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

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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*.

Key words: Decomposition; Mineralisation; Nutrient cycling; Protease activity; Soil quality indicator.

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; Jan et al., 2009). 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. 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 $\leq$ 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. 1).

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 NH_4^+ excretion back into solution (Hill and Jones, 2019; Roberts et al., 2009). Some of the NH_4^+ excreted is subsequently nitrified to 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 (ammonium, nitrate, 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 Figure 1.

2. Materials and methods

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 1). Mean annual soil surface temperate 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; Jan et al., 2009). 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 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 two 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.

2.2.1. 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 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 neutralization. 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 (v/v) soil:H_2O extracts using standard electrodes.

2.2.2. 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 Analyzer (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.

2.2.3. Determination of physical soil properties

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

2.3. 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 (v/v) soil:H_2O) and 100 µl pipetted onto a 96 well plate. Subsequently, 100 µl of substrate (500 µM L-leucine 7-amido-4-methlycoumarin hydrochloride) was added to the sample. Standards were prepared for each sample by adding 100 µl of 7-amido-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.

2.4. 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 to 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® cutoff 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. 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 (Table S1).

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 (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).

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 Supplementary information 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 to 60 d from Figures 3 and 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.

All treatments were performed in triplicate. 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. 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. 1). Correlations were carried out using the Pearson's product moment correlation using the function rcorr in the Hmisc (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 Figure S7.

3. Results

3.1. Soil properties along the grassland altitudinal gradient

We observed trends in the major characteristics of the grassland altitudinal gradient (Fig. 2). Above-ground net primary productivity (NPP), pH and protein sorption both showed a negative

trend from the lowest to highest altitude site ($p < 0.0001$; Table S2). Soil pH had little difference between the topsoil and subsoil ($p = 0.12$; 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: $p < 0.0001$; 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 ($p < 0.0001$; Table S2) though the two depths were not significantly different ($p = 0.936$; 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: $p = 0.004$; 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 (Table S2). Only microbial biomass-C showed differences between soil depths ($p < 0.0001$; Table S2). N mineralisation increased along the first half of the gradient (sites 1-5) and varied between sites ($p = 0.15$; 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 ($p = 0.02$; Table S2). Overall, leucine aminopeptidase activity varied significantly along the altitudinal gradient ($p < 0.0001$; Table S2). However, there was no significant difference in leucine aminopeptidase activity with soil depth ($p = 0.41$; 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 analysis are presented in Figure S4.

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 Figures S5 and S6 respectively. A rapid linear phase of mineralisation was observed up until 3 h for protein and amino acids ($r^2 = 0.91$

± 0.01 and r^2 = 0.85 ± 0.01, respectively) (Fig. 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. 3). There was no trend in the subsoil, but sites varied significantly (p = 0.0001; Table 2). Overall, the initial rate was lower in the subsoil compared to the topsoil (p = 0.0001; Table 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 2; Fig. 4).

The initial phase of amino acid mineralisation doubled in rate along the altitudinal gradient but halved in the subsoil (Fig. 3). However, between sites 1 and 10 the initial rate varied significantly (p < 0.0001; Table 2). The initial rate varied at each depth and was not significant (p = 0.24; Table 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 2). The differences between the second rate of amino acid mineralisation and soil depth were not significant (p = 0.45, Table 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; Fig. S3). There was little difference in CUE between the topsoil and subsoil (Two-way ANOVA: $F_{(1,39)}$ = 0.2, p = 0.66 respectively; 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; 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; Fig. S1). Overall, the sorption of protein was 2.2-fold greater than for amino acids ($p < 0.001$).

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. 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.

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. Discussion

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 3-100 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, in our study we 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 communites 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 is 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 are 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., 2018; 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., 2018).

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 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 ammonium, nitrate, 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 have not measured are also influencing 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 well 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, ammonium 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, 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.

4.3. 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 to 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), this focus should be on net belowground productivity.

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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Table 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

Table 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

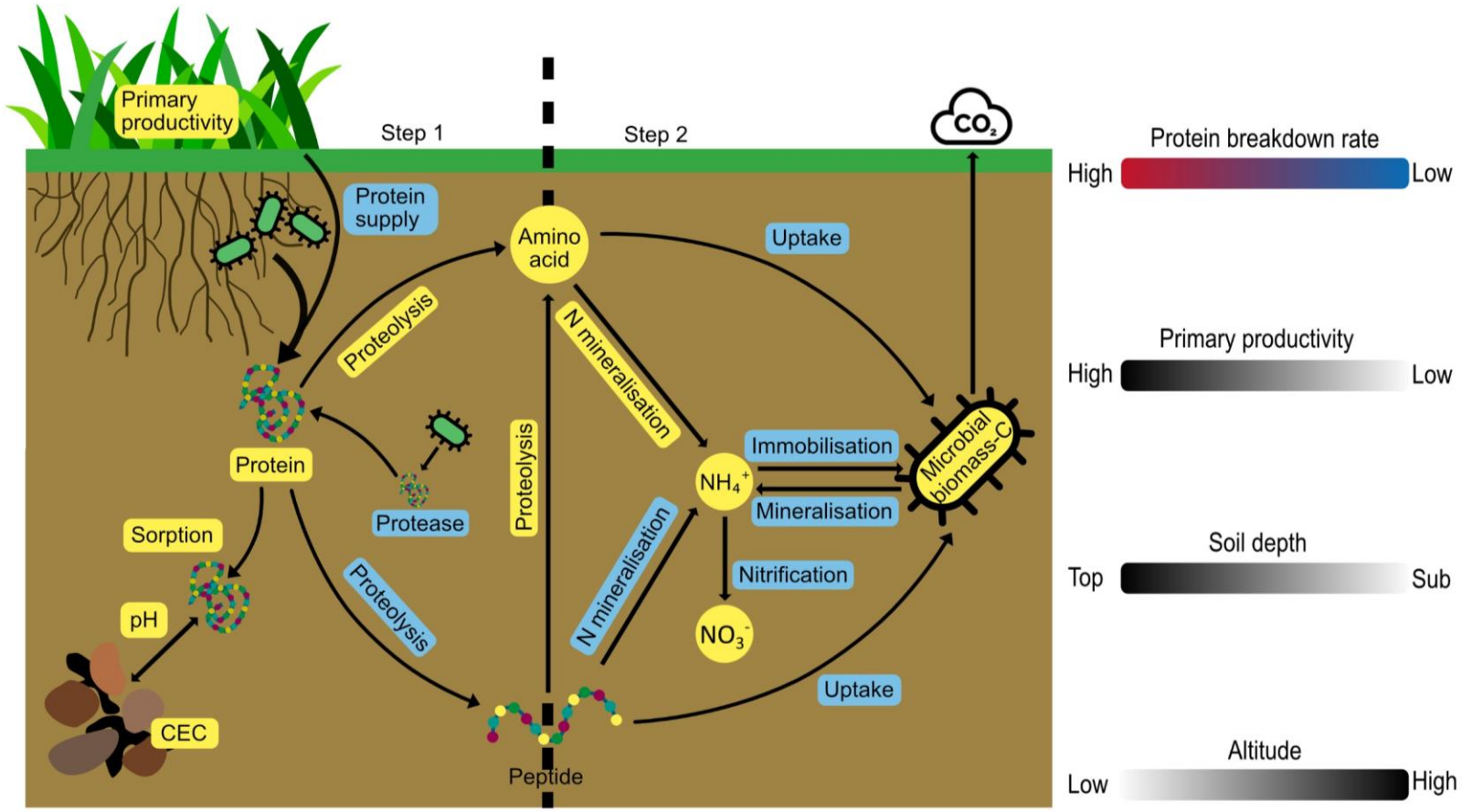

Fig. 1. Schematic diagram showing the main soil properties and processes regulating the microbially-mediated mineralisation of protein in soil. Step 1 represents the depolymerisation of protein to peptides and amino acids by proteolysis, catalysed by extracellular protease enzymes, and step 2 represents 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.

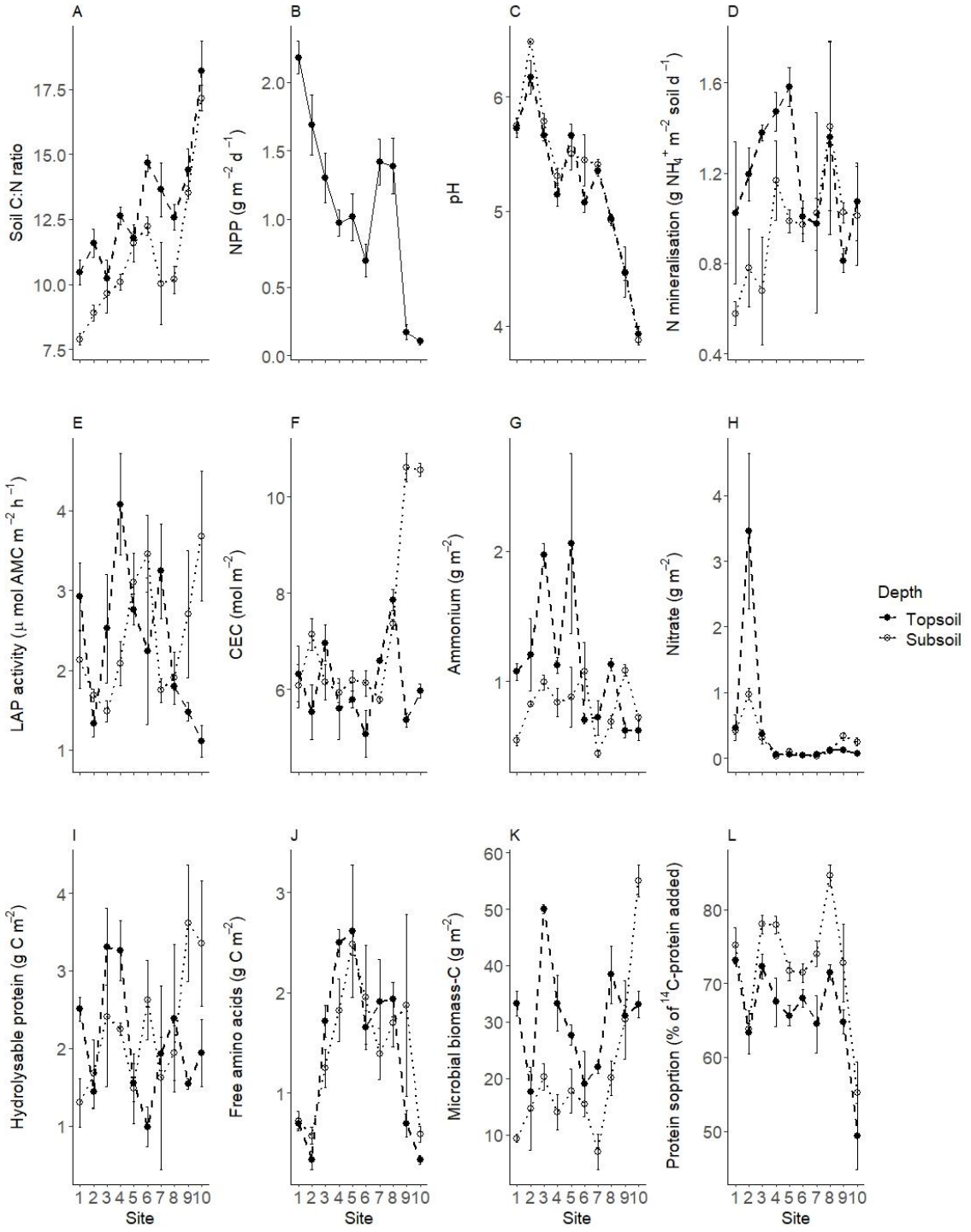

Fig. 2. Major characteristics of the grassland altitudinal catena sequence. A) soil C:N ratio, B) net primary productivity (NPP) (g m^{-2} d^{-1}), C) soil pH, D) N mineralisation (g NH_4^+ m^{-2} soil d^{-1}), E) leucine aminopeptidase activity (LAP) (μmol AMC m^{-2} h^{-1}), F) cation exchange capacity (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).

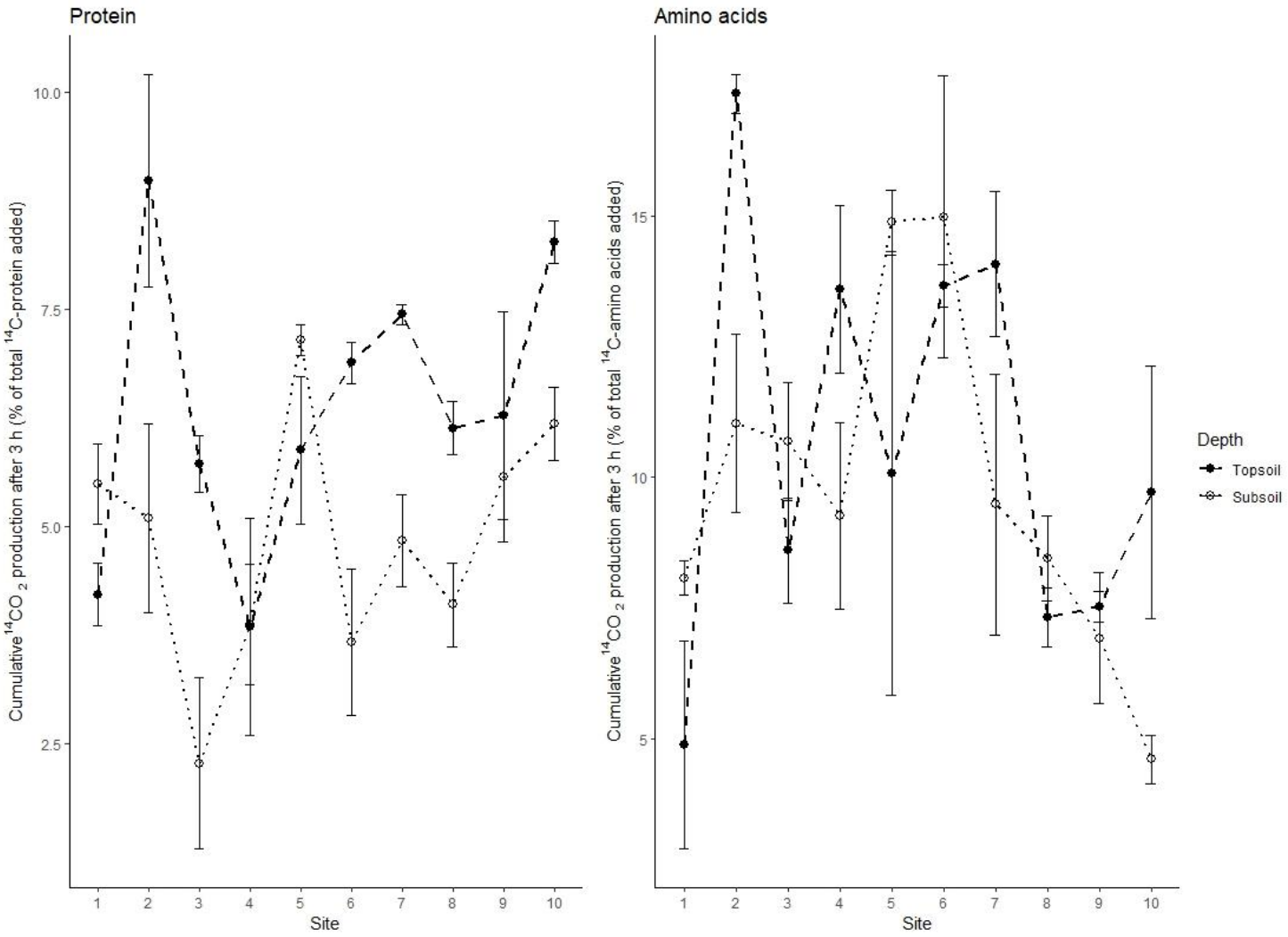

Fig. 3. Cumulative $^{14}CO_2$ production arising from the mineralisation of ^{14}C-labelled protein (left) and amino acids (right) measured between 0 and 3 h (initial phase) for ten sites along the grassland altitudinal gradient in the topsoil and subsoil (expressed as a % of total ^{14}C-substrate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

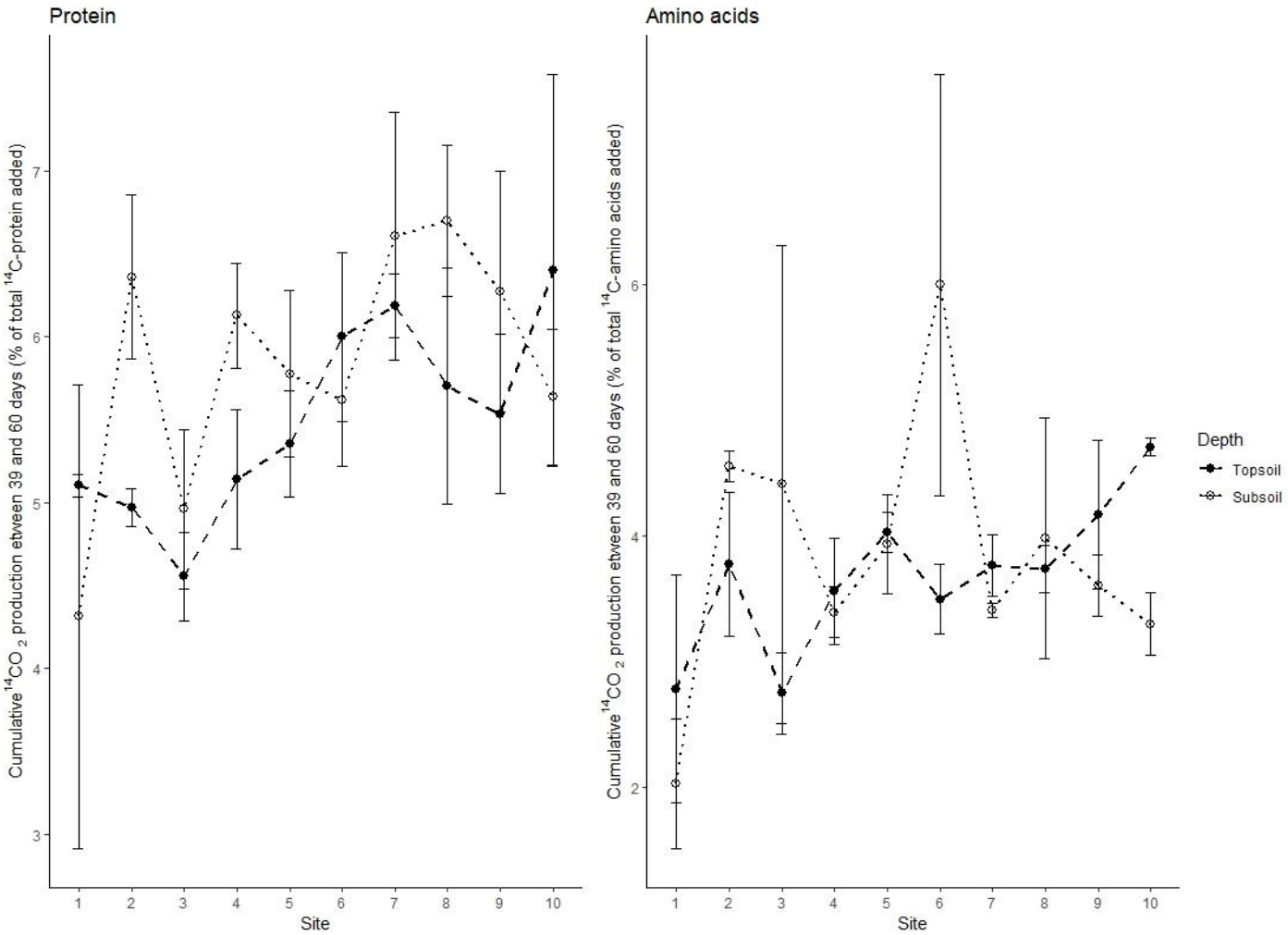

Fig. 4. Cumulative $^{14}CO_2$ production arising from the mineralisation of ^{14}C-labelled protein (left) and amino acids (right) measured between 39 and 60 d (second, slower phase) for ten sites along the grassland altitudinal gradient in the topsoil and subsoil (expressed as a % of total ^{14}C-substrate added). Values represent mean ± SEM (n = 3).

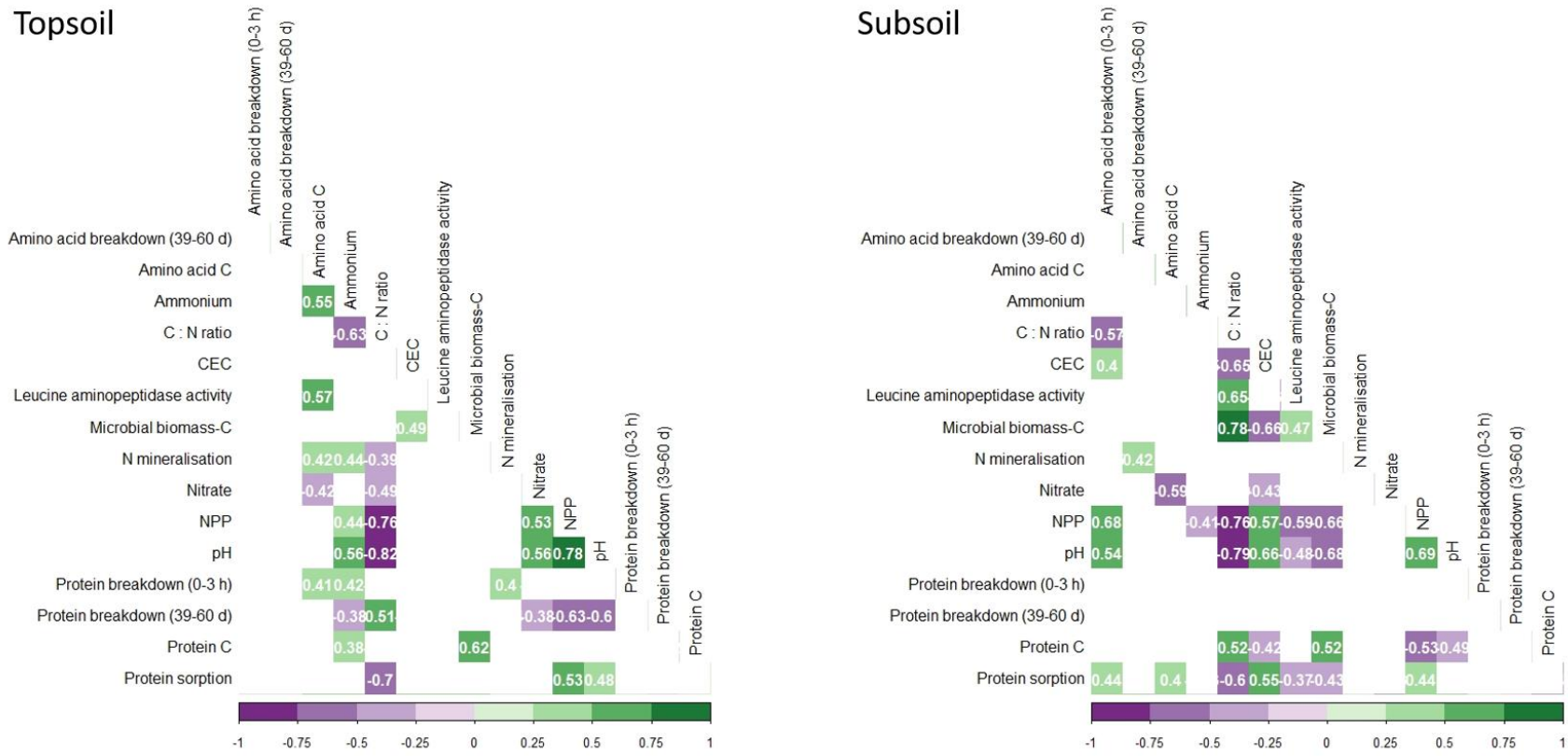

Fig. 5. Correlation matrix of soil properties and protein mineralisation rates with significance of $p < 0.05$ in the topsoil (left) and subsoil (right). No corrections were made for the *p* values to account for multiple comparisons (see Fig. S7 for *p* values). Values and colour of the squares represent correlation coefficients.