 2008
Casein	Colorimetric	Trypsin	Endopeptidase	3.4.21-25	Tyrosine	0.002	Ladd & Butler 1972 Nannipieri et al. 1980 Guan 1986
N-benzoyl L-arginine amide (BAA)	Colorimetric	Trypsin	Endopeptidase	3.4.21-25	NH_4^+	0.3	Nannipieri et al. 1980 Ladd & Butler 1972
Glycine *p*-nitroaniline (pNA)	Colorimetric	Glycine aminopeptidase	Exopeptidase (N-terminus)	3.4.11.-	pNA	0.4	Sinsabaugh et al. 1993 Allison & Jastrow 2006
Benzyloxycarbonyl phenylalanyl leucine (Z-phe-leu)	Colorimetric	Carboxypeptidase	Exopeptidase (C-terminus)	3.4.21-25	Leucine	0.13	Ladd & Butler 1972 Hayano 1993

*Costs per sample was calculated according to the cost of the substrate (£) from Merck and the typical amount used per sample.

7.3.2. Laboratory-optimised versus field-relevant protease assay conditions

Assay pH versus soil pH

The difference between assay pH and field soil pH (δpH) is shown in Figure 7.3a. Except for BAA and Z-phe-leu, most assays were measured using an assay pH within 0-0.5 units of the actual soil pH (303 assays). For BAA and z-phe-leu, the most common δpH was between 0.5-1 unit. For BAA and casein, ca. half of the assays measured a δpH greater than 1 and around a third of AMC and pNA and 16% of Z-phe-leu assays. A large proportion of studies (*n* = 121) did not report either assay pH or soil pH, meaning δpH could not be calculated. Generally, δpH was positive meaning that the assay pH used was higher than the actual soil pH. The difference between assay pH and the pH optima (δpH) is shown in Figure 7.3b. Except for BAA where two thirds of the assays were recorded at the pH optima (0-0.5 δpH), the majority of assays were measured at greater than 1 unit of pH from the optima (66-89%). Generally, δpH was positive for BAA, casein and z-phe-leu meaning that the assay pH used was higher than the optimum pH measured and negative for AMC and pNA assays. This was probably due to the large number of AMC assays following Saiya-Cork et al. (2002) which used an assay pH of 5, whilst the majority of casein assays followed the method of Ladd and Butler (1972) which used an assay pH of 8.1.

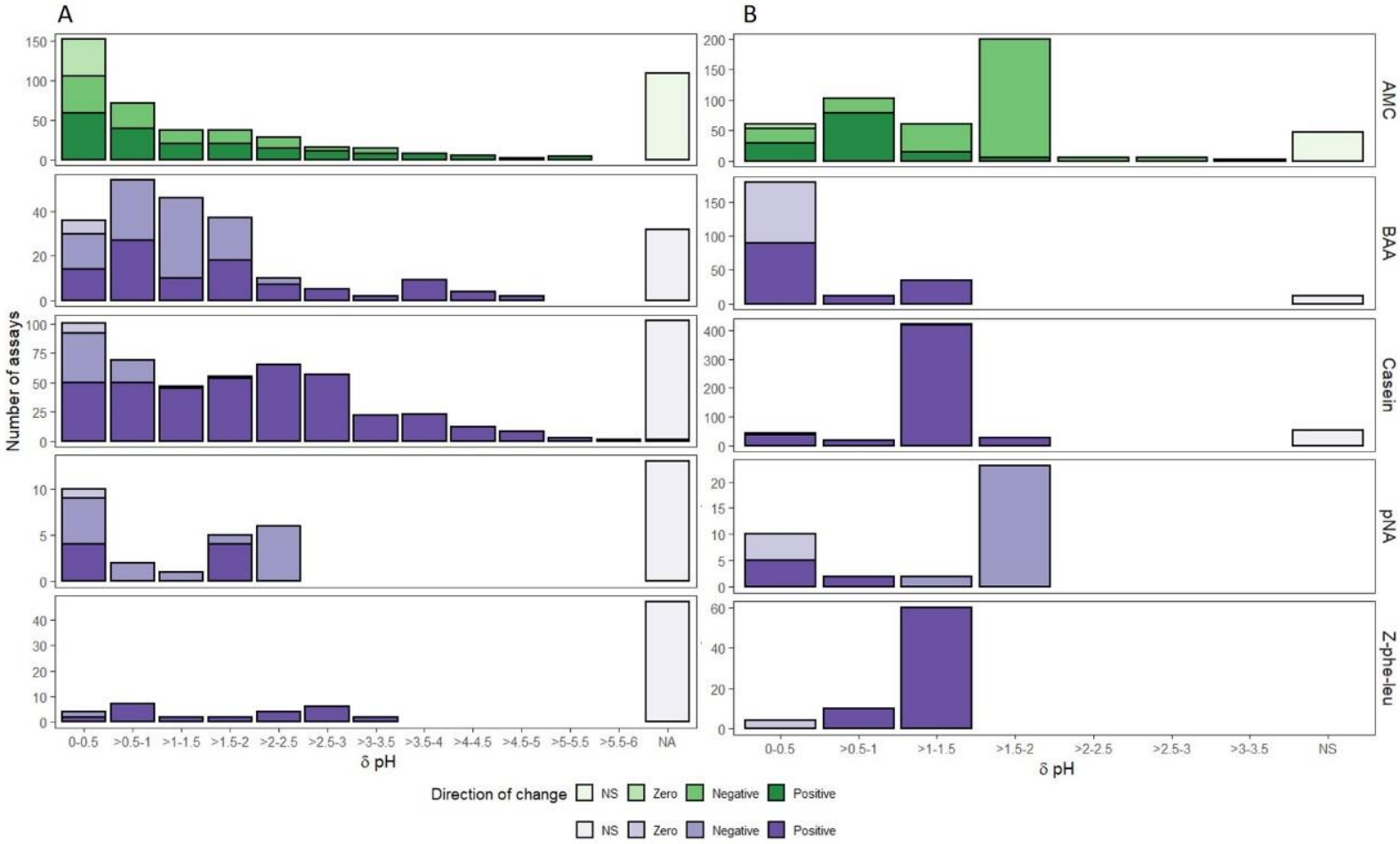

Figure 7.3. Number of assays for each δpH for the A) difference between the assay pH and soil pH, and B) difference between the assay pH and pH optima of 7 (*n* = 173, 99, 262, 16, 21 studies for AMC, BAA, casein, pNA and Z-phe-leu substrates, respectively). Colorimetric substrates are shown in purple and fluorimetric substrates are shown in green.

Assay temperature versus soil temperature

The difference between assay temperature and MAT (δtemperature) is shown in Figure 7.4. A small proportion of assays (*n* = 71) measured protease activity at a temperature close to their MAT (0-5˚C difference). Of these, no assays involved either BAA or Z-phe-leu. Generally, δtemperature was positive, meaning that the assay temperature used was higher than the actual MAT at the site where the sample was collected. Between 60-95% of assays for all substrates were measured at an assay temperature >10˚C higher than the soil's MAT. Up to 24% of assays did not report either assay temperature or MAT meaning δtemperature could not be calculated.

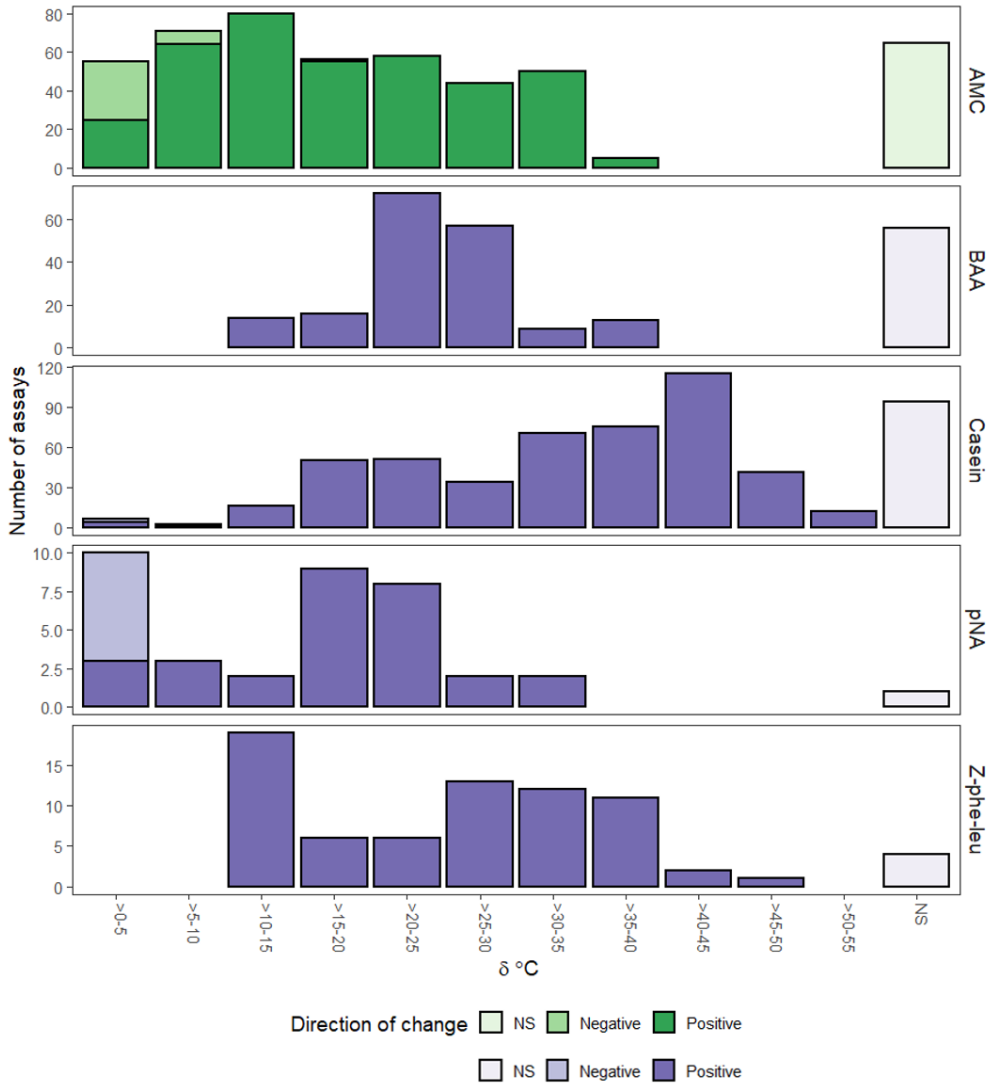

Figure 7.4. Number of assays for each δtemperature (°C) between the assay temperature and mean annual temperature (MAT) (*n* = 173, 99, 262, 16, 21, for AMC, BAA, casein, pNA and Z-phe-leu substrates, respectively). Colorimetric substrates are shown in purple and fluorimetric substrates are shown in green.

7.3.3. Numerical range of protease activity at a global scale

Mean soil protease activity varied widely between, illustrated when plotted on a log_{10} scale (Fig. 7.5). Whilst all four colorimetric substrates showed a density peak at ca. 1000 nmol product g^{-1} h^{-1}, the fluorimetric substrate AMC had a lower density peak at ca. 100 nmol product g-1 h^{-1}. Across all substrates, around 60% of the data lay between 0-1000 nmol product g^{-1} h^{-1} and 80% lay between 0-5000 nmol product g^{-1} h^{-1}. In addition, we observed large interstudy variations in soil protease activity with a mean standard error of ± 74,000 nmol product g^{-1} h^{-1} and intra-study variation with a mean standard error of ± 8,500 nmol product g^{-1} h^{-1} (Table 7.2).

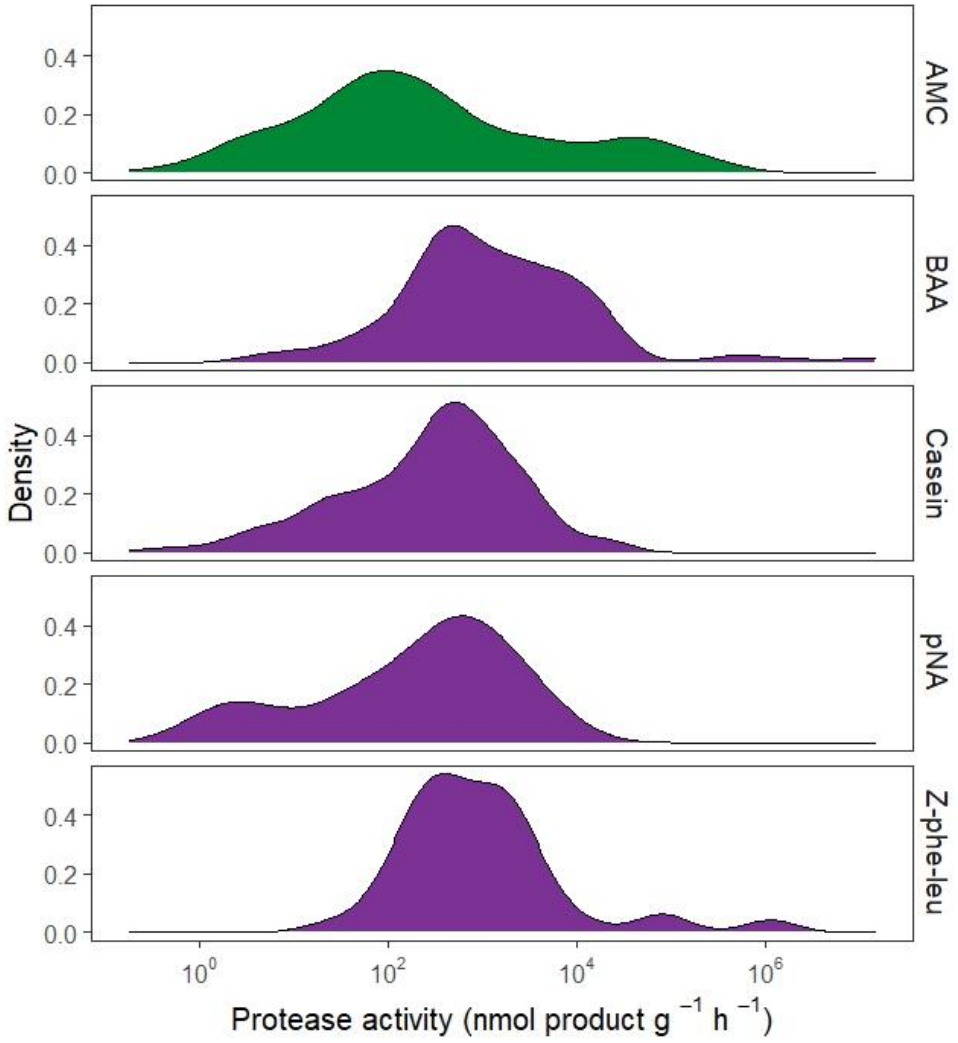

Figure 7.5. Density distribution of the mean soil protease activity (nmol product g^{-1} h^{-1}) on a log_{10} scale for each substrate with outliers removed (colorimetric substrates are shown in purple and fluorimetric substrates are shown in green).

Table 7.2. Summary statistics of protease activity and associated environmental and methodological factors used in the linear regression model across 929 assays (*n* = 105, 79, 186, 14 and 11 studies for AMC, BAA, casein, pNA and z-phe-leu respectively).

Parameter	Mean	Min	Max	Median	Lower quartile	Upper quartile
Protease activity (nmol product g^{-1} h^{-1})	18,601	0.18	4,535,469	435	79	2,252
Interstudy standard deviation	74,496	0	35,880,000	56	9	351
Intra-study standard deviation	8,517	1.6	476,141	8,079	70	1,618
Soil pH	6.2	2.6	9.3	6.2	5.2	7.5
MAT (°C)	12	-14	30	13	7.5	16
Assay temperature (°C)	38	2	55	40	30	50
Assay pH	7.2	4.5	9	7.8	4.5	8.1

7.3.4. Natural variation of protease activity at a global scale

Overall, there were few significant associations between environmental (MAT and soil pH) or methodological factors (assay temperature and pH) and protease activity and those that were significant ($p < 0.05$) had R^2 values < 0.32 (Figure 7.6, Table 7.3). The magnitude of change of any environmental or methodological factor on protease activity was small at 0.02-0.52 (on a $\log_{10}$ scale) equating to 1.0-3.3 nmol product g^{-1} h^{-1} increase or decrease in protease activity (Table 7.3) compared to the large standard error observed from studies (Table 7.2).

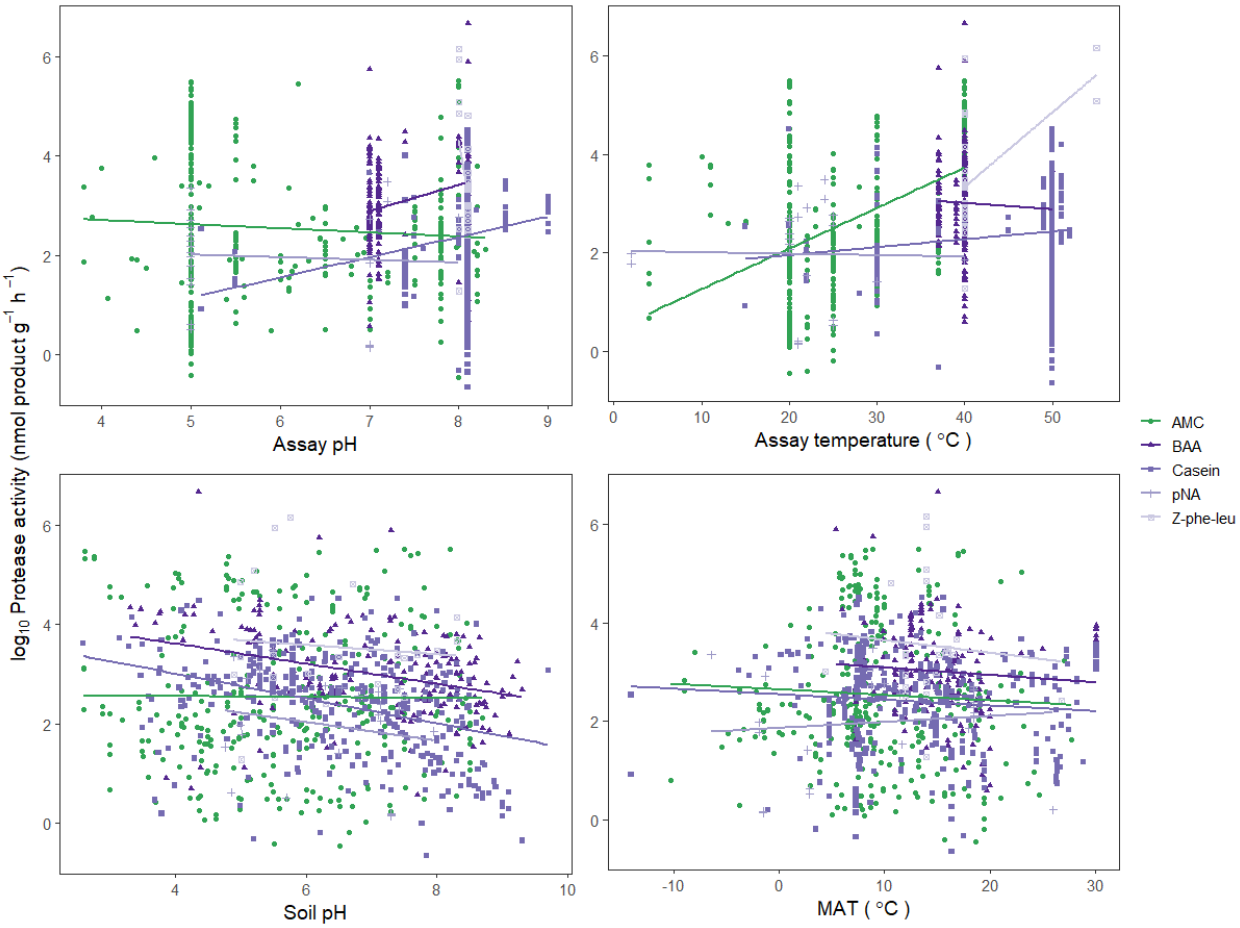

Figure 7.6. Relationship between methodological (assay pH and assay temperature [°C]) or environmental (soil pH and mean annual temperature [MAT, °C]) factors and soil protease activity ($\log_{10}$ nmol product g^{-1} h^{-1}) for five different protease substrates (n = 105, 79, 186, 14 individual studies for AMC, BAA, casein, pNA and Z-phe-leu, substrates, respectively). Solid lines trace a linear regression fit (a summary of regression analyses can be found in Table 7.3). Green symbols indicate fluorimetric substrates and purple symbols indicate colorimetric substrates.

Table 7.3. Summary of linear regression model of the relationship between environmental (MAT and soil pH) or methodological (assay pH and temperature) factors and protease activity for each substrate. Values in bold are significant at $p < 0.05$.

Factor	Substrate	Log_{10} Protease activity		
		Line equation	R^2	*p*
MAT	AMC	$y = 2.6 - 0.01x$	0.0001	0.86
	BAA	$y = 3.2 - 0.02x$	0.006	0.29
	Casein	$y = 2.5 - 0.01x$	0.007	0.09
	pNA	$y = 1.9 + 0.01x$	0.01	0.63
	Z-phe-leu	$y = 3.9 - 0.03x$	0.007	0.70
Assay temperature	AMC	$y = 0.43 + 0.08x$	0.20	**<0.001**
	BAA	$y = 3.5 - 0.01x$	0.0004	0.78
	Casein	$y = 1.6 + 0.02x$	0.01	**0.03**
	pNA	$y = 2.0 - 0.003x$	0.0004	0.92
	Z-phe-leu	$y = -2.7 + 0.15x$	0.32	**0.005**
Soil pH	AMC	$y = 2.6 - 0.01x$	0.0001	0.86
	BAA	$y = 4.4 - 0.20x$	0.12	**<0.001**
	Casein	$y = 4.0 - 0.25x$	0.14	**<0.001**
	pNA	$y = 3.2 - 0.19x$	0.04	0.38
	Z-phe-leu	$y = 4.1 - 0.09x$	0.01	0.67
Assay pH	AMC	$y = 3.0 - 0.08x$	0.01	0.21
	BAA	$y = -0.82 + 0.52x$	0.05	**0.002**
	Casein	$y = -0.90 + 0.41x$	0.03	**<0.001**
	pNA	$y = 2.3 - 0.05x$	0.003	0.08
	Z-phe-leu	$y = 87 - 10x$	0.16	0.06

7.4. Discussion

7.4.1. Colorimetric- versus fluorimetric-based protease assays

Only a few studies assessed more than one substrate simultaneously and so it was not possible to reliably determine correlations between protease activity measurements from different substrates. However, the small sample of data we obtained showed strong correlations between leucine-AMC, alanine-AMC and tyrosine-AMC but weak correlations between colorimetric substrates (Appendix 5, Fig. S3). All AMC substrates measure aminopeptidase activity and thus it is not surprising that the different substrates show strong agreement, while in colorimetric substrates a range of different proteases are measured. Typically, one protease-specific substrate is used as a proxy for total soil protease activity, yet all proteases will not have the same abundancy, activity, kinetic parameters, catalytic mechanism or ecological function (Vranová et al., 2013). With the measurement of soil protease activity becoming increasingly popular in soil enzyme studies (Fig. 7.1) it shows the importance of choosing the right protocol. In terms of ease, accuracy, reliability and increasing popularity, fluorimetric assays using AMC are considered the best choice (German et al., 2011; Nannipieri et al., 2017). In addition, the optimal pH and temperature conditions of the substrates that target multiple proteases and proteases with broad specificity (e.g. BAA and casein) are limited and centre around a couple of soil studies (Ladd, 1972; Ladd and Butler, 1972). In contrast, more recent work has been conducted on leucine aminopeptidase (e.g. Niemi and Vepsäläinen, 2005; Puissant et al., 2019). However, as this method only targets aminopeptidases it could be missing key soil biochemical pathways that involve other proteases. The use of microarrays to determine the activity of many types of proteases simultaneously would allow for a more holistic overview and quantitative assessment of protein turnover in soil (Sieber et al., 2004; Uttamchandani et al., 2005).

7.4.2. Laboratory-optimised versus field-relevant protease assay conditions

Numerous reviews have recommended that soil enzyme activity is best measured under field-relevant conditions (Burns et al., 2013; German et al., 2011; Nannipieri et al., 2017). Since the advice of German et al. (2011) was published, 30% of assays were neither conducted within 1 unit of pH of field conditions nor optimised for pH. However, Nannipieri et al. (2017) pointed out problems that occur when mimicking soil pH: 1) pH is heterogenous at the microscale (e.g. mineral surfaces) and

macroscale (e.g. rhizosphere vs. bulk soil), and 2) pH is commonly measured in a soil/water suspension which can range markedly depending on season, land use and water source. Therefore, they suggest it would be best practise to measure soil protease activity at both field pH and optimised pH conditions. Unfortunately, to date, no published studies collected in our metadata have heeded this advice. Several studies have observed microbial adaption to the edaphic environment, suggesting that optimised conditions may vary environmentally too (Puissant et al., 2019). Therefore, it is important to measure field soil protease activity under the environmental conditions when sampled in order to minimise variations that occur temporally. Best practice would be to determine V_{max} along a pH range in order to integrate enzyme activities into a model to evaluate the effect of environmental change of soil pH on soil protease activity. However, this is time consuming and thus the minimum practice is to 1) always report the pH of the assay and soil to allow for correction of the effect of using a different pH to the field condition and 2) report the objective of the protease assay stating whether it aims to mimic field conditions or optimum enzyme conditions.

Temperature of the assay follows a similar trend to the assay pH with majority of studies not using field-relevant or laboratory-optimised conditions. We see a similar trend to pH that assay temperatures fall in the realm between field and optimised. We used MAT as a proxy for soil temperature due to the lack of recording of field soil temperatures. However, MAT does not always represent the seasonal fluctuations in temperature which can affect protease activity (Koch et al., 2007; Puissant et al., 2015). Thus, when measuring soil protease activity under field conditions we suggest using soil temperature recorded as close to the assay as possible. Most of studies try to estimate protease activity to understand and quantify soil processes, therefore; measuring activity at the temperature as close as the field is essential. This is far from the case based on our observed results. Most assays are measured at higher temperatures than the field in order to increase the rate of reaction to be closer to optimum conditions. However, this leads to an inflated measurement far from what is happening *in situ*. This can lead to false or misleading conclusions being drawn, especially if the activity of a isoenzymes adapted to cold versus hot temperature are compared at the same lab temperature assay (Wallenstein et al., 2009). Crude estimations of field-temperature protease activity, calculated from mean soil protease activity using MAT reported in the studies and assuming a Q_{10} of 2, suggest that protease activity measured at field temperature would be more than 5 times lower

(Appendix 5, Supplementary Information 1). Some studies have determined the optimum temperature of the protease they are measuring at around 40-60˚C (Ladd and Butler, 1972; Nannipieri et al., 1982). Ideally, the temperature response of protease activity should be measured along a temperature gradient to determine the temperature sensitivity of the enzyme which is driven by many environmental variables e.g. interaction with the soil structure and ecological niche of the microbial pool that produce isoenzymes (Wallenstein et al., 2010). Again, this is time consuming and so, when measuring protease activity in relation to soil processes, activity should be measured at temperatures which best reflect field conditions at the time of sampling.

In the two years following the last major review into soil enzyme activity methods by Nannipieri et al. (2017), one-third of studies did not report one or more of: assay temperature, assay pH and soil pH and only a handful reporting soil temperature. This is despite Nannipieri et al.'s (2017) reiteration of the work of Dick (2011) stating that these factors are key for establishing accurate and standardised methods in soil enzymology. Therefore, we once again reiterate points made in previous reviews of enzyme activity methods; studies must be transparent in the reporting key factors that will influence the accuracy and interpretation of soil enzyme activity. From the studies obtained in this meta-analysis, it is possible that as the number of studies measuring protease activity and soil enzymes has increased each year, soil protease activity is more often being used as a soil quality indicator and a basic soil property amongst a range of key enzyme activities involved in soil C-N-P cycling (e.g. phosphatases and β-glucosidase; Boafo et al., 2020). Therefore, as the focus of these studies was not of soil protease activity, it might be that time has not been taken to research and develop the most appropriate protocol. It is fundamental to report soil and assay parameters of temperature and pH that are known to influence protease activity. Furthermore, research on how pH and temperature regulate soil protease activity by measuring response curves will aid the determination of potential field-relevant protease activity and estimates of ecological flux and feed models. This will reduce the variation in soil protease activity measurements due to methodological bias and help us better understand ecological drivers.

The choice between field-relevant or laboratory-optimised conditions depends on the aim of the study. For example, a focus on proteases and their properties would warrant an optimised approach whilst studies measuring proteases from an ecological perspective would be best opting for field conditions (Burns et al., 2013; Nannipieri et

al., 2017). Although, the adoption of field conditions represents the soil pH and temperature more accurately, it is still not a measure of *in situ* protease activity due to the use of substrates at high concentrations that saturate the system. Determining V_{max} and K_m kinetic parameters allows the user to see how enzymes react to low and high substrate concentrations (Miller et al., 2001). The main point here is to explicitly state which approach was used in a study. However, regardless of the approaches used justification must be made, which was rare for the studies reviewed here.

7.4.3. Natural variation of protease activity at a global scale

The variation of protease activity was not accounted for by environmental or methodological factors analysed in this study. Our study focused on pH and temperature as key environmental variables; however, other studies have found relationships between soil organic carbon, clay content and soil moisture with enzyme activities (e.g. Bonmati et al., 1991; Geisseler et al., 2010). Futhermore, several studies have reported seasonal effects on enzyme activities due to temperature and precipitation changes (Brzostek and Finzi, 2012; Wallenstein et al., 2009). Based on a review by Kallenbach and Grandy (2011), microbial biomass-C ranges between 43-2125 mg C kg^{-1} with an average of 365 mg C kg^{-1} in agricultural soils. As microorganisms exude proteases into soil, it would be expected that the number of proteases would limit activity to a smaller range than observed in this meta-analysis of 4 million nmol product g^{-1} h^{-1} even after the exclusion of invalid results (Noll et al., 2019). This leads to the conclusion that many values reported in the studies we collected are unreliable.

As our environmental and methodological factors have shown no relationship with soil protease activity it suggests there is a study bias. This bias is likely to be due to both intra-study and interstudy variation. Interstudy standard deviation is eight times more than intra-study variation suggesting that the interstudy variation is causing the most variation on soil protease activity. The lack of standardisation and the assortment of conditions that lie neither with field nor optimised conditions could be the reason for the interstudy variation observed (Burns et al., 2013; Nannipieri et al., 2017). Therefore, we stress the importance of developing an international standardised method, explicitly stating soil and assay properties and carefully choosing field or optimised conditions.

7.5. Conclusions

Many studies assessing enzyme activity protocols have concluded that standardised methods must be used (Burns et al., 2013; Deng et al., 2013; Dick et al., 2018; Nannipieri et al., 2017). However, we present evidence that a wide range of modified (non-standardised) methods continue to be used in most studies. When compared to average microbial biomass-C in soil, it is clear that a significant number of studies presented results that are invalid. Thus, we hope our quantitative evidence, showing the range of methods used to date and the variation and error this has caused in measurements, encourages the soil enzyme research community to adopt the standardised practice we recommend. Also, we stress the importance of fully disclosing the assay protocol conducted with all the conditions stated in the methods. When measuring soil protease activity, we recommend the following:

1. Transparent reporting of assay conditions and soil characteristics particularly pH and temperature.
2. Conduct the assay at either field or optimised pH and temperature based on the aims of the study.
3. Check that measurements lie between 0-1000 nmol product g^{-1} h^{-1}.

Future methodological developments should focus on creating microarrays that can assay multiple types of proteases simultaneously under the same pH and temperature. This will allow standardisation of protease activity measurements. Furthermore, microarrays reduce the need to use different substrates that use different protocols and conditions that make comparing protease activity unreliable.

7.6. Acknowledgements

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7.7. References

Bell, C.W., Fricks, B.E., Rocca, J.D., Steinweg, J.M., McMahon, S.K., Wallenstein,

M.D., 2013. High-throughput fluorometric measurement of potential soil extracellular enzyme activities. Journal of Visualized Experiments.

Bivand, R.., Pebesma, E., Gomez-Rubio, V., 2013. Applied spatial data analysis with R, Second edition. Springer, NY.

Boafo, D.K., Kraisornpornson, B., Panphon, S., Owusu, B.E., Amaniampong, P.N., 2020. Effect of organic soil amendments on soil quality in oil palm production. Applied Soil Ecology 147, 103358.

Bonmati, M., Ceccanti, B., Nanniperi, P., 1991. Spatial variability of phosphatase, urease, protease, organic carbon and total nitrogen in soil. Soil Biology & Biochemistry 23, 391–396.

Brzostek, E.R., Finzi, A.C., 2012. Seasonal variation in the temperature sensitivity of proteolytic enzyme activity in temperate forest soils. Journal of Geophysical Research: Biogeosciences 117, 1–10.

Burns, R.G., DeForest, J.L., Marxsen, J., Sinsabaugh, R.L., Stromberger, M.E., Wallenstein, M.D., Weintraub, M.N., Zoppini, A., 2013. Soil enzymes in a changing environment: Current knowledge and future directions. Soil Biology & Biochemistry 58, 216–234.

Deng, S., Popova, I.E., Dick, L., Dick, R., 2013. Bench scale and microplate format assay of soil enzyme activities using spectroscopic and fluorometric approaches. Applied Soil Ecology 64, 84–90.

Dewan, R.K., Chudgar, A., Mead, R., Bloomfield, V.A., Morr, C. V., 1974. Molecular weight and size distribution of bovine milk casein micelles. BBA - Protein Structure 342, 313–321.

Dick, R.P., Dick, L.K., Deng, S., Li, X., Kandeler, E., Poll, C., Freeman, C., Jones, T.G., Weintraub, M.N., Esseili, K.A., Saxena, J., 2018. Cross-laboratory comparison of fluorimetric microplate and colorimetric bench-scale soil enzyme assays. Soil Biology & Biochemistry 121, 240–248.

Dick, W.A., 2011. Development of a soil enzyme reaction assay, in: Dick, R.P. (Ed.), Methods of Soil Enzymology. Soil Science Society of America, Madison, WI, USA, pp. 71–84.

Fick, S.E., Hijmans, R.J., 2017. WorldClim 2: new 1km spatial resolution climate surfaces for global land areas. International Journal of Climatology 37, 4302–4315.

Fornasier, F., Dudal, Y., Quiquampoix, H., 2011. Enzyme Extraction from Soil, in: Dick,

R.P. (Ed.), Methods of Soil Enzymology. Soil Science Society of America, Madison, WI, U.S.A, pp. 371–383.

Geisseler, D., Horwath, W.R., Scow, K.M., 2010. Soil moisture and plant residue addition interact in their effect on extracellular enzyme activity. Pedobiologia 54, 71–78.

German, D.P., Weintraub, M.N., Grandy, A.S., Lauber, C.L., Rinkes, Z.L., Allison, S.D., 2011. Optimization of hydrolytic and oxidative enzyme methods for ecosystem studies. Soil Biology & Biochemistry 43, 1387–1397.

Greenfield, L.M., Hill, P.W., Paterson, E., Baggs, E.M., Jones, D.L., 2020. Do plants use root-derived proteases to promote the uptake of soil organic nitrogen? Plant and Soil 459, 355-367.

Hijmans, R.J., 2020. raster: Geographic Data Analysis and Modeling. R package version 3.1-5.

Jan, M.T., Roberts, P., Tonheim, S.K., Jones, D.L., 2009. Protein breakdown represents a major bottleneck in nitrogen cycling in grassland soils. Soil Biology & Biochemistry 41, 2272–2282.

Kallenbach, C., Grandy, A.S., 2011. Controls over soil microbial biomass responses to carbon amendments in agricultural systems: A meta-analysis. Agriculture, Ecosystems and Environment 144, 241–252.

Koch, O., Tscherko, D., Kandeler, E., 2007. Temperature sensitivity of microbial respiration, nitrogen mineralization, and potential soil enzyme activities in organic alpine soils. Global Biogeochemical Cycles 21, 4017.

Ladd, J.N., 1972. Properties of proteolytic enzymes extracted from soil. Soil Biology & Biochemistry 4, 227–237.

Ladd, J.N., Butler, J.H.A., 1972. Short-term assays of soil proteolytic enzyme activities using proteins and dipeptide derivatives as substrates. Soil Biology & Biochemistry 4, 19–30.

Landi, L., Renella, G., Giagnoni, L., Nannipieri, P., 2011. Activities of Proteolytic Enzymes, in: Dick, R.P. (Ed.), Methods of Soil Enzymology. Soil Science Society of America, Madison, USA, pp. 247–260.

Lowry, O.H., Rosebrough, N.J.., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. The Journal of Biological Chemistry 193, 265–275.

Miller, M., Nielsen, T.H., Bonde, T., Sorensen, J., 2001. Fluctuations of formamide hydrolase activity in a barley field soil after drying and rewetting. Applied Soil

Ecology 16, 159–167.

Moore, S., Stein, W.H., 1954. A modified ninhydrin reagent for the photometric determination of amino acids and related compounds. Journal of Biological Chemistry 211, 907–913.

Nannipieri, P., Ceccanti, B., Conti, C., Bianchi, D., 1982. Hydrolases extracted from soil: Their properties and activities. Soil Biology & Biochemistry 14, 257–263.

Nannipieri, P., Trasar-Cepeda, C., Dick, R.P., 2017. Soil enzyme activity: a brief history and biochemistry as a basis for appropriate interpretations and meta-analysis. Biology & Fertility of Soils 54, 1–9.

Niemi, R.M., Vepsäläinen, M., 2005. Stability of the fluorogenic enzyme substrates and pH optima of enzyme activities in different Finnish soils. Journal of Microbiological Methods 60, 195–205.

Noll, L., Zhang, S., Zheng, Q., Hu, Y., Wanek, W., 2019. Wide-spread limitation of soil organic nitrogen transformations by substrate availability and not by extracellular enzyme content. Soil Biology & Biochemistry 133, 37–49.

Pancholy, S.K., Lynd, J.Q., 1972. Quantitative fluorescence analysis of soil lipase activity. Soil Biology & Biochemistry 4, 257–259.

Pebesma, E., Bivand, R.S., 2005. Classes and methods for spatial data in R. R News 5 (2).

Peterson, G.L., 1979. Review of the folin phenol protein quantitation method of lowry, rosebrough, farr and randall. Analytical Biochemistry 100, 201–220.

Puissant, J., Cécillon, L., Mills, R.T.E., Robroek, B.J.M., Gavazov, K., De Danieli, S., Spiegelberger, T., Buttler, A., Brun, J.J., 2015. Seasonal influence of climate manipulation on microbial community structure and function in mountain soils. Soil Biology & Biochemistry 80, 296–305.

Puissant, J., Jones, B., Goodall, T., Mang, D., Blaud, A., Gweon, H.S., Malik, A., Jones, D.L., Clark, I.M., Hirsch, P.R., Griffiths, R., 2019. The pH optimum of soil exoenzymes adapt to long term changes in soil pH. Soil Biology & Biochemistry 138, 1076012.

R Core Team, 2018. R: A language and environment for statistical computing.

Reiskind, J.B., Lavoie, M., Mack, M.C., 2011. Kinetic studies of proteolytic enzyme activity of arctic soils under varying toluene concentrations. Soil Biology & Biochemistry 43, 70–77.

Schloter, M., Nannipieri, P., Sørensen, S.J., van Elsas, J.D., 2018. Microbial indicators

for soil quality. Biology and Fertility of Soils 54, 1–10.

Sieber, S.A., Mondala, T.S., Head, S.R., Cravatt, B.F., 2004. Microarray Platform for Profiling Enzyme Activities in Complex Proteomes. Journal of the American Chemical Society 126, 15640–15641.

Sinsabaugh, R.L., Lauber, C.L., Weintraub, M.N., Ahmed, B., Allison, S.D., Crenshaw, C., Contosta, A.R., Cusack, D., Frey, S., Gallo, M.E., Gartner, T.B., Hobbie, S.E., Holland, K., Keeler, B.L., Powers, J.S., Stursova, M., Takacs-Vesbach, C., Waldrop, M.P., Wallenstein, M.D., Zak, D.R., Zeglin, L.H., 2008. Stoichiometry of soil enzyme activity at global scale. Ecology Letters 11, 1252–1264.

Slessarev, E.W., Lin, Y., Bingham, N.L., Johnson, J.E., Dai, Y., Schimel, J.P., Chadwick, O.A., 2016. Water balance creates a threshold in soil pH at the global scale. Nature 540, 567–569.

Tabatabai, M.A., 1994. Soil Enzymes, in: Weaver, R.W., Bottomley, S.A.P., Bezdicek, D., Smith, S., Tabatabai, M.A., Wollum, A. (Eds.), Methods of Soil Analysis. Part 2, Microbiological and Biochemical Properties. Soil Science Society of America, Madison, WI, USA, pp. 775–833.

Trasar-Cepeda, C., Leirós, M.C., Gil-Sotres, F., 2008. Hydrolytic enzyme activities in agricultural and forest soils. Some implications for their use as indicators of soil quality. Soil Biology & Biochemistry 40, 2146–2155.

Uttamchandani, M., Huang, X., Chen, G.Y.J., Yao, S.Q., 2005. Nanodroplet profiling of enzymatic activities in a microarray. Bioorganic and Medicinal Chemistry Letters 15, 2135–2139.

Vazquez, S., Ruberto, L., Mac Cormack, W., 2005. Properties of extracellular proteases from three psychrotolerant Stenotrophomonas maltophilia isolated from Antarctic soil. Polar Biology 28, 319–325.

Vranová, V., Rejsek, K., Formanek, P., 2013. Proteolytic activity in soil: A review. Applied Soil Ecology 70, 23–32.

Wallenstein, M., Allison, S.D., Ernakovich, J., Steinweg, J.M., Sinsabaugh, R., 2010. Controls on the Temperature Sensitivity of Soil Enzymes: A Key Driver of In Situ Enzyme Activity Rates, in: G., S., A., V. (Eds.), Soil Enzymology. Springer, Berlin, Heidelberg, pp. 245–258.

Wallenstein, M.D., McMahon, S.K., Schimel, J.P., 2009. Seasonal variation in enzyme activities and temperature sensitivities in Arctic tundra soils. Global Change Biology 15, 1631–1639.

Watanabe, K., Hayano, K., 1995. Seasonal variation of soil protease activities and their relation to proteolytic bacteria and Bacillus spp in paddy field soil. Soil Biology & Biochemistry 27, 197–203.

Weintraub, M.N., Schimel, J.P., 2005. Seasonal protein dynamics in Alaskan arctic tundra soils. Soil Biology & Biochemistry 37, 1469–1475.

Wickham, H., 2016. ggplot2: Elegant Graphics for Data Analysis.

Chapter 8

Discussion & Conclusion

Soil protease activity: Summary & the next steps

8.1. Introduction

A detailed discussion of the results from each experiment are described in Chapters 3-7. In this chapter, I synthesize the main findings of these experiments and discuss them in relation to the thesis aims, commons themes and wider implications. Finally, I will suggest future research as a result of new questions that have evolved from the findings of this thesis.

8.2. Synthesis of findings

This thesis aimed to assess a) the methods used to quantify protein mineralisation and soil protease activity, and b) the soil, plant and microbial factors that affect soil protease activity.

In Chapter 3, my findings supported my hypothesis that the choice of method and soil type affect the amount of protein that could be recovered from soil. Evidence from this Chapter found 0.1 M NaOH to be the most effective extractant, however, NaOH denatures proteins and thus is not recommended for uses where intact proteins are needed for subsequent analyses. To analyse intact proteins, 0.05 M Na-pyrophosphate is recommended, but it co-extracts humic substances. If coextraction is problematic for subsequent analysis, deionised water is recommended. Despite the experiment being limited to three soil types, the contrasting properties of the soils (i.e. clay, organic matter and pH) show that the extraction efficiency of protein depends on the soil type. The implications are that quantitative comparisons between soils are inaccurate and thus, studies should determine the recovery of a known mixture of proteins (i.e. spiked controls) that are of interest to the study, for each soil type analysed. Deionised water proved the best extractant in that it does not have the negative effects of denaturing proteins or co-extraction of interfering substances.

Chapter 4 determined how key edaphic factors affect protein mineralisation along an altitudinal gradient that varied with soil type and land use and, thus, the limiting factors on the soil N cycle. My hypothesis that key regulators (NH_4^+, NO_3^-, protein, amino acid, microbial biomass-C, pH, CEC, N mineralisation, sorption and primary productivity) will predict protein mineralisation rates as these drive or limit degradation processes was partially correct. Our results showed that protease activity was not regulated by a small number of factors but a wide range of interacting factors including soil pH, NO_3^-, CEC, C:N ratio and above-ground NPP). The hypothesis: the rate of protein mineralisation will decrease along the grassland altitudinal gradient

(from low to high altitude) as primary productivity, pH and C and N availability reduce microbial activity was not correct with no trend in protein mineralisation rates with altitude. My hypothesis that protein mineralisation is negatively correlated with depth as protein inputs and microbial biomass C decreases in the subsoil relative to the topsoil was correct. Inputs of C (e.g. from plant roots) into the subsoil are lower and, therefore, microbial biomass-C is less abundant (Loeppmann et al., 2016). Microorganisms utilise the C and N from protein in the soil and, so, a smaller biomass results in lower turnover rates. In contrast to other studies (e.g. Jan et al., 2009), we found that protein mineralisation rate was similar to that of amino acids. This was likely due to the soluble nature of our protein, the realistic concentrations used, a high degree of functional redundancy in the microbial community and microbial enzyme adaptation to their ecological niche. The influence of these factors ranged with altitude and soil depth. In answer to the Chapter's title: 'Is soluble protein mineralisation and protease activity in soil regulated by supply or demand?' I concluded that the soil N cycling is limited by supply of protein rather than demand shown by protease activity and protein turnover.

In Chapter 5, I explored whether plants use root-derived proteases to promote the uptake of soil organic N. My hypothesis that plants will both secrete proteases from their roots but also retain surface-bound protease activity to maximise protein-N capture from soils was partially correct. I found little evidence for the secretion of free proteases, rather that protease activity was associated with the root surface. Root protease activity did not appear to be for the purpose of enhancing plant N nutrition due to the lack of up-regulation under N deficiency. My second hypothesis that protease activity from rhizosphere soil will be proportionally higher than for roots as it is more energetically favourable for the soil microbial community to use the products of protein hydrolysis rather than inorganic N was correct. Despite plant root-derived proteases contributing one-fifth of total rhizosphere protease activity (i.e. both microbial- and plant-derived proteases), it is likely that plant root protease activity plays a minor role in plant N nutrition and so does not regulate N mineralisation in soil.

In Chapter 6, I hypothesised correctly that protein addition and root hairs would increase leucine aminopeptidase activity in the rhizosphere due to an increase in substrate. My findings from Chapter 6, determined the effect of root hairs and soluble protein addition to increase leucine aminopeptidase activity. This creates an advantage for plants with root hairs to access protein hotspots in soil. The advantage

is likely indirect via microbial-derived proteases due to the findings of Chapter 5 that suggest root-derived proteases are not regulated for N acquisition. In addition, the results showed clear evidence of the disparity between *in situ* and *ex situ* rhizosphere enzyme analysis. A review on rhizosphere sampling techniques by Oburger and Jones (2018) concluded that current techniques have many problems affecting the reliability of results including damage to roots and fungal hyphae by destructive sampling. Potential artefacts of *ex situ* measurements were also highlighted in Chapter 5. It is likely that my *ex situ* measurements of rhizosphere soil were sampled outside of the active zone of protease activity in the rhizosphere measured by *in situ* zymography (i.e. most protease activity is occurring very close to the rhizoplane). Therefore, caution is needed when interpreting rhizosphere enzyme activity from *ex situ* destructive sampling.

Lastly, in Chapter 7 I carried out a critical review and meta-analysis of the common assay methods for measuring soil protease activity. I found soil protease activity to have a large variation at the global scale which far exceeds the realistic range that is expected. My hypothesis that pH and temperature would explain the variation of protease activity at a global scale was not correct. This variation is likely due to the non-standardisation of the methodology (e.g. pH, temperature, fluorometric vs. colorimetric assays) and inaccurate estimation of protease activity (e.g. incorrect calculations). The findings of the meta-analysis showed a clear lack of comprehensive reporting of assay conditions, particularly for pH and temperature, making it difficult to ascertain whether individuals have conducted their experiments under laboratory-optimised or field-relevant conditions. Critically, the choice between the two conditions greatly affects the interpretation of the results and whether they can answer the aim of the study. For example, focus on proteases and their properties would warrant an optimised approach, whilst studies looking at the ecological role of proteases (e.g. N mineralisation) would opt for field-relevant conditions (Burns et al., 2013; Nannipieri et al., 2017).

8.3. Wider Implications

The experimental chapters in this thesis have addressed many questions to further our knowledge of soil protease activity. Several studies have concluded that soil protease activity is the bottleneck of the terrestrial N cycle (Geisseler et al., 2009; Jan et al., 2009). However, the findings from Chapter 5 suggest that this is not the

case. Our results indicate that protein availability is the bottleneck in the production of NH_4^+ rather than the depolymerisation of protein. In addition, factors controlling protease activity and protein mineralisation interact depending on location, substrate type and soil depth. This is important for our future understanding and modelling of soil C and N cycling. A simplistic, 'one size fits all' approach will not accurately predict protein mineralisation and protease activity in a soil C and N cycle model. A recent study by Noll et al. (2019) that determined protein turnover rates in two soil types and three land uses under a range of temperature and moisture treatments also determined that substrate availability is the limiting factor. This gives further evidence that the consensus of soil protease activity acting as a bottleneck of the terrestrial N cycle cannot be applied to every soil type and land use.

Previously plant root release of proteases has been considered a mechanism that enhances availability of N for nutrition (Adamczyk et al., 2010; Paungfoo-Lonhienne et al., 2008). However, our results indicate that this is of limited functional significance due to the lack of up-regulation in protease activity under N deficiency. Therefore, plants themselves are not likely to use root-derived proteases to increase organic N plant acquisition but they could still be important in soil N mineralisation without acting as a primary regulator. This is important to consider when working to improve the efficiency of sustainable agriculture systems as crops in agricultural systems tend to be N limited if fertiliser additions are reduced (Rütting et al., 2018). It is also possible that other non-crop species may release proteases. If plants do not use root-derived proteases to increase supply of N from organic N compounds, this suggests that microbial production of extracellular proteases is the key regulator of protein break-down into inorganic N that plants subsequently utilise. Thus, the use of organic fertilisers would need to be optimised for soil microorganisms to utilise and make N available for plants rather than the direct utilisation by plants. The results from Chapter 6 showed soluble protein addition to enhance protease activity in the rhizosphere indicating the potential of organic fertilisers containing soluble protein to increase available N via microbial-derived proteases.

8.4. Future research

Research presented in this thesis has provided vital knowledge about protein mineralisation and protease activity in soil. However, while filling key knowledge gaps, several further questions have been raised, some of which are detailed below.

The work in this thesis has been carried out using soluble proteins (tobacco leaf protein in Chapters 3, 4 and 5 and BSA in Chapter 6). Assessments of the effect of protein hydrophobicity is needed in order to determine how these proteins interact with soil. For example, hydrophobins are a small group of proteins produced by filamentous fungi that can create a hydrophobic surface coating (Rillig et al., 2007). It is probable that they contribute to protein stabilisation in the soil due to their hydrophobic nature and ability to make both hydrophobic and hydrophilic soil surfaces hydrophobic, however, this can depend on the soil moisture content. In order to extract these proteins ethanol is needed, which recovered the least amount of protein in our study on hydrophilic protein extraction (Rillig et al., 2007 and references therein). The proportion of hydrophilic and hydrophobic proteins in soil is likely to impact N mineralisation by regulating the breakdown of protein and consequently determine C and N availability. Therefore, when measuring soil protein, it is important to develop accurate methods to quantify and characterise both these fractions. However, it should be noted that most proteins held in soil organic matter are likely to be partially degraded and therefore of limited value for soil proteomic studies. More work is needed to determine the active proteins in soil (i.e. those in the active microbial biomass) from those in the dormant community or necromass (dead biomass). This will require a combination of stable isotopic labelling (e.g. ^{15}N, ^{13}C, ^{18}O to label the active microbial pool) and proteomics.

My findings from Chapter 5 suggest that root-derived plant protease activity was associated with the root surface. However, our study did not determine the mechanism/s responsible for uptake of organic N from protein. It is likely the use of protein-derived N by plant roots is by one or a combination of three mechanisms: 1) proteases are cell wall bound allowing them to cleave proteins that come into direct contact with the root surface where smaller products can be taken up; 2) proteins diffuse through pores in the cell wall and enter the apoplast where proteases inside the cell break them into soluble products; and 3) smaller proteins are taken up by the root cell via endocytosis. In order to determine the viability and relative importance of these mechanisms further research is needed. Tracer studies similar to Paungfoo-Lonhienne et al. (2008), which used a fluorescent protein to identify the uptake and location of the protein with the plant root, could be used to experimentally test this. Gaining knowledge of the relative importance of the mechanisms would lead to a better understanding of the function of root-derived proteases. In addition, it would be

good to test the approaches used here in a wider range of plant species which are naturally adapted to growing under N-limited conditions (e.g. upland grasses). Our studies also did not include potential microbial symbionts. Further work is therefore needed to establish the potential of endo/ectomycorrhizas and dark septate endophytic (DSE) fungi in protease release. The role of these symbionts in soil protein mobilisation is well established in some ecosystems (e.g. forests, polar grasses) but their potential contribution to N nutrition in agriculture is unknown (Nath and Meena, 2018).

Furthermore, Chapter 5 results showed differences in leucine aminopeptidase activity between species, although this was not statistically tested due to differences in plant physiology. However, our study analysed only two agricultural plant species (*Triticum aestivum* and *Zea mays*) and one soil type (Cambisol). Combined with evidence from Godlewski and Adamczyk (2007) which measured root-associated protease activity of different plant species can range by factor of 14, it suggests that the contribution of plant root-derived proteases to total soil protease activity could differ between species as well as habitats. It is worth noting that Godlewski and Adamczyk (2007) measured proteases that had been exuded from the root into a solution whilst our study showed no evidence for this. It is not clear how measurements differed from the control in Godlewski and Adamczyk (2007) and, thus, could be a false positive. In addition, the findings of Chapter 4, the influence of edaphic factors on protease activity changes with altitude and depth, suggests that environment could also have indirect effect on the microbial contribution to soil protease activity due to variations in microbial community structure. Studies have shown that microbial communities are adapted to their environment in terms of temperature and soil pH (Koch et al., 2007; Puissant et al., 2019, 2015). Therefore, future work should investigate how species and soil type affect the relative contributions of plant root- and microbial-derived proteases on soil protease activity. This is possible with continued advances in soil proteomics - an analysis which offers the potential to identify enzyme function and the source organisms (Renella et al., 2014).

The ability to analyse the spatial distribution of protease activity of plant roots improves our understanding of enzyme dynamics in plant-soil-microorganism interactions. Following on from the aseptic work of Chapter 5, determining the role of plant root-derived protease activity, and also from Chapter 6, assessing the spatial distribution of protease activity in the rhizosphere, my next step would be to use *in situ*

zymography under aseptic conditions to determine the spatial distribution of plant root-derived proteases in isolation. So far there have been no studies measuring extracellular protease activity of plant roots, yet my work from chapter 5 and also by Godlewski and Adamczyk (2007) and Paungfoo-Lonhienne et al. (2008) have shown plants produce root-derived proteases. In addition, it has been shown that root morphology and spatial location within the root system (e.g. root tip) can affect enzyme activity at and near to the root surface (Ma et al., 2018; Pausch et al., 2016). Also, protein addition may affect root morphology e.g. leading to the development of root branching and increases in root surface area (Rasmussen et al., 2014). Therefore, determining the locations of protease activity along the root as a result of different root morphological traits and protein addition would provide important knowledge on the spatial dynamics of the plant fraction of soil protease activity.

It is apparent from our review in Chapter 7 that further advancements are needed in methods to measure soil protease activity. Currently, the five main substrates used to measure soil protease activity involve different protocols, specifically type of protease targeted and assay conditions, as well as lack standardisation even for assays of a single protease type. Therefore, there is an urgent need to develop a standardised microarray to analyse multiple proteases at once. Microarrays could simultaneously assay multiple substrates with fluorophores under the same conditions to gain more comprehensive information of the activity of multiple protease types. Microarrays have already been developed for use in medicine and biological applications (e.g. Sieber et al., 2004; Uttamchandani et al., 2005) which can set a basis for development of soil-specific microarrays.

To summarise the future research priorities, I have complied the above paragraphs into six bullet points:

1. Determine the proportions of hydrophobic and hydrophilic proteins in different soil types.
2. Determine the different functions and locations of hydrophobic and hydrophilic proteins in the plant-soil system.
3. Determine mechanism/s involved in protein uptake by plant root proteases.
4. Determine the contribution of plant root-derived and microbial-derived proteases to total soil protease activity in different plant species, management regimes, habitats and soil types.

5. Apply *in situ* zymography to measuring protease activity of sterile plant roots.
6. Develop a soil-specific microarray for measuring soil protease activity.

8.5. Concluding remarks

In this thesis I addressed fundamental questions related to protein mineralisation and protease activity in soil, both methodologically and mechanistically. I have found that intrinsic protein mineralisation is limited by supply rather than demand and that it is unlikely that plant-root derived proteases are produced for the purpose of N nutrition. In addition, I determined that a combination of root hairs and protein addition produced the highest leucine aminopeptidase activity creating an advantage for plants with root hairs to access protein hotspots in soil via microbial activity. This research provides a more detailed understanding of protein mineralisation and protease activity in soil.

Throughout the development of this thesis I encountered major limitations with the methods used to determine soil proteins and protease activity. I found protein recovery to be dependent on both extractant and soil type, preventing direct comparison of studies using different recovery methods. Also, I found soil protease activity to vary widely due to study-biases and a lack of reporting of key assay conditions. The need for comprehensive reporting of enzyme assay conditions is paramount in future research into soil protease activity dynamics in the soil-plant-microorganism system.

8.6. References

Adamczyk, B., Smolander, A., Kitunen, V., Godlewski, M., 2010. Proteins as nitrogen source for plants: a short story about exudation of proteases by plant roots. Plant Signaling & Behavior 5, 817–819.

Burns, R.G., DeForest, J.L., Marxsen, J., Sinsabaugh, R.L., Stromberger, M.E., Wallenstein, M.D., Weintraub, M.N., Zoppini, A., 2013. Soil enzymes in a changing environment: Current knowledge and future directions. Soil Biology & Biochemistry 58, 216–234.

Geisseler, D., Horwath, W.R., Doane, T.A., 2009. Significance of organic nitrogen uptake from plant residues by soil microorganisms as affected by carbon and nitrogen availability. Soil Biology & Biochemistry 41, 1281–1288.

Godlewski, M., Adamczyk, B., 2007. The ability of plants to secrete proteases by

roots. Plant Physiology and Biochemistry 45, 657–664.

Jan, M.T., Roberts, P., Tonheim, S.K., Jones, D.L., 2009. Protein breakdown represents a major bottleneck in nitrogen cycling in grassland soils. Soil Biology & Biochemistry 41, 2272–2282.

Koch, O., Tscherko, D., Kandeler, E., 2007. Temperature sensitivity of microbial respiration, nitrogen mineralization, and potential soil enzyme activities in organic alpine soils. Global Biogeochemical Cycles 21, 4017.

Ma, X., Liu, Y., Zarebanadkouki, M., Razavi, B.S., Blagodatskaya, E., Kuzyakov, Y., 2018. Spatiotemporal patterns of enzyme activities in the rhizosphere: effects of plant growth and root morphology. Biology and Fertility of Soils 54, 819–828.

Nannipieri, P., Trasar-Cepeda, C., Dick, R.P., 2017. Soil enzyme activity: a brief history and biochemistry as a basis for appropriate interpretations and meta-analysis. Biology & Fertility of Soils 54, 1–9.

Nath, D., Meena, V.S., 2018. Mycorrhizae: A potential microorganism and its implication in agriculture, in: Role of Rhizospheric Microbes in Soil: Stress Management and Agricultural Sustainability. Springer Singapore, pp. 251–276.

Noll, L., Zhang, S., Zheng, Q., Hu, Y., Wanek, W., 2019. Wide-spread limitation of soil organic nitrogen transformations by substrate availability and not by extracellular enzyme content. Soil Biology & Biochemistry 133, 37–49.

Oburger, E., Jones, D.L., 2018. Sampling root exudates – Mission impossible? Rhizosphere 6, 116–133.

Paungfoo-Lonhienne, C., Lonhienne, T.G.A., Rentsch, D., Robinson, N., Christie, M., Webb, R.I., Gamage, H.K., Carroll, B.J., Schenk, M., Schmidt, S., 2008. Plants can use protein as a nitrogen source without assistance from other organisms. PNAS 105, 4524–4529.

Pausch, J., Loeppmann, S., Kühnel, A., Forbush, K., Kuzyakov, Y., Cheng, W., 2016. Rhizosphere priming of barley with and without root hairs. Soil Biology & Biochemistry 100, 74–82.

Puissant, J., Cécillon, L., Mills, R.T.E., Robroek, B.J.M., Gavazov, K., De Danieli, S., Spiegelberger, T., Buttler, A., Brun, J.J., 2015. Seasonal influence of climate manipulation on microbial community structure and function in mountain soils. Soil Biology & Biochemistry 80, 296–305.

Puissant, J., Jones, B., Goodall, T., Mang, D., Blaud, A., Gweon, H.S., Malik, A., Jones, D.L., Clark, I.M., Hirsch, P.R., Griffiths, R., 2019. The pH optimum of soil

exoenzymes adapt to long term changes in soil pH. Soil Biology & Biochemistry 138, 1076012.

Rasmussen, Jay, Gilroyed, Brandon H, Reuter, Tim, Badea, Ana, Eudes, F., Graf, R., Laroche, A., Kav, N.N. V, Mcallister, T.A., Rasmussen, J, Gilroyed, B H, Reuter, T, Badea, A, Laroche, R., Kav, A., Mcallister, N.N. V, 2014. Efficiency of protein as a nitrogen source for wheat and morphological changes in roots exposed to high protein concentrations. Canadian Journal of Plant Science 94, 603–613.

Renella, G., Ogunseitan, O., Giagnoni, L., Arenella, M., 2014. Environmental proteomics: A long march in the pedosphere. Soil Biology & Biochemistry 69, 34–37.

Rillig, M.C., Caldwell, B.A., Wösten, H.A.B.B., Sollins, P., 2007. Role of proteins in soil carbon and nitrogen storage: Controls on persistence. Biogeochemistry 85, 25–44.

Rütting, T., Aronsson, H., Delin, S., 2018. Efficient use of nitrogen in agriculture. Nutrient Cycling in Agroecosystems 110, 1–5.

Sieber, S.A., Mondala, T.S., Head, S.R., Cravatt, B.F., 2004. Microarray Platform for Profiling Enzyme Activities in Complex Proteomes. Journal of the American Chemical Society 126, 15640–15641.

Uttamchandani, M., Huang, X., Chen, G.Y.J., Yao, S.Q., 2005. Nanodroplet profiling of enzymatic activities in a microarray. Bioorganic and Medicinal Chemistry Letters 15, 2135–2139

Appendix 1
Supplementary material for Chapter 3

1.1. Methods for protein recovery from soil

Table S1 below summarises the wide range of methods used to extract proteins from soil. We hypothesised that the choice of method may greatly affect the amount of protein that could be recovered from soil; however, no one has previously compared the efficiency of these different methods in a systematic way. We chose three contrasting soils to test these methods. We acknowledge that there are many other soil types that could also have been used in our experimentation (e.g. andosols, arenosols, vertisols, calcisols), however, we felt out choice was sufficient to test the general principles associated with protein extraction efficiency.

Table S1. Common extractants and methods used for protein recovery from soil. Key results are given for studies that measured extraction efficiency or the effect of soil type on protein recovery.

Study	Extractants used	Method	Soil type (classification)	Key results
Friedel et al., 2002	0.5 M K_2SO_4	- Extracted in 1:2.5 w/v ratio, shaken and centrifuged. - Chloroform-fumigation of extracts - Proteins acid hydrolysed using 10 ml 6 M HCl - Hydrolysed amino acids analysed by fluorescence	Haplic Luvisol Calcaric Regosol Fibric Histosol Haplic Podsol Mollic Planosol (FAO)	
Haney et al., 2001	0.001, 0.01, 0.01 and 0.5 M K_2SO_4 (pH 6.5)	- Chloroform-fumigation - Extracted with K_2SO_4, shaken for 1 h, and then centrifuged - Soil microbial biomass C (SMBC) measured by carbon analyser	Typic Quartzipsamments Typic Kanhapludults Torretic Paleustolls Plinthic Paleudults Udic Paleustalfs Oxyaquic Hapluderts Udifluventic Ustocherpts Udertic Haplustolls Typic Calciustolls (USDA)	Decreasing extractant molarity resulted in more SMBC in soils with low pH and lower SMBC in soils with high pH.
Masciandaro et al., 2008	0.1 M Na-pyrophosphate (pH 7) 67 mM phosphate buffer (pH 6) 0.5 M K_2SO_4 (pH 6.6)	- Na-pyrophosphate extracted at 1:5 w/v ratio at 37°C and shaken for 24 h. Phosphate buffer and K_2SO_4 extracted at 1:3 w/v ratio at room temperature and shaken for 1 h. Both centrifuged. - Proteins analysed by tyrosine and tryptophan determination with Folin–Ciocalteu's reagent and SDS-PAGE	Lithic Calcixeroll Inceptisol (FAO)	Overall K_2SO_4 extracted more protein. Extracted protein differed between the two soils. Similar pattern of proteins found with phosphate buffer and K_2SO_4 but pyrophsopahte differed.

Makarov et al., 2015	0.05 and 0.5 M K_2SO_4	- Extracted in 1:5 w/v ratio and shaken for 1 h then centrifuged. - Chloroform-fumigated - Organic C/N and microbial C/N measured	Luvisol Chernozem Kastanozem Umbrisol (FAO)	0.5 M K_2SO_4 extracted more organic C/N but similar quantities of microbial C/N. The highest amounts of organic C/N and microbial C/N were extracted from Umbrisol.
Bremner and Lees, 1949	Sodium salts of inorganic acids including 0.1 and 0.5 M pyrophosphate and 0. 5 M chlroide Sodium salts of organic acids including 0.2 M citrate 0.5 M sodium hydroxide	- Extracted in 1:5 w/v ratio and shaken then centrifuged. - N extracted determined by the micro-Kjeldahl method	Endoleptic Regosol Chromic Luvisol (FAO)	More N extracted by 0.5 M Na-pyrophosphate compared 0.1 M Na-pyrophosphate. Extraction of N differed with soil type and extractant.
Nannipieri et al., 1974	0.1 M Na-pyrophosphate (pH 7.1)	- Extracted in 1:10 w/v ratio, shaken then centrifuged for 30 min at 18,000 *g* - Urease activity measured	Humic podzol (FAO)	
Busto and Perez-Mateos, 1995	0.01, 0.05, 0.1, 0.2 M Na-pyrophosphate (pH 5-9)	- Extracted in 1:2 or 1:4 w/v ratio, shaken and centrifuged - β-glucosidase activity measured by colorimetric assay	Umbric Dystrochrept (USDA)	β-glucosidase activity decreased with increasing molarity of Na-pyrophosphate. Highest β-glucosidase activity was between pH 7 and 8.
Bonmati et al., 1998	140 mM Na-pyrophosphate (pH 7.1)	- Extracted in 1:10 w/v ratio and shaken for 24 h at 37°C then centrifuged for 30 min at 18,000 *g* - Protease activity assayed with Folin–Ciocalteu's reagent - Soil extracts analysed by pyrolysis-gas chromatography	Calcaric Fluvisol Dystric Cambisol	Soils extracted differing amounts of proteins especially glycoproteins.
Criquet et al., 2002	0.1 M $CaCl_2$, 0.1 M Na-pyrophosphate, 0.1 M Na-	- Extracted in 1:8.75 w/v ratio and shaken for 6 h at room	Evergreen oak litter	Highest protein concentrations extracted

	citrate, 0.1 M phosphate buffer, 0.1 M *bis*-TRIS (all at pH 6.0)	temperature then centrifuged for 20 min at 12,000 *g* - Protein concentration was measured by the Bradford method		by *bis*-TRIS and Na-pyrophosphate. Lowest protein concentrations extracted by $CaCl_2$ but had the least amount of interfering substances.
Halvorson and Gonzalez, 2006	50 mM Na-citrate, 50 mM Na-pyrophosphate, 50 mM Na-oxalate, 50 mM, Na-formate and 50 mM Na-orthophosphate (all at pH 8.0)	- Protein concentration measured by the Bradford method	Ultic Dystrudepts Typic Paleudults Typic Hapludalfs Typic Dystrudepts (USDA)	Highest protein concentrations extracted from soils with more organic matter. Na-pyrophosphate and Na-citrate extracted the most protein.
Bastida et al., 2018	Modified universal buffer (MUB), composed of tris (hydroxymethyl) aminomethane, maleic acid, citric acid, boric acid, NaOH, HCl and distilled water at pH 6.5. 0.1 M Na-pyrophosphate (pH 7.1)	- Extracted in 1:4 w/v ratio and shaken for 1 h then centrifuged for 15 min at 13,000 rpm. Protein pelleted by TCA - Proteins measured by tryptic digestion of protein pellets followed by mass spectrometric analysis	Gypsic xerosol Calcaric regosol (FAO)	Higher protein concentration when extracted by Na-pyrophosphate.
Murase et al., 2003	67 mM Na-phosphate buffer (pH 6.0)	- Extracted in 1:3 w/v ratio and shaken for 1 h then centrifuged at 12,000 rpm for 30 min - Protein analysed by SDS-PAGE	Entisol (FAO)	
Matsumoto et al., 2000	1/15 M phosphate buffer (pH 7.0)	- Extracted in 1:4 w/v ratio and shaken for 1 h then filtered - Protein measured by HPLC analyses and SDS-PAGE	Andosol Cambisol Fluvisol Gleysol Regosol (FAO)	Highest protein concentrations in the Andosol and lowest in the Regosol.
Kanerva et al., 2013	0.05 M phosphate buffer (pH 6 and 8) and 0.1 M Tris-SDS (pH 6.8)	- Extracted in 1:20 w/v, shaken for 3 h, and then centrifuged for 20 min at 10,000 *g*	Haplic Podzol Stagnic Cambisol	Protein recovery depended on extractant and soil type.

		- BSA added to determine protein recovery - Protein concentration measured by the Bradford method - MALDI-TOF-MS analysis of protein	Vertic Stagnosol (FAO)	Overall, sandy soils had highest protein recoveries.
Benndorf et al., 2007	0.1 M NaOH	- Extracted in 1:2 w/v ratio, shaken for 30 min, and then centrifuged for 10 min at 16,000 *g* - Humic substances removed by phenol extraction. - Protein analysed by SDS-PAGE	Compost soil	
Chen et al., 2009	0.05 M citrate (pH 8.0), 0.1 M NaOH, and 0.1 M Tris-SDS (pH 6.8)	- Extracted in 1:10 w/v ratio, shaken for 1 h, and centrifuged for 15 min at 15,000 *g* - Phenol phase to precipitate proteins - Protein analysed by SDS-PAGE	Soils with ranging texture, organic matter and pH	Different protein bands depending on soil type and extractant.
Keiblinger et al., 2012	0.1 M NaOH, 50 mM Tris-SDS and 50 mM Tris-SDS-phenol	- Extracted in 1:3 w/v ratio, shaken for 30 min and centrifuged for 20 min at 3220 *g* - Protein analysed by SDS-PAGE	Dystric Cambisol (FAO) Standard potting soil	The largest number of proteins was extracted by Tris-SDS-phenol. More proteins were extracted in Cambisol than potting soil.
Wright and Upadhyaya, 1998	20 mM and 50 mM citrate (pH 7.0 and 8.0)	- Extracted in 1:8 w/v ratio, shaken and centrifuged for 5 min at 10,000 *g* - Glomalin measured by enzyme-linked immunosorbent assay	Typic Fragiudults Typic Dystrochrepts Mollic Haplaudalfs Typic Endoaquolls Aridic Ustochrepts Calciorthidic Paleustalfs Typic Upidsamments (USDA)	Amount of glomalin extraction differed with soil type.

Rosier et al., 2006	20 mM Na-citrate (pH 7.0)	- Autoclaved with extractant and then centrifuged at 5000 *g* for 15 min. - Glomalin measured by enzyme-linked immunosorbent assay - BSA added measured by the Bradford method		34-85 % of BSA added recovered.
Marchetti et al., 2007	20 mM Na-citrate (pH 7.0) followed by 50 mM Na-citrate (pH 8.0)	- Extracted in 1:5 w/v ratio, shaken for 6 h, and then centrifuged for 15 min at 10,000 *g* - Cry toxins measured by enzyme-linked immunosorbent assay	Aquic Ustipsamments Sulfic Endoaquepts (USDA)	Higher extraction of Cry toxins in Sulfic Endoaquepts.
Schneider et al., 2012	50 mM Tris-SDS (pH 7.0)	- Extracted in 1:5 w/v ratio, shaken for 1 h, and then centrifuged for 5 min at 14,000 *g* - Protein analysed by SDS-PAGE	Rendzix Leptosols Chromic Cambisol Dystric Cambisol	

1.2. Cation exchange capacity

Cation exchange capacity was measured on the fresh soil and heat-sterilised soil (80°C; 1 h). The cation exchange capacity was measured as stated in the main manuscript text (Rhoades, 1982). There were no significant differences between cation exchange capacity in the fresh and heat-sterilised for all of the soil types (Table S2). This suggests that heat sterilisation did not affect cation exchange capacity of the three soils and, therefore, is unlikely to influence the sorption or recovery of proteins by the different extractants.

Table S2. Mean cation exchange capacity (mmol $kg^{-1} \pm$ SEM) of fresh and heat-sterilised soil for each soil type. Different letters indicate significant differences between treatment for each soil type (paired t test; $p<0.05$).

Soil Type	**Treatment**	
	Fresh	**Heat-sterilised**
Cambisol	145 ± 6^a	104 ± 30^a
Ferralsol	90 ± 8^a	72 ± 6^a
Histosol	334 ± 6^a	350 ± 6^a

1.3. Soluble protein measurements

We assessed protein content of the soil extractants in 0.5 M K_2SO_4 using the Coomassie Blue method (Bradford, 1976). We acknowledge that this method can be subject to some interference e.g. from humic substances (Roberts and Jones, 2008). As Table S3 shows, total protein did not differ significantly between the soil types. Thus ^{14}C-labelled protein added to the three soil types was added in a similar ratio. Each gram of soil contained 0.086 mg of ^{14}C-labelled protein. Therefore, the rates of ^{14}C-labelled protein addition are reflective of natural soil levels.

Table S3. Mean soluble protein (mg g^{-1}) ± SEM of the three soil types. Different letters indicate significant differences of protein between soil types (one-way ANOVA, Tukey HSD; $p<0.05$).

	Soil type		
	Cambisol	Ferralsol	Histosol
Soluble protein (mg g^{-1})	0.035 ± 0.005^{a}	0.044 ± 0.004^{a}	0.033 ± 0.013^{a}

1.4. pH of the different extraction solutions

The extraction efficiency of many inorganic and organic compounds in soil are known to be highly pH dependent. As proteins carry pH dependent charge, it is likely that this may affect their binding to soil particles and, therefore, recovery from soil. Consequently, we measured the pH of our different extraction solutions as shown in Table S4 below.

Table S4. pH of extractants that were not adjusted.

Extractant	Concentration	pH
$CaCl_2$	0.01 M	6.07
	0.05 M	6.13
	0.1 M	6.06
	0.5 M	6.39
K_2SO_4	0.01 M	5.42
	0.05 M	5.87
	0.1 M	5.49
	0.5 M	5.66
NaOH	0.01 M	12.00
	0.05 M	12.70
	0.1 M	13.00
	0.5 M	13.69
Methanol	25% v/v	5.45
	50% v/v	5.44
	75% v/v	5.60
	100% v/v	6.88
Ethanol	25% v/v	6.59
	50% v/v	6.62
	75% v/v	6.69
	100% v/v	7.51

1.5. Protein mineralisation

We have tested the sterility of the soils after heat-sterilisation. Soil (1 g) was sterilised at 80°C for 1 h in 50 ml polypropylene centrifuge tubes. In order to measure the rate of protein breakdown to $^{14}CO_2$ (Jan et al., 2009), ^{14}C-labelled protein (100 µl; 1.2 kBq ml^{-1}) was added to the soil and a NaOH trap (1 M; 1 ml) was placed above the soil and the tubes sealed. At various times after ^{14}C-protein addition (0.5, 1, 3, 6 and 24 h) the NaOH trap was taken out and replaced with a fresh trap and the $^{14}CO_2$ adsorbed in the NaOH was counted on a Wallac 1404 liquid scintillation counter. Protein mineralisation, as measured by $^{14}CO_2$ production, was significantly reduced by heat-sterilisation compared to the fresh soil in all soil types (one-way ANOVA: Cambisol: $F_{(1,28)} = 17.7$; $p<0.001$, Ferralsol: $F_{(1,28)} = 14.6$; $p<0.001$, Histosol: $F_{(1,28)} = 19.0$; $p<0.001$) (Fig. S1). Therefore, heat-sterilisation of the soil was deemed satisfactory for the purposes of our experiment. The results also indicated that minimal microbial activity would have occurred in the 30 min incubation time used in the main protein extraction assays.

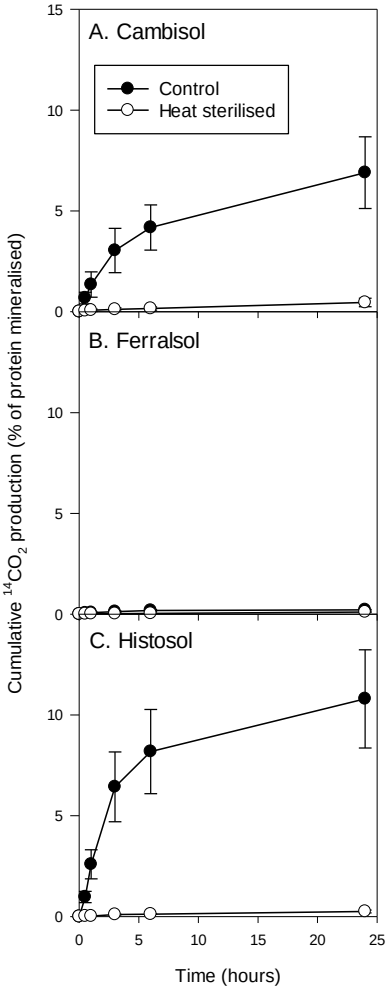

Fig S1. Cumulative $^{14}CO_2$ production after the addition of ^{14}C-labelled protein to fresh and heat sterilised soil (means ± SEM, *n* = 3). The legend is the same for all panels.

1.6. Protease activity in soil

Although we have discounted the microbial breakdown of our added ^{14}C-labelled protein (see above), it is still possible that our added protein could be broken down into peptide fragments by exoenzymes. To address this, protease activity was measured on the fresh and heat-sterilised soil (80°C; 1 h). Soil was extracted in deionised water (1:5 w/v soil:extractant ratio). An alanine aminopeptidase and leucine aminopeptidase assay were carried out according to Vepsäläinen et al. (2001). Due to the use of deionised water as an extractant, total aminopeptidase activity may be an underestimate. Deionised water may be incapable of removing proteases that have been adsorbed onto soil surfaces (e.g. organic matter and clay minerals). The results demonstrated that protease was very low in the soils relative to the amount of protein we added. However, mild heat sterilisation did not eliminate protease activity (Table

S5). Therefore, some very limited proteolysis of ^{14}C-labelled proteins may have taken place leading to the production of very small amounts of ^{14}C-enriched peptides. We do not expect this, however, to bias our findings. Further, in the main experiment the protein was incubated with soil over 30 min allowing minimal time for proteolysis to take place (especially given the amount of protein added relative to protease activity). This is further supported by the ^{14}C mineralisation data presented in Table S5 which shows limited breakdown of the protein even under non-sterile conditions (0.97 ± 0.28% of the total added after 30 min incubation).

Table S5. Mean alanine aminopeptidase and leucine aminopeptidase activity (nmol AMC g^{-1} h^{-1}) ± SEM (n = 3) of fresh and heat-sterilised soil for each soil type. Letters indicate significant differences between treatment for each soil type and assay (paired t test; p<0.05).

Soil type	Alanine aminopeptidase		Leucine aminopeptidase	
	Fresh	Heat-sterilised	Fresh	Heat-sterilised
Cambisol	9.4 ± 0.7^{a}	8.8 ± 1.1^{a}	8.4 ± 0.5^{a}	3.8 ± 0.6^{b}
Ferralsol	26.8 ± 11.4^{a}	9.7 ± 1.9^{a}	7.5 ± 1.1^{a}	11.9 ± 4.3^{a}
Histosol	9.2 ± 0.8^{a}	7.8 ± 1.8^{a}	7.5 ± 1.5^{a}	8.0 ± 1.9^{a}

1.7. Effect of incubation time on protein sorption

We have tested the effect of a prolonged contact time of the protein with the solid phase and its subsequent recovery. ^{14}C-labelled protein (100 µl; 1.2 kBq ml^{-1}) was added to 1 g of heat-sterilised soil (80°C; 1 h) and incubated for 30 min, 1 h, 3 h, 6 h and 24 h. After incubation, the ^{14}C-labelled protein was extracted by deionised water following the same procedure as described in the main manuscript. ^{14}C-labelled protein recovery was unaffected by incubation time for the Ferralsol. Incubation for 24 h reduced recovery relative to the other times for the Histosol and Cambisol (Table S6). We conclude that 0.5 h is an appropriate length of incubation for protein in the soil, limiting protease activity which would cause the ^{14}C-labelled protein to be broken down. Longer incubation times (>0.5 h) would have increased the risk of microbial regrowth and exoenzyme degradation of the added protein.

Table S6. Effect of incubation time on the recovery of ^{14}C-labelled protein from soil (% of total added) mean ± SEM (n = 3). Different letters indicate significant differences between extraction efficiencies of different incubation times for each soil type (one-way ANOVA with Tukey HSD; p<0.05).

Soil type	Incubation time (h)				
	0.5	**1**	**3**	**6**	**24**
Cambisol	26.9 ± 1.8^{ab}	31.8 ± 2.0^{a}	31.5 ± 2.2^{a}	25.0 ± 2.1^{ab}	18.7 ± 1.4^{b}
Ferralsol	10.4 ± 1.1^{a}	11.4 ± 0.6^{a}	11.1 ± 1.3^{a}	10.1 ± 0.4^{a}	11.3 ± 0.8^{a}
Histosol	59.9 ± 3.1^{a}	48.6 ± 7.2^{ab}	42.4 ± 7.1^{ab}	39.5 ± 2.1^{ab}	31.1 ± 3.0^{b}

1.8. Confirmation of effective phase separation by centrifugation

We used high-speed centrifugation to ensure rapid phase separation of the solid and liquid phases. This protocol followed many previous published studies from our laboratory. However, to confirm this was effective, firstly we extracted the soil with deionised water as described in the main manuscript. We then centrifuged this extract (18 000 *g*; 60 s). To check if any solid remained in the supernatant after centrifugation, we pipetted 0.5 ml of the supernatant into a ceramic crucible. The crucibles were weighed prior to the addition of 0.5 ml of supernatant and then again after 24 h when all the water had evaporated. The maximum weight left in the crucible was 0.0005 g (Table S7) showing quasi-complete phase separation was achieved. Based on the initial weight of soil in the extract we calculate that centrifugation removed 99.80 ± 0.03% of the total solid. This value could be even higher when the weight of dissolved salts in the supernatant are accounted for.

Table S7. Crucible weights before and after the addition of 0.5 ml of water extractant supernatant for each soil type.

Soil type	Repetition	Empty crucible weight (g)	Weight after drying (g)	Difference in weight (g)
Cambisol	1	24.2103	24.2108	0.0005
	2	17.6983	17.6985	0.0002
	3	11.5214	11.5215	0.0001
Ferralsol	1	16.2720	16.2721	0.0001
	2	18.9231	16.9235	0.0004
	3	18.4251	18.4251	0.0000
Histosol	1	11.6769	11.6770	0.0001
	2	16.1895	16.1897	0.0002
	3	12.7913	12.7915	0.0002

1.9. Effect of humic acids on ^{14}C counting efficiency

To determine if there was a quenching effect of humic acids in the supernatants of the protein extractants on the counting efficiency of ^{14}C within our samples. A stock solution of humic substances (1 g l^{-1}) was made using a commercial source of water soluble humic acid (Humic acid-Na salt, Cat. No. H16752; Sigma-Aldrich, Poole, Dorset, UK). From this stock solution, a serial dilution was performed to obtain a range of humic acid concentrations. Two sets of serial dilutions were performed. One dilution series had ^{14}C-labelled protein added (100 µl; 114 Bq) to each dilution and the solutions mixed. From this, 0.5 ml was subsequently counted on a Wallac 1404 liquid scintillation counter with automatic quench correction. The quench correction was based on the manufacturer's algorithm contained within their WinSpectral® software. No significant difference was measured between the activities of samples at different humic acid concentrations showing that humic substances did not affect ^{14}C counting efficiency of our added protein (Table S8).

Table S8. Mean activity produced by humic acids and humic acids with added ^{14}C-labelled protein (Bq) ± SEM (n = 3). Different letters indicate significant differences between humic acid concentration for humic acid only dilution and dilution with added ^{14}C-labelled protein (one-way ANOVA with Tukey HSD; $p < 0.05$).

	Humic acid concentration (g l^{-1})					
	0	**0.0625**	**0.125**	**0.25**	**0.5**	**1**
Humic acid only	0.2 ± 0.0^{a}	0.2 ± 0.3^{a}	0.2 ± 0.0^{a}	0.3 ± 0.1^{a}	0.2 ± 0.1^{a}	0.2 ± 0.0^{a}
+ 100 µl ^{14}C-labelled protein	71.8 ± 8.6^{a}	75.0 ± 9.9^{a}	77.1 ± 11.6^{a}	77.4 ± 12.0^{a}	77.0 ± 10.9^{a}	74.7 ± 9.8^{a}

1.10. Effect of solvents on ^{14}C counting efficiency

We determined if there was a quenching effect of methanol and ethanol on the counting efficiency of ^{14}C within our extracts. In this experiment, we used ^{14}C-labelled guanidine as a model compound as it is soluble in water and organic solvents. Mirroring the protocol in the main manuscript, we added a known amount of ^{14}C-labelled guanidine (0.36 kBq) to either 0.5 ml of deionised water (control), HPLC-grade methanol or HPLC-grade ethanol. The amount of ^{14}C was then subsequently counted on a Wallac 1404 liquid scintillation counter with automatic quench correction. All treatments were run in triplicate. The results showed that there was no significant difference in the measured ^{14}C activity for the different solvents showing that they had minimal effect on the ^{14}C counting efficiency (one-way ANOVA: $F_{(2,8)} = 5.14$; $p = 0.17$) as shown below in Table S9 below.

Table S9. Mean activity after the addition of ^{14}C-labelled guanidine (kBq) ± SEM (n = 3) to either water, ethanol or water. Different letters indicate significant differences between the different solvents (one-way ANOVA with Tukey HSD; $p<0.05$).

Solvent	**^{14}C content (kBq sample^{-1})**	**Counting efficiency (% relative to water)**
Water	0.359 ± 0.007^{a}	100 ± 1.9
Ethanol	0.369 ± 0.001^{a}	103 ± 0.4
Methanol	0.370 ± 0.001^{a}	103 ± 0.3

1.11. Salt induced protein precipitation

The hypothesis that protein precipitation was induced at high salt concentrations was tested in the absence of soil. ^{14}C-labelled tobacco leaf protein (100 µl; 1.2 kBq ml^{-1}) was added to deionised water and two simple salt extractants of varying concentration: K_2SO_4 (0.01, 0.05, 0.1, 0.5 M) and $CaCl_2$ (0.01, 0.05, 0.1, 0.5 M). The solutions were shaken for 30 min (200 rev min^{-1}) and then centrifuged for 60 s (18,000 *g*). ^{14}C that remained in the solution was determined by liquid scintillation counting as described above. The extraction efficiency (%) is defined as the percentage of ^{14}C-labelled protein recovered from the soil after extraction compared to the baseline value of ^{14}C-labelled protein added to the soil. Overall, we found significant differences between the extraction efficiency of K_2SO_4 and $CaCl_2$ in comparison to deionised water (one-way ANOVA: $F_{(2, 24)} = 15.63$; $p<0.001$) (Table S10). This supports the hypothesis that large salt concentrations cause precipitation of protein is correct.

Table S10. The effect of salt concentration on protein recovery from soil. Values represent the mean (*n* = 3) ± SEM.

	Concentration (M)	**Extraction efficiency (%)**
Deionised water	N/A	50.4 ± 13.5
K_2SO_4	0.01	21.8 ± 1.1
	0.05	25.9 ± 1.3
	0.1	28.1 ± 1.3
	0.5	28.3 ± 6.3
$CaCl_2$	0.01	17.1 ± 2.6
	0.05	19.5 ± 1.8
	0.1	20.1 ± 2.0
	0.5	24.7 ± 3.0

1.12. Co-extraction of humic substances during protein recovery

UV-Visible spectroscopy provides a cheap, quick but reliable proxy of dissolved organic carbon (DOC) concentrations. Absorbance at 254 nm in the UV spectrum detects aromatic humic substances (Edzwald et al., 1985) and 400 nm detects colour in the visible spectrum (Wallage and Holden, 2010). The ratio of these two wavelengths (E2:E4) provides a measure of humification (Carter et al., 2012; Peacock et al., 2014).

Table S11. Mean absorbance (RAU* cm^{-1}) ± SEM of supernatant from extractant solutions at 254 nm and 400 nm for 1:100 dilution (*n* = 3)
*Relative absorbance units

		Cambisol			**Ferralsol**			**Histosol**		
Extractant	Concentration (M)	**254**	**400**	**254/400**	**254**	**400**	**254/400**	**254**	**400**	**254/400**
Na-pyrophosphate	0.1	0.315 ± 0.009	0.041 ± 0.001	7.643 ± 0.311	0.429 ± 0.059	0.048 ± 0.003	8.954 ± 1.169	0.752 ± 0.127	0.146 ± 0.020	5.123 ± 0.366
	0.05	0.204 ± 0.026	0.044 ± 0.001	4.625 ± 0.493	0.466 ± 0.027	0.052 ± 0.003	9.009 ± 0.166	0.578 ± 0.147	0.105 ± 0.019	5.328 ± 0.560
	0.01	0.097 ± 0.005	0.030 ± 0.0007	3.203 ± 0.149	0.439 ± 0.044	0.067 ± 0.012	6.747 ± 0.544	0.204 ± 0.044	0.044 ± 0.005	4.476 ± 0.533
$CaCl_2$	0.5	0.056 ± 0.0003	0.027 ± 0.0003	2.113 ± 0.039	0.169 ± 0.029	0.126 ± 0.0003	1.341 ± 0.231	0.057 ± 0.001	0.026 ± 0.0006	2.180 ± 0.036

	0.1	0.058 ± 0.002	0.028 ± 0.001	2.099 ± 0.030	0.180 ± 0.033	0.126 ± 0.0006	1.426 ± 0.256	0.054 ± 0.0003	0.026 ± 0.0003	2.117 ± 0.024
	0.05	0.055 ± 0.0006	0.026 ± 0.0003	2.089 ± 0.013	0.299 ± 0.007	0.155 ± 0.011	1.945 ± 0.098	0.055 ± 0.0003	0.026 ± 0.0006	2.104 ± 0.036
	0.01	0.056 ± 0.001	0.025 ± 0.0003	2.212 ± 0.059	0.288 ± 0.009	0.151 ± 0.020	1.954 ± 0.186	0.054 ± 0.0003	0.026 ± 0.0003	2.117 ± 0.024
Na-citrate	0.5	0.310 ± 0.021	0.050 ± 0.003	6.166 ± 0.239	0.381 ± 0.045	0.045 ± 0.002	8.639 ± 1.406	0.854 ± 0.189	0.123 ± 0.020	6.775 ± 0.640
	0.1	0.219 ± 0.018	0.044 ± 0.002	4.924 ± 0.189	0.527 ± 0.042	0.054 ± 0.003	9.757 ± 0.971	0.814 ± 0.076	0.124 ± 0.011	6.561 ± 0.380
	0.05	0.166 ± 0.049	0.037 ± 0.005	4.949 ± 1.725	0.337 ± 0.060	0.055 ± 0.008	6.069 ± 0.279	0.532 ± 0.152	0.093 ± 0.018	5.600 ± 0.613
	0.01	0.076 ± 0.003	0.029 ± 0.0007	2.650 ± 0.027	0.241 ± 0.009	0.061 ± 0.006	4.114 ± 0.652	0.175 ± 0.033	0.044 ± 0.004	3.882 ± 0.453
K_2SO_4	0.5	0.059 ± 0.000	0.027 ± 0.0006	2.187 ± 0.047	0.058 ± 0.0007	0.025 ± 0.0003	2.277 ± 0.049	0.057 ± 0.0009	0.025 ± 0.0003	2.238 ± 0.063
	0.1	0.060 ± 0.002	0.028 ± 0.0003	2.131 ± 0.083	0.064 ± 0.005	0.026 ± 0.0006	2.481 ± 0.216	0.057 ± 0.002	0.026 ± 0.0009	2.177 ± 0.013
	0.05	0.059 ± 0.003	0.027 ± 0.0003	2.172 ± 0.118	0.051 ± 0.010	0.027 ± 0.0009	1.890 ± 0.394	0.057 ± 0.0007	0.026 ± 0.0003	2.152 ± 0.002

	0.01	0.059 ± 0.002	0.028 ± 0.0007	2.120 ± 0.023	0.053 ± 0.0009	0.025 ± 0.0003	2.105 ± 0.013	0.063 ± 0.001	0.027 ± 0.002	2.344 ± 0.102
K-phosphate buffer pH 6	0.5	0.073 ± 0.003	0.030 ± 0.002	2.410 ± 0.109	0.131 ± 0.005	0.031 ± 0.0003	4.179 ± 0.111	0.208 ± 0.049	0.044 ± 0.005	4.614 ± 0.577
	0.1	0.063 ± 0.001	0.029 ± 0.001	2.204 ± 0.088	0.110 ± 0.002	0.030 ± 0.0003	3.615 ± 0.033	0.086 ± 0.012	0.030 ± 0.002	2.828 ± 0.283
	0.05	0.062 ± 0.001	0.027 ± 0.0006	2.309 ± 0.014	0.079 ± 0.008	0.032 ± 0.003	2.544 ± 0.448	0.082 ± 0.012	0.029 ± 0.001	2.837 ± 0.298
	0.01	0.064 ± 0.002	0.028 ± 0.0007	2.262 ± 0.088	0.076 ± 0.009	0.028 ± 0.001	2.652 ± 0.191	0.068 ± 0.005	0.029 ± 0.002	2.395 ± 0.248
K-phosphate buffer pH 8	0.5	0.128 ± 0.052	0.037 ± 0.008	3.218 ± 0.599	0.211 ± 0.030	0.042 ± 0.005	4.996 ± 0.173	0.551 ± 0.104	0.104 ± 0.013	5.184 ± 0.421
	0.1	0.065 ± 0.002	0.028 ± 0.0006	2.333 ± 0.050	0.133 ± 0.002	0.033 ± 0.000	4.030 ± 0.063	0.356 ± 0.063	0.070 ± 0.007	4.977 ± 0.451
	0.05	0.066 ± 0.005	0.029 ± 0.002	2.261 ± 0.045	0.138 ± 0.010	0.033 ± 0.001	4.213 ± 0.421	0.228 ± 0.036	0.051 ± 0.002	4.404 ± 0.533
	0.01	0.065 ± 0.004	0.028 ± 0.001	2.331 ± 0.064	0.098 ± 0.009	0.035 ± 0.002	2.756 ± 0.069	0.086 ± 0.008	0.029 ± 0.0009	2.926 ± 0.230
NaOH	0.5	0.401 ± 0.014	0.078 ± 0.003	5.116 ± 0.073	0.483 ± 0.013	0.092 ± 0.004	5.260 ± 0.110	2.902 ± 0.153	0.553 ± 0.035	5.262 ± 0.228

	0.1	0.398 ± 0.017	0.088 ± 0.003	4.503 ± 0.032	0.981 ± 0.152	0.240 ± 0.047	4.148 ± 0.146	1.552 ± 0.309	0.346 ± 0.066	4.518 ± 0.256
	0.05	0.274 ± 0.026	0.064 ± 0.005	4.271 ± 0.122	1.252 ± 0.240	0.328 ± 0.072	3.861 ± 0.101	0.960 ± 0.263	0.200 ± 0.042	4.692 ± 0.322
	0.01	0.090 ± 0.0009	0.031 ± 0.0006	2.916 ± 0.052	0.284 ± 0.056	0.084 ± 0.015	3.370 ± 0.078	0.191 ± 0.022	0.049 ± 0.003	3.858 ± 0.229
Tris-SDS	0.1	0.093 ± 0.003	0.034 ± 0.0003	2.711 ± 0.117	0.076 ± 0.002	0.030 ± 0.0009	2.524 ± 0.130	0.126 ± 0.017	0.037 ± 0.004	3.412 ± 0.182
	0.05	0.068 ± 0.001	0.029 ± 0.0003	2.330 ± 0.043	0.070 ± 0.003	0.030 ± 0.002	2.323 ± 0.053	0.102 ± 0.009	0.035 ± 0.003	2.919 ± 0.039
	0.01	0.085 ± 0.002	0.032 ± 0.002	2.632 ± 0.125	0.058 ± 0.001	0.028 ± 0.0003	2.108 ± 0.020	0.066 ± 0.002	0.029 ± 0.001	2.319 ± 0.099
Methanol	100%	0.054 ± 0.0003	0.026 ± 0.000	2.064 ± 0.013	0.054 ± 0.0003	0.026 ± 0.0003	2.117 ± 0.024	0.080 ± 0.004	0.027 ± 0.0003	2.984 ± 0.134
	75%	0.056 ± 0.001	0.028 ± 0.001	1.999 ± 0.120	0.056 ± 0.001	0.026 ± 0.0006	2.141 ± 0.013	0.074 ± 0.004	0.030 ± 0.002	2.468 ± 0.050
	50%	0.056 ± 0.0006	0.027 ± 0.0006	2.075 ± 0.023	0.055 ± 0.001	0.027 ± 0.001	2.067 ± 0.056	0.064 ± 0.002	0.026 ± 0.0003	2.431 ± 0.079
	25%	0.057 ± 0.002	0.028 ± 0.001	2.025 ± 0.012	0.055 ± 0.0003	0.025 ± 0.0003	2.158 ± 0.025	0.063 ± 0.004	0.026 ± 0.0003	2.377 ± 0.117

Ethanol	100%	0.054 ± 0.0007	0.027 ± 0.001	1.994 ± 0.066	0.054 ± 0.001	0.025 ± 0.000	2.147 ± 0.048	0.083 ± 0.009	0.027 ± 0.0006	3.051 ± 0.252
	75%	0.055 ± 0.001	0.026 ± 0.0009	2.091 ± 0.036	0.053 ± 0.0003	0.025 ± 0.0003	2.106 ± 0.036	0.076 ± 0.007	0.027 ± 0.0007	2.842 ± 0.172
	50%	0.061 ± 0.004	0.029 ± 0.004	2.163 ± 0.159	0.054 ± 0.0003	0.026 ± 0.0003	2.117 ± 0.024	0.073 ± 0.003	0.027 ± 0.0009	2.660 ± 0.096
	25%	0.055 ± 0.0003	0.026 ± 0.0006	2.104 ± 0.036	0.054 ± 0.0009	0.025 ± 0.0003	2.146 ± 0.049	0.066 ± 0.005	0.027 ± 0.0003	2.475 ± 0.171
Deionised water	0	0.069 ± 0.007	0.029 ± 0.002	2.341 ± 0.087	0.056 ± 0.002	0.026 ± 0.001	2.131 ± 0.050	0.068 ± 0.005	0.030 ± 0.002	2.296 ± 0.062

Figure S2. Image to demonstrate colour produced in supernatant from extraction of the Cambisol with different extractants. From left to right: deionised water; 0.5 M Na-citrate; 0.1 M Na-pyrophosphate; 0.5 M NaOH.

References

Bastida, F., Jehmlich, N., Torres, I.F., García, C., 2018. The extracellular metaproteome of soils under semiarid climate: A methodological comparison of extraction buffers. Science of The Total Environment 619–620, 707–711.

Benndorf, D., Balckem, G.U., Harms, H., von Bergen, M., 2007. Functional metaproteome analysis of protein extracts from contaminated soil and groundwater. ISME Journal 1, 224–234.

Bonmati, M., Ceccanti, B., Nannipieri, P., 1998. Protease extraction from soil by sodium pyrophosphate and chemical characterization of the extracts. Soil Biology & Biochemistry 30, 2113–2125.

Bradford, M.M., 1976. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. Analytical Biochemistry 72, 248–254.

Bremner, J.M., Lees, H., 1949. Studies on soil organic matter: Part II. The extraction of organic matter from soil by neutral reagents. The Journal of Agricultural Science 39, 274–279.

Busto, M.D., Perez-Mateos, M., 1995. Extraction of humic-β-glucosidase fractions from soil. Biology & Fertility of Soils 20, 77–82.

Carter, H.T., Tipping, E., Koprivnjak, J.-F., Miller, M.P., Cookson, B., Hamilton-Taylor, J., 2012. Freshwater DOM quantity and quality from a two-component

model of UV absorbance. Water Research 46, 4532–4542.

Chen, S., Rillig, M.C., Wang, W., 2009. Improving soil protein extraction for metaproteome analysis and glomalin-related soil protein detection. Proteomics 9, 4970–4973.

Criquet, S., Farnet, A.M., Ferre, E., 2002. Protein measurement in forest litter. Biology & Fertility of Soils 35, 307–313.

Edzwald, J.K., Becker, W.C., Wattier, K.L., 1985. Surrogate Parameters for Monitoring Organic Matter and THM Precursors. Research and Technology 77, 122–132.

Friedel, K., Scheller, E., Èrgen, J., 2002. Composition of hydrolysable amino acids in soil organic matter and soil microbial biomass. Soil Biology & Biochemistry 34, 315–325.

Halvorson, J.J., Gonzalez, J.M., 2006. Bradford reactive soil protein in Appalachian soils: distribution and response to incubation, extraction reagent and tannins. Plant Soil 286, 339–356.

Haney, R.L., Franzluebbers, A.J., Hons, F.M., Hossner, L.R., Zuberer, D.A., 2001. Molar concentration of K_2SO_4 and soil pH affect estimation of extractable C with chloroform fumigation-extraction. Soil Biology & Biochemistry 33, 1501–1507.

Jan, M.T., Roberts, P., Tonheim, S.K., Jones, D.L., 2009. Protein breakdown represents a major bottleneck in nitrogen cycling in grassland soils. Soil Biology & Biochemistry 41, 2272–2282.

Kanerva, S., Smolander, A., Kitunen, V., Ketola, R.A., Kotiaho, T., 2013. Comparison of extractants and applicability of MALDI-TOF-MS in the analysis of soil proteinaceous material from different types of soil. Organic Geochemistry 56, 1–9.

Keiblinger, K.M., Wilhartitz, I.C., Schneider, T., Roschitzki, B., Schmid, E., Eberl, L., Riedel, K., Zechmeister-Boltenstern, S., 2012. Soil metaproteomics - Comparative evaluation of protein extraction protocols. Soil Biology & Biochemistry 54, 14–24.

Makarov, M.I., Malysheva, T.I., Menyailo, O. V, Soudzilovskaia, N.A., P Van Logtestijn, R.S., C Cornelissen, J.H., 2015. Effect of K_2SO_4 concentration on extractability and isotope signature ($\delta^{13}C$ and $\delta^{15}N$) of soil C and N fractions. European Journal of Soil Science 66, 417–426.

Marchetti, E., Accinelli, C., Epifani, R., 2007. Persistence of Cry toxins and cry genes

from genetically modified plants in two agricultural soils. Agronomy for Sustainable Development 27, 231–236.

Masciandaro, G., Macci, C., Doni, S., Elena Maserti, B., Calvo-Bado Leo, A., Ceccanti, B., Wellington, E., 2008. Comparison of extraction methods for recovery of extracellular β-glucosidase in two different forest soils. Soil Biology & Biochemistry 40, 2156–2161.

Matsumoto, S., Ae, N., Yamagata, M., 2000. Extraction of mineralizable organic nitrogen from soils by a neutral phosphate buffer solution. Soil Biology & Biochemistry 32, 1293–1299.

Murase, A., Yoneda, M., Ueno, R., Yonebayashi, K., 2003. Isolation of extracellular protein from greenhouse soil. Soil Biology & Biochemistry 35, 733–736.

Nannipieri, P., Ceccanti, B., Crevelli, S., Sequi, P., 1974. Use of 0.1 M pyrophosphate to extract urease from a podzol. Soil Biology & Biochemistry 6, 359–362.

Peacock, M., Evans, C.D., Fenner, N., Freeman, C., Gough, R., Jones, T.G., Lebron, I., 2014. UV-visible absorbance spectroscopy as a proxy for peatland dissolved organic carbon (DOC) quantity and quality: considerations on wavelength and absorbance degradation. Environmental Science: Processes & Impacts.

Rhoades, J.D., 1982. Cation exchange capacity, in: Page, A.L., Miller, R.H., Keeney, D.R. (Eds.), Methods of Soil Analysis. Part 2. Chemical and Microbiological Properties. Soil Science Society of America, Madison, Wisconsin, pp. 149–157.

Roberts, P., Jones, D.L., 2008. Critical evaluation of methods for determining total protein in soil solution. Soil Biology & Biochemistry 40, 1485–1495.

Rosier, C.L., Hoye, A.T., Rillig, M.C., 2006. Glomalin-related soil protein: Assessment of current detection and quantification tools. Soil Biology & Biochemistry 38, 2205–2211.

Schneider, T., Keiblinger, K.M., Schmid, E., Sterflinger-Gleixner, K., Nther Ellersdorfer, G., Roschitzki, B., Richter, A., Eberl, L., Zechmeister-Boltenstern, S., Riedel, K., 2012. Who is who in litter decomposition? Metaproteomics reveals major microbial players and their biogeochemical functions. ISME Journal 6, 1749–1762.

Vepsäläinen, M., Kukkonen, S., Vestberg, M., Sirviö, H., Maarit Niemi, R., 2001. Application of soil enzyme activity test kit in a field experiment. Soil Biology & Biochemistry 33, 1665–1672.

Wallage, Z.E., Holden, J., 2010. Spatial and temporal variability in the relationship between water colour and dissolved organic carbon in blanket peat pore waters. Science of The Total Environment 408, 6235–6242.

Wright, S.F., Upadhyaya, A., 1998. A survey of soils for aggregate stability and glomalin, a glycoprotein produced by hyphae of arbuscular mycorrhizal fungi. Plant and Soil 198, 97–107.

Appendix 2

Supplementary material for Chapter 4

Table S1. Two-step extraction of ^{14}C-labelled protein and ^{14}C-labelled amino acid mixture remaining (% of total ^{14}C-labelled substrate added) in either the topsoil (Top) or subsoil (Sub) at the end of the $^{14}CO_2$ evolution experiment following the addition of either ^{14}C-labelled protein or amino acid mixture to soil. Values represent mean ± SEM (*n* = 3).

		^{14}C-labelled protein (% of total ^{14}C-labelled protein added)			**^{14}C-labelled amino acid mix (% of total ^{14}C-labelled amino acid mixture added)**		
Site	**Soil depth**	**^{14}C extracted by deionised water**	**^{14}C extracted by 0.05 M Na-pyrophosphate**	**Total ^{14}C extracted**	**^{14}C extracted by deionised water**	**^{14}C extracted by 0.05 M Na-pyrophosphate**	**Total ^{14}C extracted**
1	Top	3.3 ± 0.4	17.0 ± 1.5	20.2 ± 1.4	1.5 ± 0.2	2.3 ± 0.3	3.8 ± 0.2
	Sub	5.4 ± 0.6	13.0 ± 4.0	18.3 ± 3.3	1.6 ± 0.3	5.1 ± 0.7	8.2 ± 0.8
2	Top	3.4 ± 0.6	16.3 ± 1.3	19.6 ± 0.8	2.3 ± 0.4	5.0 ± 0.6	7.3 ± 0.4
	Sub	4.4 ± 0.2	15.3 ± 1.6	19.7 ± 1.4	3.1 ± 0.5	5.1 ± 0.1	8.2 ± 0.4
3	Top	2.6 ± 0.2	24.1 ± 0.9	26.7 ± 1.0	1.4 ± 0.1	4.4 ± 0.2	5.8 ± 0.2
	Sub	5.9 ± 0.8	21.3 ± 1.5	27.2 ± 1.6	3.8 ± 1.6	4.7 ± 1.0	8.5 ± 0.6
4	Top	6.1 ± 1.0	18.8 ± 0.5	25.0 ± 1.5	2.1 ± 0.1	6.1 ± 0.1	8.2 ± 0.1
	Sub	5.8 ± 0.2	23.1 ± 1.7	28.9 ± 1.6	3.6 ± 1.3	6.1 ± 0.4	9.7 ± 1.7
5	Top	4.3 ± 1.0	17.7 ± 0.5	22.0 ± 1.4	2.0 ± 0.3	4.1 ± 0.9	6.1 ± 0.9
	Sub	5.4 ± 0.6	16.0 ± 4.0	21.4 ± 4.6	2.7 ± 0.4	3.7 ± 0.7	6.4 ± 0.8
6	Top	7.5 ± 3.2	10.1 ± 2.1	17.6 ± 1.1	1.8 ± 0.4	3.8 ± 1.4	5.7 ± 1.3
	Sub	7.5 ± 0.2	10.1 ± 1.5	17.6 ± 1.5	4.4 ± 2.4	5.4 ± 1.6	9.8 ± 1.1

7	Top	5.0 ± 0.6	9.5 ± 1.9	14.5 ± 1.5	1.6 ± 0.2	1.4 ± 0.1	3.0 ± 0.1
	Sub	6.6 ± 0.4	17.4 ± 1.8	24.0 ± 1.5	2.4 ± 0.3	4.2 ± 0.8	6.6 ± 0.5
8	Top	2.8 ± 0.5	16.8 ± 0.7	19.7 ± 0.5	1.3 ± 0.6	4.2 ± 1.0	5.4 ± 1.2
	Sub	4.8 ± 0.4	23.2 ± 1.9	28.0 ± 2.2	1.8 ± 0.5	4.9 ± 1.0	6.7 ± 0.6
9	Top	2.7 ± 0.3	16.0 ± 1.9	18.7 ± 2.0	1.3 ± 0.1	4.7 ± 0.3	6.0 ± 0.4
	Sub	4.0 ± 0.6	10.1 ± 1.8	14.1 ± 1.3	1.7 ± 0.3	3.4 ± 0.6	5.1 ± 0.6
10	Top	4.9 ± 0.7	11.1 ± 1.8	16.0 ± 1.2	1.4 ± 0.1	4.1 ± 0.9	5.5 ± 0.8
	Sub	9.6 ± 2.6	4.9 ± 0.8	14.5 ± 1.9	0.9 ± 0.1	2.6 ± 0.1	2.7 ± 0.2

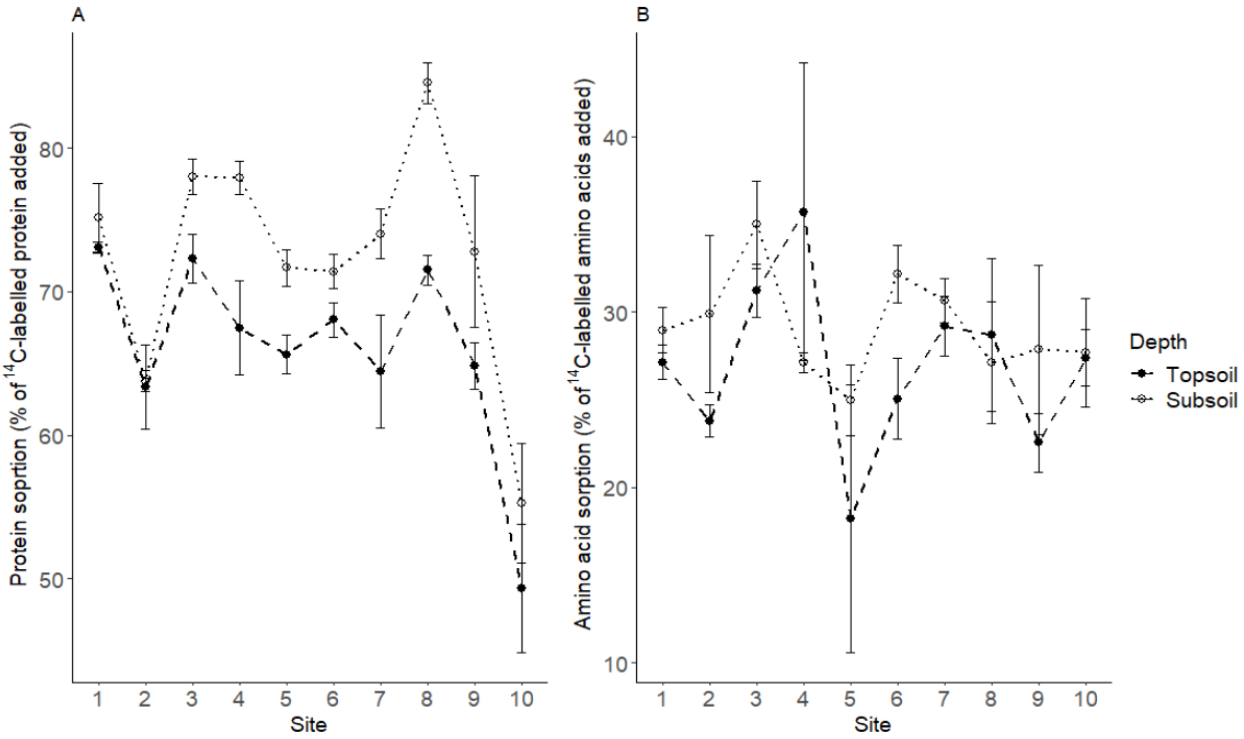

Figure S1. Sorption of either ^{14}C-labelled protein (A) and ^{14}C-labelled amino acid mixture (B) to either the topsoil or subsoil from across the grassland altitudinal gradient. Values are expressed as the amount of substrate sorbed to the solid phase as a percentage of the total ^{14}C-labelled substrate added to the soil. Values represent means ± SEM (*n* = 3).

Supplementary Information 3

The breakdown of amino acids was generally biphasic, consistent with many previous studies investigating the turnover of low molecular weight solutes in soil (Jones et al., 2005; Hill et al., 2008; Glanville et al., 2016). Therefore, we described the process in a two-phase double first order kinetic decay model:

$$S = [a_1 \times \exp(-k_1 t)] + [a_2 \times \exp(-k_2 t)] \quad (1)$$

Where S is the ^{14}C-label remaining in the soil, k_1 is the exponential coefficient describing the initial breakdown by the microbial biomass, k_2 is the exponential coefficient describing the secondary, slower phase breakdown, a_1 and a_2 describe the proportion of ^{14}C associated with the pools of exponential coefficients k_1 and k_2 and t is time (Boddy et al., 2008). The first rapid phase of $^{14}CO_2$ production is attributable to the immediate use of the substrate in catabolic processes (i.e. respiration; Glanville et al., 2016). The half-life ($t_{½}$) of the C pool a_1 can be calculated as:

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

The slower second phase (k_2) of $^{14}CO_2$ production is attributable to the subsequent turnover of ^{14}C taken up and immobilised within the soil microbial community (i.e. C that is used in cell maintenance and growth, but which is subsequently mineralised during cell death or secondary maintenance). The half-life of C pool a_2 can be calculated as:

$$t_{1/2} = \ln(2) / k_2 \quad (3)$$

The values obtained for the half-life of C pool a_2 has some degree of uncertainty due to difficulties in quantifying the level of C pool connectivity within the microbial food web and different amounts of isotopic pool dilution (see Glanville et al., 2016 for further details). The cumulative $^{14}CO_2$ mineralisation data was transformed to give plots of ^{14}C remaining in the soil versus time. Equation (1) was then fitted to the transformed experimental mineralisation results using a least sum of squares iteration routine with Sigmaplot 13.0 (SPSS Inc., Chicago, IL). Microbial substrate carbon use efficiency (CUE) is defined as the proportion of ^{14}C-amino acid that is immobilised relative to the total amount taken up by the biomass. CUE was calculated as:

$$CUE = a_2 / (a_1 + a_2). \quad (4)$$

A full description and validation of the approach used to calculate CUE is provided in the Supplementary Information of Jones et al. (2018a) while the general principles of using ^{14}C to calculate microbial CUE are described in Jones et al. (2018b).

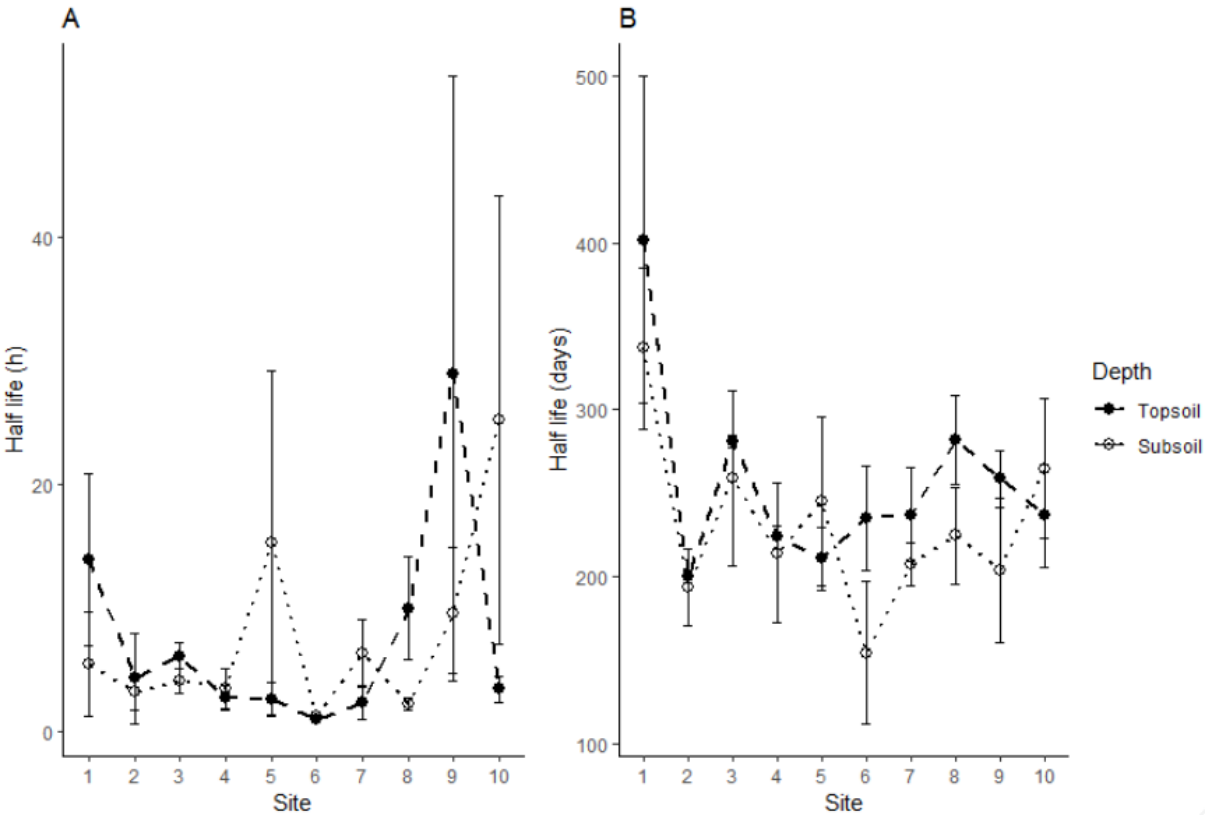

Figure S2. Half-lives of amino acid breakdown along the grassland altitudinal gradient in the topsoil and subsoil. A) Half-life of pool *a*1 (h) and B) half-life of pool *a*2 (days). Values represent mean ± SEM (*n* = 3).

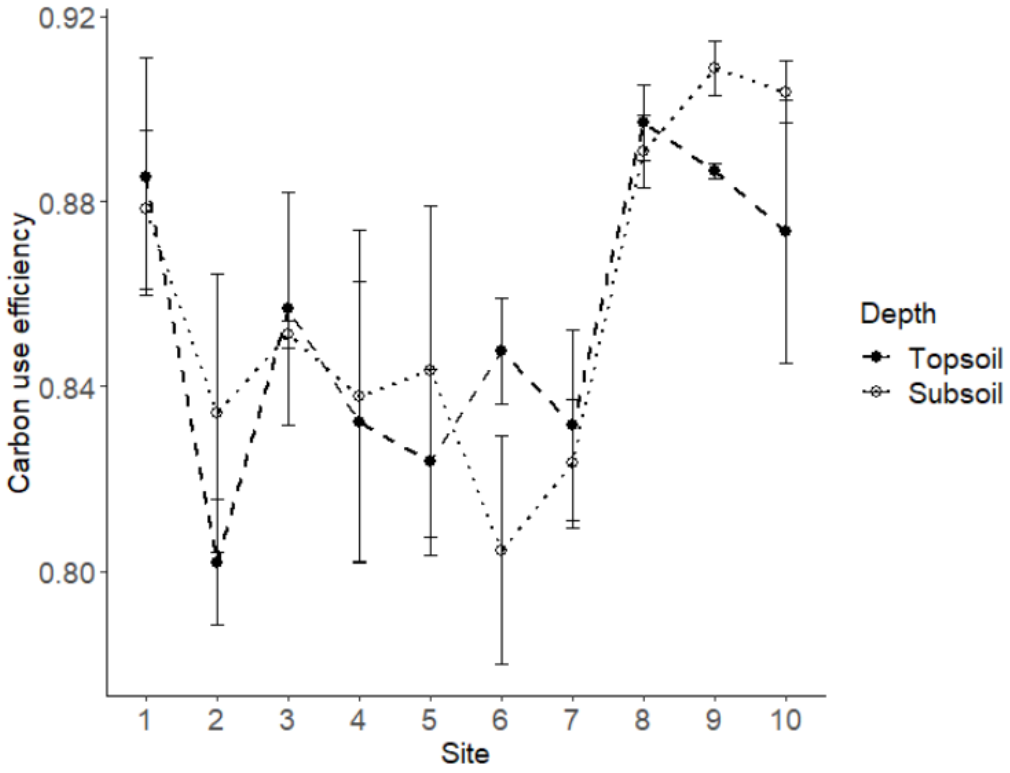

Figure S3. Microbial carbon use efficiency of amino acid-derived C along the grassland altitudinal gradient in the topsoil and subsoil. Values represent mean ± SEM (*n* = 3).

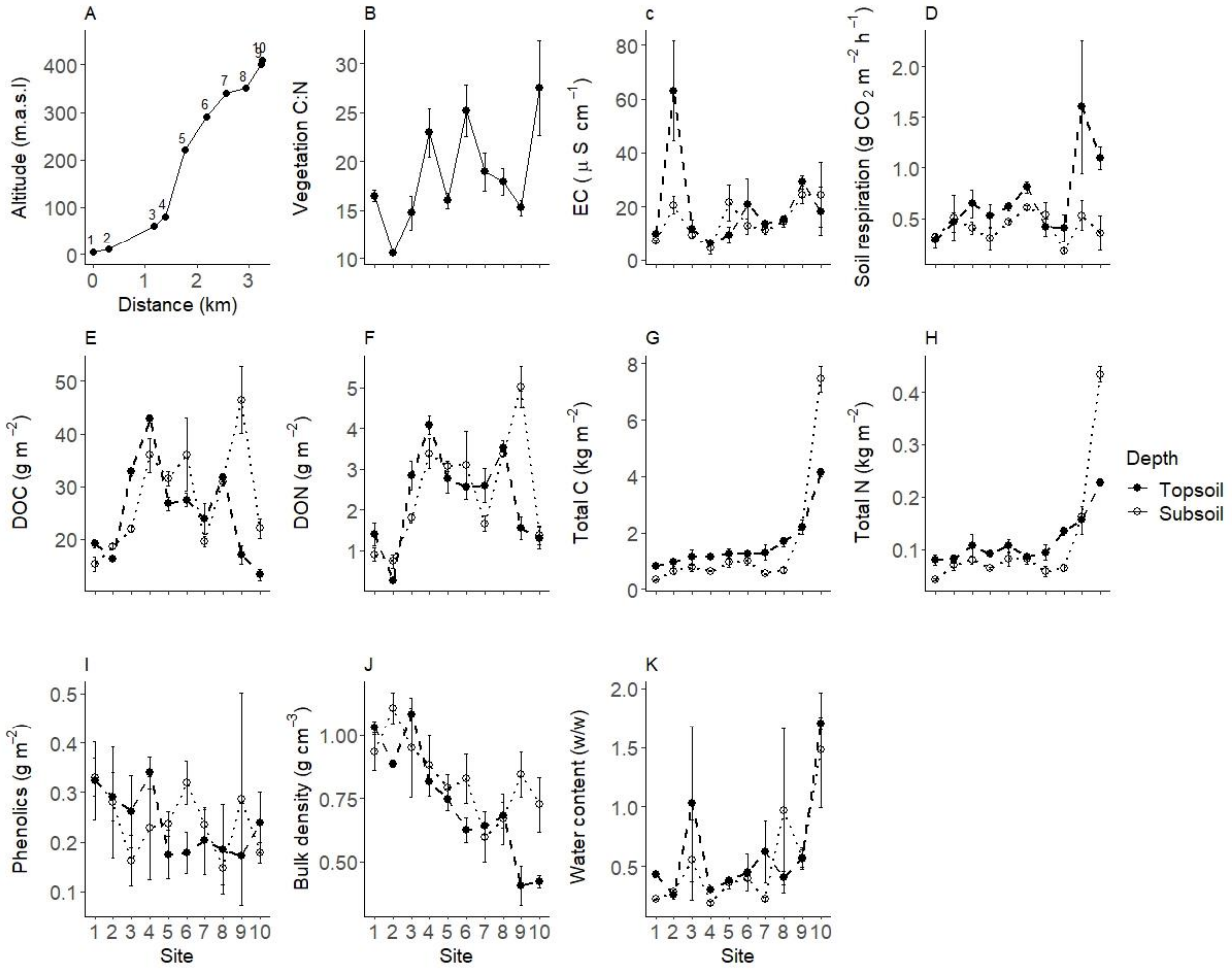

Figure S4. Soil properties of the grassland altitudinal catena sequence. A) location of sites over distance (km) and altitude (m.a.s.l), B) above-ground vegetation C:N ratio, C) electrical conductivity (EC) (µS cm^{-1}), D) soil respiration (g CO_2 m^{-2} h^{-1}), E) dissolved organic C (DOC) (g m^{-2}), F) dissolved organic N (DON) (g m^{-2}), G) total C (kg m^{-2}), H) total N (kg m^{-2}), I) soluble phenolics (g m^{-2}), J) bulk density (g cm^{-3}), and K) volumetric water content (w/w).Values represent mean ± SEM (*n* = 3) and are expressed on a dry weight basis except for A.

Table S2. Two-way ANOVA results for soil properties and the one-way ANOVA result for and plant C:N net primary productivity. *P* values in bold are significant (p <0.05).

Soil property	***Residuals***	**Site**			**Depth**		
		df	***F***	***P value***	***df***	***F***	***P value***
Amino acid C*		9	38.9	**<0.0001**	1	0.06	0.813
Ammonium	40	9	7.26	**<0.0001**	1	14.6	**0.0004**
Bulk density	40	9	8.35	**<0.0001**	1	7.16	**0.011**
Cation exchange capacity	40	9	15.8	**<0.0001**	1	51.3	**<0.0001**
C:N ratio	40	9	26.4	**<0.0001**	1	39.1	**<0.0001**
DOC	40	9	26.2	**<0.0001**	1	7.79	**0.008**
DON*		9	36.6	**<0.0001**	1	1.16	0.281
Electrical conductivity*		9	33.2	**0.0001**	1	0.66	0.416
Microbial C	40	9	12.6	**<0.0001**	1	37.4	**<0.0001**
N mineralisation*		9	11.6	0.245	1	5.89	**0.015**
Nitrate	40	9	21.4	**<0.0001**	1	0.006	0.936
pH	40	9	94.5	**<0.0001**	1	2.49	0.122
Phenols	40	9	0.82	0.592	1	0.00	0.995
Plant C:N	28	1	6.52	**0.016**	-	-	-
Protease activity	40	9	2.68	**0.016**	1	0.35	0.555
Protein C	40	9	1.74	0.111	1	0.32	0.574
Soil respiration	40	9	3.88	**0.001**	1	13.7	**0.0007**

Total C	40	9	152	**<0.0001**	1	0.70	0.407
Total N	40	9	95.7	**<0.0001**	1	0.20	0.655
Water content*		9	28.2	**0.0009**	1	3.81	0.051
Net primary productivity	28	1	35.2	**<0.0001**	-	-	-

*Soil properties did not have a normal distribution after transformation; therefore, a Kruskal-Wallis test was performed to determine the difference with site and depth separately. In these cases, *F* value refers to the chi-squared value.

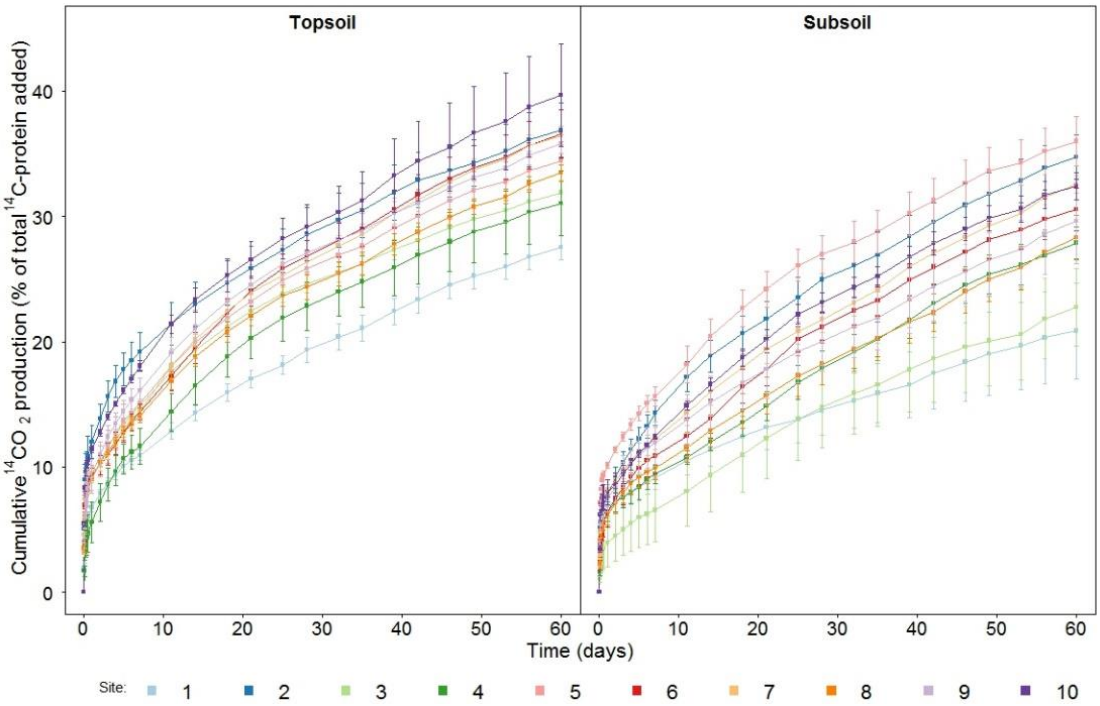

Figure S5. Cumulative $^{14}CO_2$ production arising from the mineralisation of ^{14}C-labelled protein for ten sites along the grassland altitudinal gradient in the topsoil and subsoil. Values represent mean ± SEM (*n* = 3).

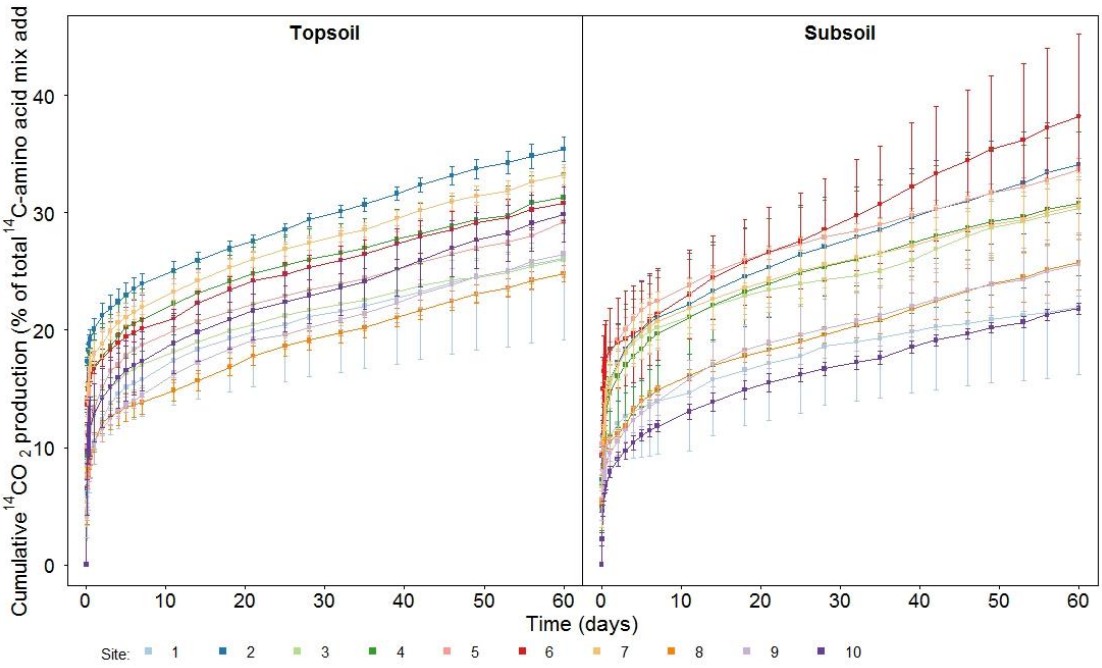

Figure S6. Cumulative $^{14}CO_2$ production arising from the mineralisation of a mixture of ^{14}C-labelled amino acids for ten sites along the grassland altitudinal gradient in the topsoil and subsoil. Values represent mean ± SEM (*n* = 3).

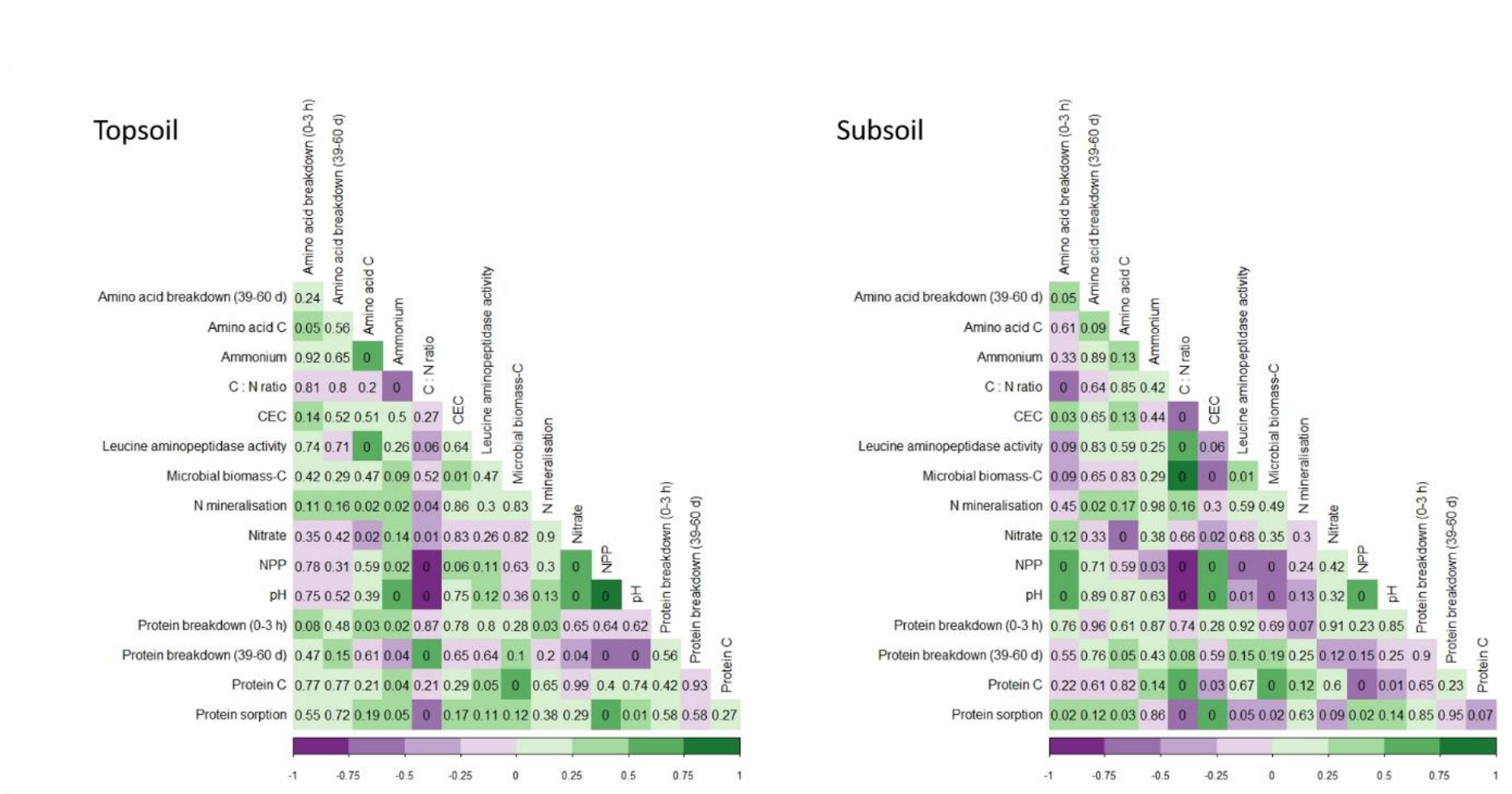

Figure S7. Correlation matrix (Corrplot) for the different measured parameters in the topsoil and subsoil of all sites along the grassland altitudinal gradient. *P* values are stated for each correlation and colours relate to the correlation coefficient.

References

Boddy, E., Roberts, P., Hill, P.W., Farrar, J., Jones, D.L., 2008. Turnover of low molecular weight dissolved organic C (DOC) and microbial C exhibit different temperature sensitivities in Arctic tundra soils. Soil Biology & Biochemistry 40, 1557-1566.

Glanville, H.C., Hill, P.W., Schnepf, A., Oburger, E., Jones, D.L., 2016. Combined use of empirical data and mathematical modelling to better estimate the microbial turnover of isotopically labelled carbon substrates in soil. Soil Biology & Biochemistry 94, 154–168.

Hill, P.W., Farrar, J.F., Jones, D.L., 2018a. Decoupling of microbial glucose uptake and mineralization in soil. Soil Biology & Biochemistry 40, 616-624.

Jones, D.L., Kemmitt, S.J., Wright, D., Cuttle, S.P., Bol, R., Edwards, A.C., 2005. Rapid intrinsic rates of amino acid biodegradation in soils are unaffected by agricultural management strategy. Soil Biology & Biochemistry 37, 1267-1275.

Jones, D.L., Olivera-Ardid, S., Klumpp, E., Knief, C., Hill, P.W., Lehndorff, E., Bol, R., 2018a. Moisture activation and carbon use efficiency of soil microbial communities along an aridity gradient in the Atacama Desert. Soil Biology & Biochemistry 117, 68-71.

Jones, D. L., Hill, P.W., Smith, A.R., Farrell, M., Ge, T., Banning, N.C., Murphy, D. V., 2018b. Role of substrate supply on microbial carbon use efficiency and its role in interpreting soil microbial community-level physiological profiles (CLPP). Soil Biology & Biochemistry 123, 1–6.

Appendix 3

Supplementary material for Chapter 5

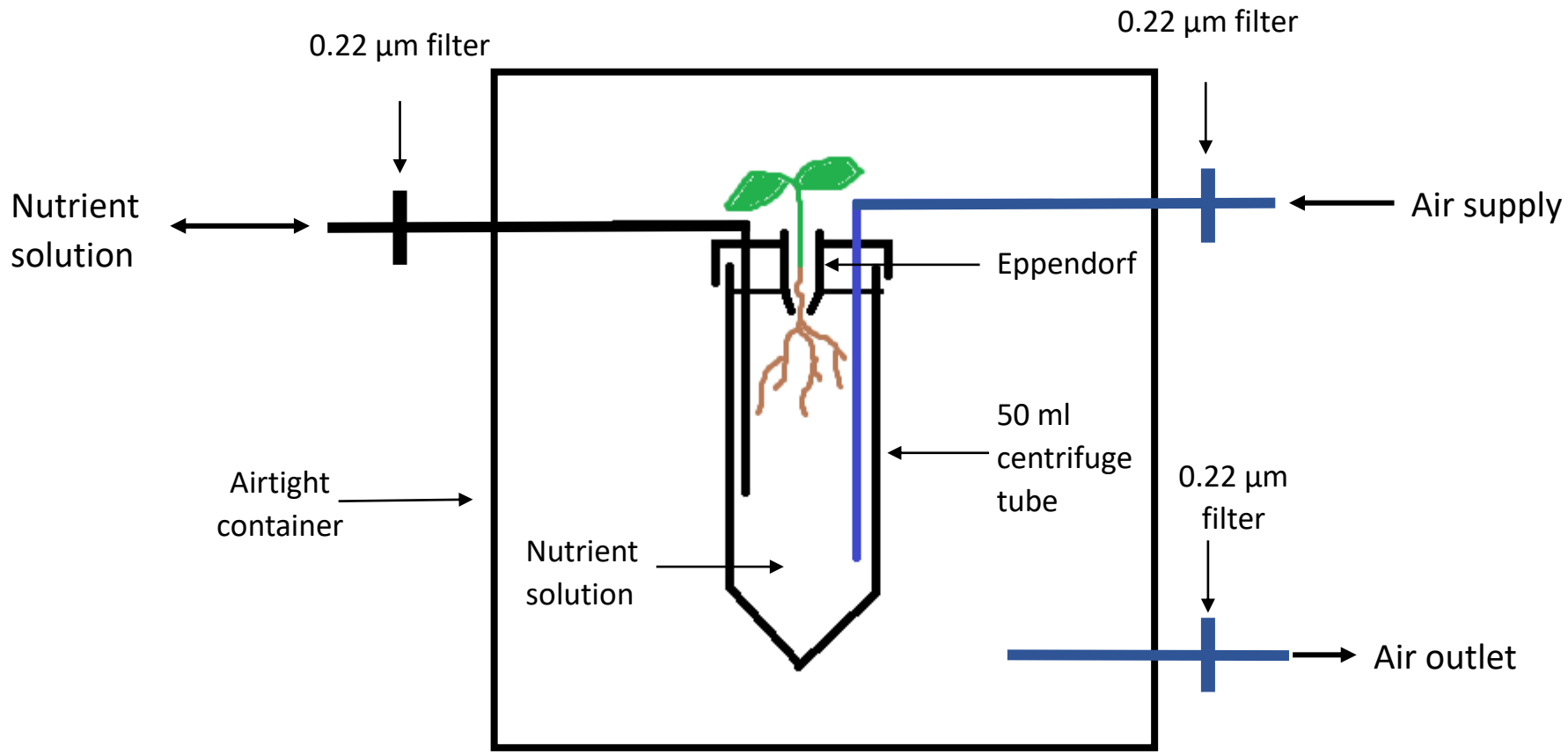

Figure S1. Diagram of the sterile hydroponics setup used for the experiments.

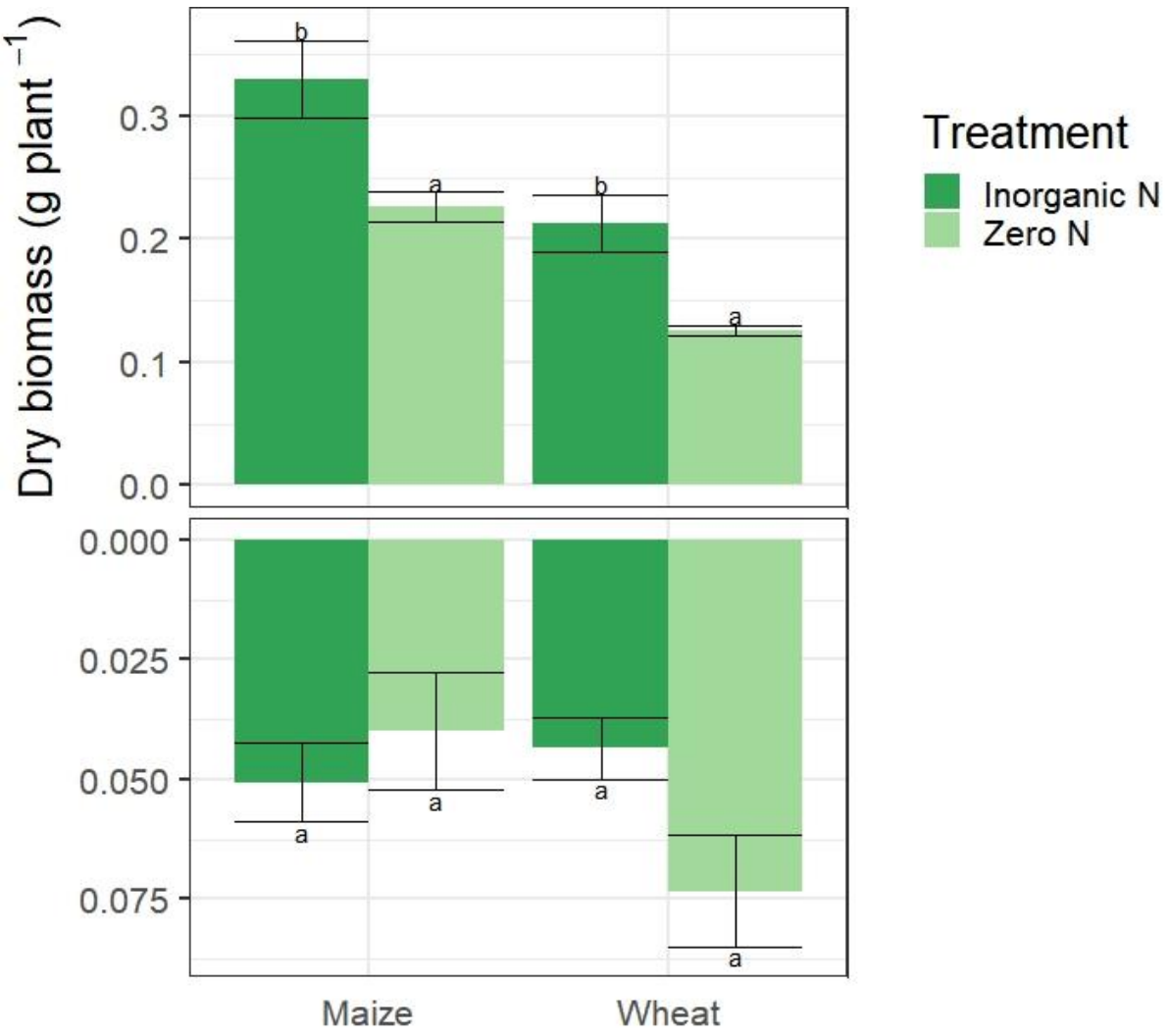

Figure S2. Shoot (upper graph) and root (lower graph) dry biomass of maize and wheat under different N treatments after two weeks of growth. Different letters represent significant difference between N treatments for each plant ($p < 0.05$). Values represent mean ± SEM ($n = 4$).

Appendix 4

Supplementary material for Chapter 6

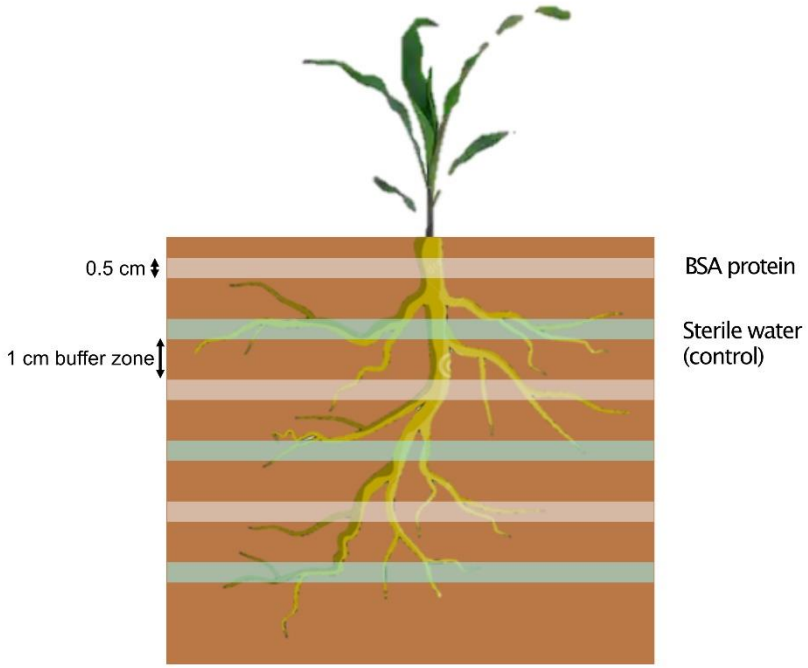

Figure S1. Rhizobox set-up showing the position of the protein and control (distilled water) horizontal bands in soil.

Appendix 5

Supplementary material for Chapter 7

Table S1. Search term strategy for Web of Science. Search term selected is shaded.

Search term	Results	Comments
TS = (protease activity soil)	1667	707 in field of microbiology
TS = (protease activity AND soil OR aminopeptidase activity AND soil)	1856	746 in field of microbiology
TS = (soil? activity protease OR *peptidase OR proteinase)	115392	Too many and unrelated fields
TS = (soil activity protease OR *peptidase OR proteinase)	116351	Too many and unrelated fields
TI = (soil AND activity AND protease OR *peptidase OR proteinase)	39137	Too many and no soil topics
TI = (soil?) AND TS= (soil AND activit* AND protease OR *peptidase? OR proteinas?)	173	Too few
TI = (soil?) AND TS= (soil AND protease OR *peptidase? OR proteinas? activit*)	186	Too few
TS = (protease activity soil NOT bacteria?)	1294	257 in biotechnology applied microbiology
TI = (soil?) AND TS= (soil AND activit* AND protease OR *peptidase OR proteinase)	197	Too few
TI = (soil?) AND TS= (soil AND activit* AND protease OR *peptidase OR proteinase OR enzyme assay)	397	Key authors not found
TS = ((protease? OR *peptidase? OR proteinase?) NEAR/5 soil AND activit*)	43	Too few
TS = ((protease? OR *peptidase? OR proteinase?) NEAR/10 soil AND activit*)	61	Too few
TS = (soil NEAR/5 (protease* OR peptidase* OR aminopeptidase* OR proteinase*))	431	119 in field of microbiology
TS = (soil NEAR/5 (protease* OR peptidase* OR aminopeptidase* OR proteinase*) AND (activit* OR assay?))	384	Key authors not found
TS = (soil NEAR/10 (protease* OR peptidase* OR aminopeptidase* OR proteinase*) AND (activit* OR assay? OR "enzyme assay"))	668	Stursova NOT Saiya-cork, Marx, Vapsalainen and Nannipieri

TS = (soil NEAR/10 (protease* OR peptidase* OR aminopeptidase* OR proteinase*) AND (activit* OR assay? OR "enzyme assay" OR "enzyme activity"))	668	Stursova NOT Saiya-cork, Marx, Vapsalainen and Nannipieri
TS = (soil NEAR/5 (protease* OR peptidase* OR aminopeptidase* OR proteinase*) AND soil NEAR/10 (activit* OR assay? OR "enzyme assay" OR "enzyme activity"))	307	Key authors not found
TS = (soil NEAR/10 (protease* OR peptidase* OR aminopeptidase* OR proteinase* OR activit* OR assay? OR enzyme?))	32490	Too many and unrelated fields
TS = (soil* NEAR/10 (protease* OR peptidase* OR aminopeptidase* OR proteinase* OR proteolytic OR assay? OR enzyme?))	4,756	Includes Marx, Nannipieri, Vepsalainen, Stursova NOT Saiya-cork
TS = (soil* NEAR/10 (protease* OR peptidase* OR aminopeptidase* OR proteinase* OR proteolytic OR assay? OR enzyme? OR "enzyme activity"))	5,918	Includes Saiya-Cork, Marx and Nannipieri, Stursova and Vepsalainen

Table S2. List of search terms used to search in ScienceDirect including common synonyms and acronyms.

Search term	Results	Journals selected
soil AMC	209	Soil Biology & Biochemistry, Applied Soil Ecology, Science of the Total Environment, CATENA, Geoderma, Agricultural Water Management
soil 7-Amino-4-Methylcoumarin	136	Soil Biology & Biochemistry, Geoderma, Applied Soil Ecology, Science of the Total Environment, Agriculture Ecosystems& Environment, Fungal Ecology
soil BAA-protease	92	Soil Biology & Biochemistry, Science of the Total Environment, Applied Soil Ecology, Geoderma, European Journal of Soil Biology, Soil and Tillage Research
soil N-benzoyl L-arginine amide	19	Soil Biology & Biochemistry, Science of the Total Environment, Methods in Enzymology
soil casein	394	Soil Biology & Biochemistry, Plant science, Journal of Plant Physiology
soil caseinate	63	Soil Biology & Biochemistry, Applied Soil Ecology, Geoderma, Agriculture Ecosystems & Environment
soil gelatin	126	Soil Biology & Biochemistry
soil p-nitroanilide	16	Soil Biology & Biochemistry
soil p-nitroaniline	27	Soil Biology & Biochemistry and Science of the Total Environment
soil hemoglobin/haemoglobin	154	Science of the Total Environment
soil benzyloxycarbonyl glycyl L-phenylalanine	2	Soil Biology & Biochemistry
soil benzyloxycarbonyl phenylalanyl leucine	9	Soil Biology & Biochemistry and Advances in Agronomy

Table S3. Selection criteria of studies for use in the systematic review.

Criteria	Included
Protease activity measured in a soil medium	✓
Protease activity was measured from soil aggregates	✗
Unobtainable studies	✗
Studies determining effect of assay condition on protease activity e.g. pH and temperature	✓
No control	✗

Table S4. Criteria for data exportation of soil protease activity for use in the meta-analysis.

Criteria
For multiple soil depths the maximum protease activity was recorded
Unable to convert into nmol product g^{-1} h^{-1}
For time series data the maximum activity was taken
Protease activity reported on a dry weight basis
Protease activity measurement taken from control
Protease activity measurement was collected for all soil types/location assessed in the study

Table S5. Common forms of substrates for AMC, z-phe-leu and pNA groups used for measuring protease activity in soil.

Substrate group	Common forms	No. of published studies using the substrate
AMC	Alanine-AMC	20
	Alanine-alanine-phenylalanine-AMC	5
	Arginine-AMC	5
	Glycine-AMC	1
	Leucine-AMC	278
	Lysine-alanine-AMC	3
	Proline-AMC	2
	Serine-AMC	2
	Succinyl-AMC	1
	Tyrosine-AMC	17
Z-phe-leu	Z-phenylalanine-leucine	24
	Z-glycine-leucine	1
	Z-phenylalanine-glycine	2
	Z-phenylalanine-tyrosine-leucine	4
pNA	Glycine-pNA	17
	Leucine-pNA	12

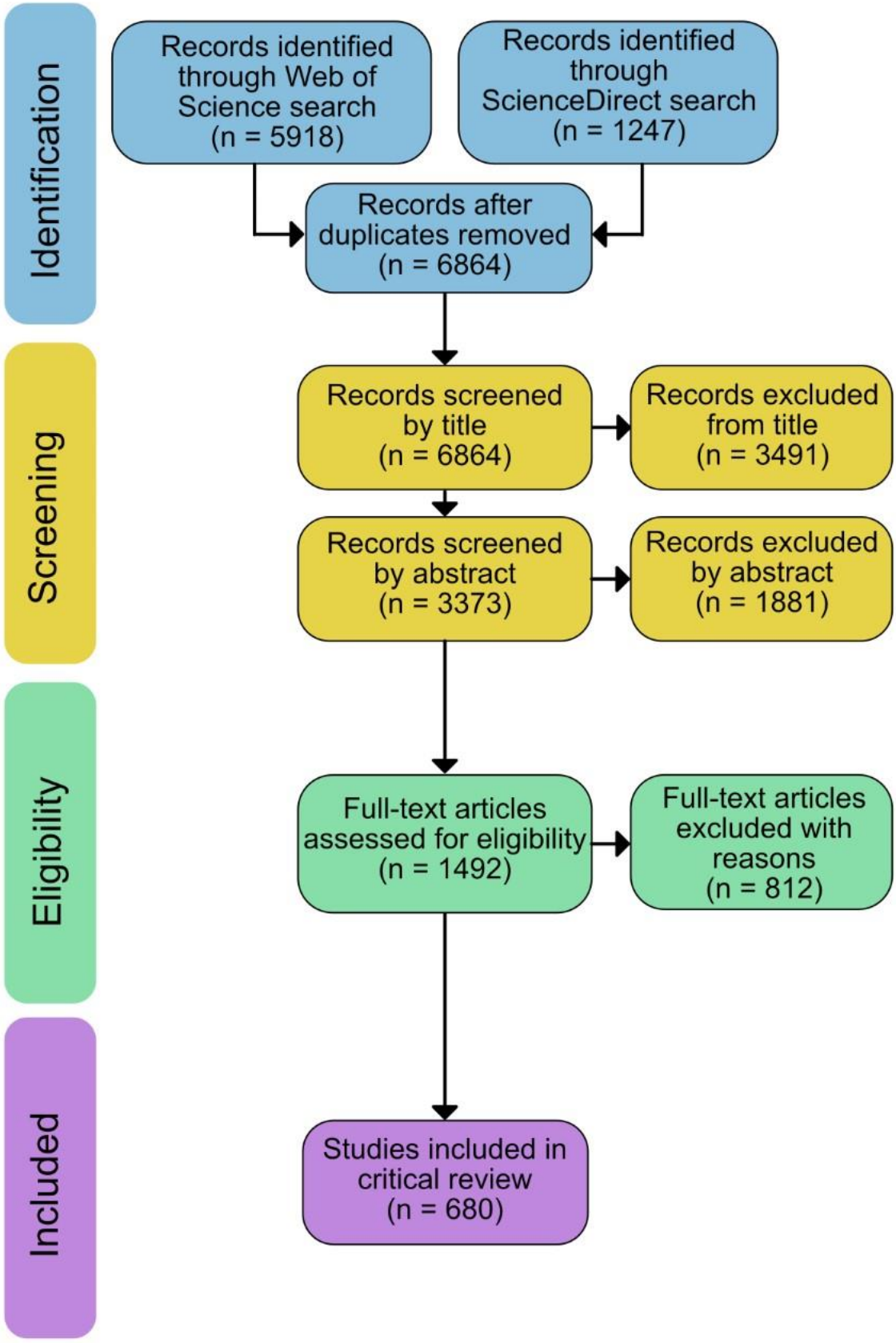

Figure S1. PRISMA diagram of the systematic review process.

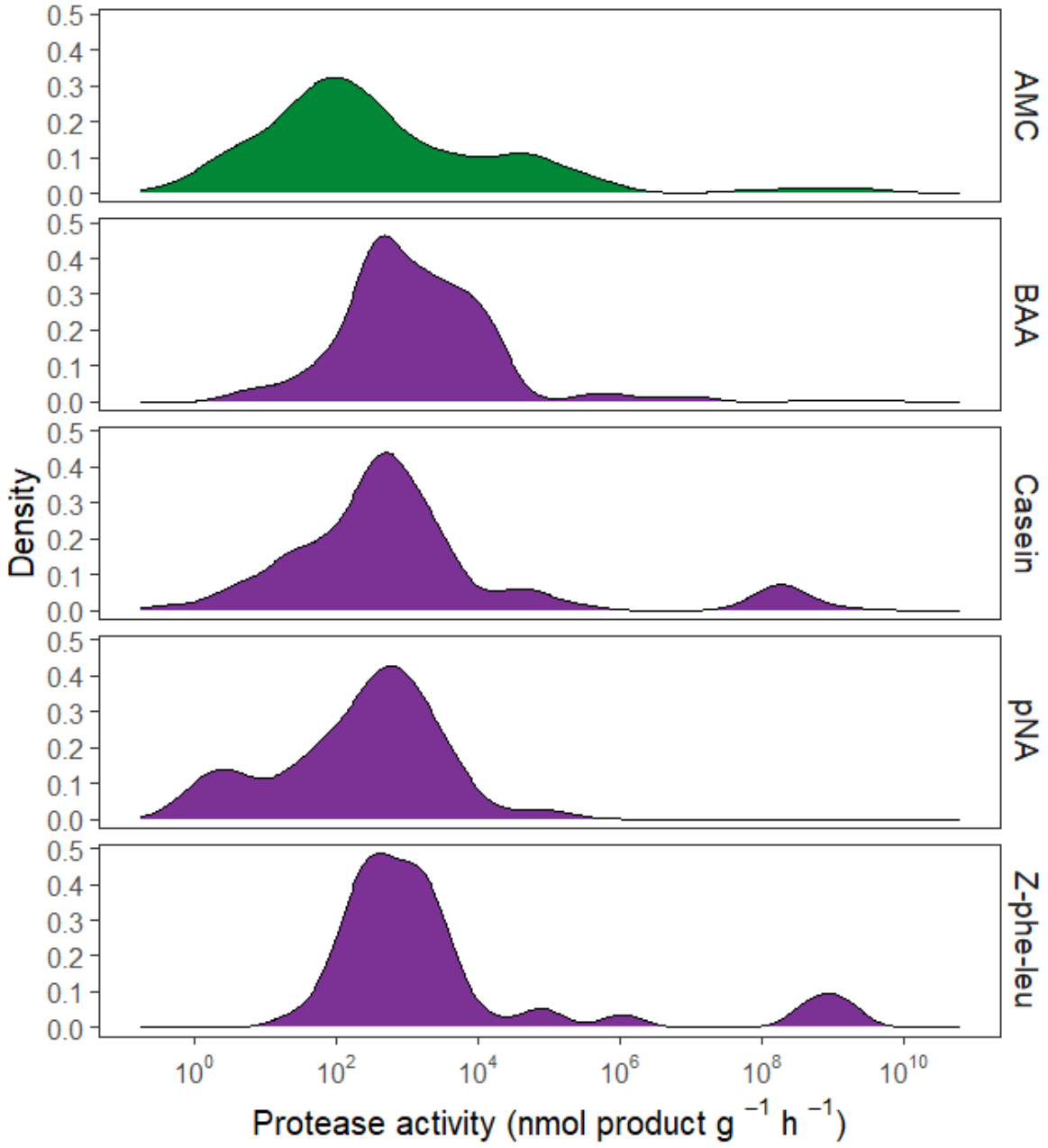

Figure S2. Density distribution of the mean soil protease activity (nmol product g^{-1} h^{-1}) on a log_{10} scale for 5 different protease substrates (colorimetric = purple and fluorimetric = green).

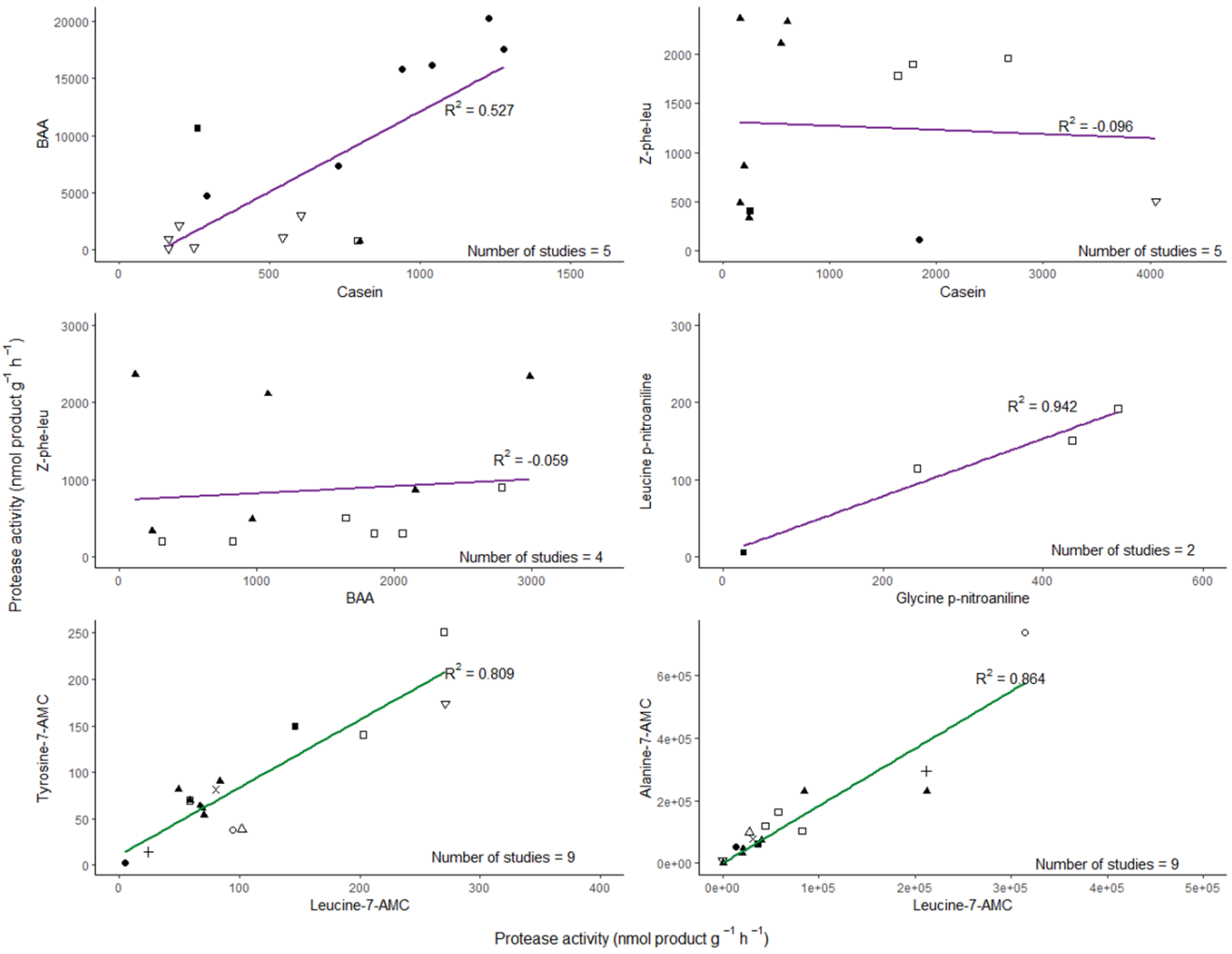

Figure S3. Correlations between mean protease activity (nmol product g^{-1} h^{-1}) obtained from studies that measured it with two different substrates. Points with the same shape represent mean protease activity measurements from the same study.

Supplementary Information 1

In order to estimate enzyme activity under field conditions, mean protease activity was converted to mean protease activity at the MAT (Protease activity$_{MAT}$) according to the soil geographical location using equation 1 and assuming a Q10 value of 2 (von Lützow and Kögel-Knabner, 2009).

$$k_1 = k_2 Q_{10}^{(\frac{t_1 - t_2}{10})} \quad (1)$$

Where k_1 is the is the rate at MAT, k_2 is the rate at the assay temperature, t_1 is the MAT, t_2 is the assay temperature and Q_{10} is the temperature coefficient. Next, the MAT-corrected protease activity was converted to the mean protease activity at the soil pH recorded by the study. Table S6 shows summary statistics of Protease activity$_{MAT}$.

Table S6. Summary statistics of protease activity$_{MAT}$ across 929 assays (*n* = 105, 79, 186, 14 and 11 studies for AMC, BAA, casein, pNA and z-phe-leu respectively).

	Mean	**Median**	**Minimum**	**Maximum**
Protease activity$_{MAT}$ (nmol product g^{-1} h^{-1})	3324	72	0.02	80,7342

References

von Lützow, M., Kögel-Knabner, I., 2009. Temperature sensitivity of soil organic matter decomposition-what do we know? Biology and Fertility of Soils 46, 1–15.