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Transformation of peptides and amino acids in soil and plants

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Philosophiae Doctor

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Abbreviations

AMPs	Antimicrobial peptides
ANOVA	Analysis of variance
AOA	Ammonia oxidising archaea
AOB	Ammonia oxidising bacteria
ATP	Adenosine triphosphate
C	Carbon
$CaCl_2$	Calcium chloride
CO_2	Carbon dioxide
d	Day(s)
DIC	Dissolved inorganic carbon
DIN	Dissolved inorganic nitrogen
DNA	Deoxyribonucleic acid
DOC	Dissolved organic carbon
DON	Dissolved organic nitrogen
EC	Electrical conductivity
EON	Extractable organic nitrogen
g	Gravitational acceleration
H_2O	Water
ha^{-1}	Per hectares(s)
HCl	Hydrochloric acid
HMW	High molecular weight

KBq	Kilobecquerel
KCl	Potassium chloride
KNO_3	Potassium nitrate
L	Litre(s)
LMW	Low molecular weight
LSD	Least significant differences
M	Molar
mg	Milligrams
ml	Millilitre(s)
mM	Millimolar
mm	Millimetre(s)
µL	Microliter
µM	Micromolar
µmol	Micromole
mRNA	Messenger ribonucleic acid
MPa	Matric potential values
N	Nitrogen
N_2O	Nitrous oxide
NaOH	Sodium hydroxide
NH_2OH	Hydroxyl amine
NH_3	Ammonia
NH_4^+	Ammonium

NH_4Cl	Ammonium chloride
NO	Nitric Oxide
NO_2	Nitrogen dioxide
NO_3^-	Nitrate
NRPSs	Non-ribosomal peptide synthesises
O_2	Oxygen
OH	Hydroxide
OPT	Oligopeptide transporter
P	Phosphorous
PAR	Photosynthetically active radiation
PMF	Proton motive force
PTR	Peptide transporter
RLD	Root length density
RNA	Ribonucleic acid
S	Sulphur
SEM	Standard error of the mean
SOM	Soil organic matter
SON	Soil organic nitrogen
TDN	Total dissolved nitrogen
TFAA	Total free amino acids
Tg N/yr	Teragram of nitrogen per year
TOC	Total organic carbon

TON	Total organic nitrogen
v/v	Volume to volume
w/v	Weight to volume
WFPS	Water filled pore space

Abstract

Nitrogen (N) is an essential nutrient for plant growth and therefore a detailed mechanistic understanding of soil N cycling and plant N uptake is necessary to ensure sustainable food production. In addition, N availability can have significant effects on vegetation community structure and therefore climate-change induced shifts in N availability could affect ecosystem functioning and climate change feedbacks. The potential importance of organic N for plant nutrition has only recently been realised, and knowledge of its cycling and the competition for this resource in plant-soil systems is lacking.

The first aim of this PhD thesis was to investigate the potential for plants to compete with soil microbes for N at an early stage in protein breakdown, rather than having to rely on the breakdown of proteins to amino acids and subsequent mineralization to NH_4^+/NO_3^-. The second aim of this thesis was to study N cycling processes in subsoil environments and their potential importance in plant N acquisition. The final aim was to examine how increased N inputs affected C and N cycling in Arctic tundra ecosystems, in order to gain a better understanding of potential climate change impacts on this vulnerable area.

Experimentation with isotopically labelled N forms revealed differential mobility of the different N forms in soil. Firstly, NO_3^- proved extremely mobile in comparison to NH_4^+, which was retained both on the exchange phase and rapidly captured by soil microorganisms. High molecular weight (HMW) dissolved organic nitrogen (DON) showed greater potential for leaching than its low molecular weight (LMW) counterpart, which was also rapidly assimilated by soil microbes. Soil microbes showed a preference for peptide-N over other N forms, whilst wheat plants showed a greater affinity for NH_4^+ when grown under sterile conditions. When in competition with microbes, wheat mainly took up NO_3^-, with the capture of alanine and peptides being comparatively low. The presence of peptide-N decreased the ability of wheat to use NH_4^+, whilst the presence of other N forms depressed wheat's use of peptide-N. Studies of arable topsoil and subsoil revealed that C limitation was limiting microbial processes at depth, which was likely caused in part by low root length density and low rates of C input. This caused slower C and N mineralization processes at depth. Deep soils therefore appear unlikely to be a major N source for plants, but this could be stimulated by a labile C input. Surface moisture limitation appeared unlikely to cause a reliance on subsoil N, as there was no significant difference in the change in soil water content with depth during drought conditions. Increased organic N inputs to the Arctic significantly affected plant composition and increased plant litter degradation rates. This has implications for the Arctic's ability to store C and suggests a positive feedback to climate change.

In conclusion, this thesis has shown that DON inputs are highly important for plant and microbial nutrition, and that DON can influence C degradation in the Arctic. Subsoil could potentially contribute to plant nutrition, but low inputs of organic material limits microbial activity, so wheat's main N supply appears to come from surface horizons where N cycling is greatest. Plants can readily uptake peptides, although the results suggest that agricultural plants may struggle to compete with microbes for this resource. Furthermore, when added in combination with other reduced forms of N, the uptake of both peptide and the other N forms decreases.

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Microbial activity differentially regulates the vertical mobility of nitrogen compounds in soil. Published in Soil Biology and Biochemistry

Nutritional competition between plants and soil microorganisms for different nitrogen forms as influenced by peptides and vice versa. Unpublished

Nitrogen availability down the soil profile in a wheat cropping system: Relationships with root density, soil N reserves and microbial activity. Unpublished

Long-term nutrient enrichment drives soil and vegetation community development in Arctic habitats. Unpublished

Chapter 1

Overview of thesis

1.1. General introduction

In biological terms nitrogen (N) occupies a unique position among the periodic table. It represents an essential element for plant growth and is required in large amounts by most agricultural crops. It is also a primary regulator of plant productivity in most terrestrial ecosystems (Paul and Clark, 1989). Due to the economic importance of food production, N cycling in agricultural ecosystems has been extensively studied over the last century. In recent years, understanding the impacts of anthropogenically-mediated pollution (e.g. acid rain) and climate change has led to a renewed interest in understanding N cycling in natural and semi-natural ecosystems. For example, climate-induced changes in growing season length, temperature and CO_2 have led to changes in soil N availability, and in some ecosystems are thought to be responsible for shifts in vegetation community structure and productivity leading to changes in ecosystem functioning (Hill et al., 2011a). Despite the vast number of research papers on N in the plant-soil system, the cycling and competition for N resources in terrestrial ecosystem remains poorly understood. This is exemplified by the progressive shift in the N paradigm over the last 20 years from a focus solely on inorganic N (i.e. NO_3^- and NH_4^+) uptake to one which now includes various forms of dissolved organic nitrogen (DON) (Schimel and Bennett , 2004).

The soil N cycle is considered to be an integral part of the overall terrestrial N cycle. Therefore the subject of soil N is broad and deals not only with the distribution and transformation of organic and inorganic forms of N in soil but also the

interrelationships which exist between the atmosphere, hydrosphere and soil. N can enter soil from wet and dry deposition (i.e. NH_4^+ and NO_3^-), fertilisers (i.e. DON, NH_4^+ and NO_3^-), animal residues (dissolved and solid N), through N-fixation (i.e. N_2), with losses mainly occurring via crop removal, leaching (mainly DON and NO_3^-), and volatilization (i.e. NH_3). The initial conversion to NH_4^+ is referred to as ammonification whilst the subsequent oxidation of this compound to NO_3^- is termed nitrification. The utilization of NH_4^+ and NO_3^- by plants and microorganisms constitutes assimilation and immobilization, respectively. Combined N is ultimately returned to the atmosphere through biological denitrification (i.e. N_2O, NO, N_2) or via NH_3 volatilization, thereby completing the cycle.

With respect to insoluble organic N in soil (SON), vast reserves exist in soil organic matter (SOM) and associated with mineral material, often exceeding 4,000 kg ha^{-1} within the surface (0-30 cm) horizons (Tan, 1998). Typically, 95% of the N held in the surface layer of soil is organically combined. The importance of this organic N reservoir in supplying NO_3^- and NH_4^+ from the standpoint of soil fertility has long been recognized, and our knowledge concerning the nature and chemical composition of organic N is extensive (Jones et al., 2004). Recent evidence, however, has suggested that plants may be able to acquire low molecular weight (MW) organic N directly from soil in the form of amino acids circumnavigating the need to wait for mineralization to NH_4^+ and NO_3^- (Chapin et al., 1993; Jones et al., 2005). Furthermore, it has been shown that different plant species within a community may have contrasting preferences for organic and inorganic N suggesting that the relative abundance of DON and dissolved inorganic N (DIN) may be important in shaping plant communities (Näsholm et al., 2000; McKane et al., 2002). The recognition that plants can take up DON is not new

(McKee, 1962) but its ecological significance has only just been realised recently (Bardgett et al., 2003). From a soil perspective we can assume that root turnover and shoot residue incorporation represents the major source of organic matter entering soil and start of the N breakdown pathway. Therefore the dominant form of N entering soil is protein and peptides and to a lesser extent free amino acids (the latter typically being 100-fold lower in abundance). While the multiple routes involved in protein breakdown are well known from a biochemical perspective, they have been surprisingly few mechanistic studies on protein, and particularly peptide, behaviour in soil (Paul and Clark, 1989; Jones et al., 2005a). The overarching aim of this PhD is to challenge the conventional paradigm of N cycling which views amino acids and DIN as the main forms of N available for plant and microbial assimilation. Specifically, I wish to test the assertion that competition between plants and microbes primarily occurs at a higher point in the N cycle (i.e. at the peptide level). For this reason a large proportion of the literature review will be dedicated to peptide form and functioning in biological systems.

1.2. Aims and objectives

The thesis objectives are:

1- To test the the influence of microbial activity and chemical form on the potential for organic and inorganic N to move vertically in soil.

2- To characterize rates of microbial uptake and mineralization of amino acids and peptides in soils in relation to rates of inorganic N cycling.

3- To determine the influence of peptide addition on the uptake of inorganic forms of N and vice versa.

4- To determine how much N can be taken up as peptides by plants grown in soil

5- To determine what forms of N microorganisms prefer.

6- To determine the utilization of protein-N by plants in comparison to other N forms.

7- To investigate microbial N cycling in relationship to root growth in wheat cropping soils.

1.3. Plan of the thesis

Chapter 1: Introduction

This will be an introduction to the subject area and will detail the Aims and Objectives of the thesis.

Chapter 2: Literature review

This is a critical review of the current evidence surrounding N cycling in soil. In addition it will evaluate the different N uptake mechanisms by microorganisms.

Chapter 3: Microbial activity differentially regulates the vertical mobility of N compounds in soil

The aim of this laboratory study was to focus on the influence of microbial activity and N form on the potential for organic and inorganic N to be vertically transported down the soil profile.

Chapter 4: Nutritional competition between plants and soil microorganisms for different forms of N as influenced by peptides and vice versa

Information on the interactive effects of peptides, amino acids and inorganic N on plant and microbial competition for N the rhizosphere is not available. To understand the preference for available forms of N, it is imperative to study the interactions of various N forms. To follow the dynamics of N uptake, I used stable- (^{15}N, ^{13}C) and radio-isotope (^{14}C) labelled peptides. To eliminate the influence of microorganisms, sterile plants were used for part of the study. This study aimed to answer the following questions? 1. Can peptides be taken up intact by plants growing under sterile conditions? 2. How much N can be taken up as peptides by plants grown in soil in comparison to those grown under sterile conditions? 3. What forms of N do microorganisms prefer? 4. What is the effect of the availability of different N forms on peptide uptake, and similarly the effect of peptides on the uptake of alternative N forms?

Chapter 5: The ability of sterile plant to take up protein

The aim of this study was to confirm whether plant could use forms of N above peptides in the soil N cycle.

Chapter 6: Evaluation of soil N turnover in relationship to root growth in a wheat cropping system

Most work on plant N uptake is performed on seedlings. This chapter aimed to look at the distribution of roots down the soil profile in a mature wheat crop and to link this to the forms and turnover of N forms down the soil profile.

Chapter 7: Long-term nutrient enrichment drives soil and vegetation community development in Arctic habitats

This chapter presents laboratory experiments investigating plant and microbial N availability/use along a long-term N deposition gradient in the Arctic. It will use radiotracers as well as a range of analytical techniques and will focus on soils collected from Svalbard. The aim of this study was to investigate the potential feedbacks between soil development, plant community nutrient content and herbivore grazing along two contrasting nutrient enrichment gradients in the high Arctic.

Chapter 8: Discussion, conclusions and future work

This represents a general discussion of all the results presented in the experimental chapters, highlighting the key conclusions and identifying areas for further work.

Chapter 2

2.1. Nitrogen

The Earth's atmosphere contains 78.1% N_2 by volume, but although it is present in large amounts, it is biologically inert unless combined with other elements (e.g. C, H, or O). N is fundamental to life, as it represents the building blocks of nucleic acids (e.g. DNA, RNA), cell structural elements (e.g. tubulin, actin) and all enzymes. In most cells, N is present in amino acids, peptides and proteins, and is probably the most important biochemical component of cells (by weight) after carbohydrate moieties (Brady, 1984; Paul & Clark, 1989).

In soil, N is predominantly present in four forms:

- Dissolved organic N (DON) and NH_4^+, which are fixed or held on exchange surfaces (e.g. clays);
- DON, NH_4^+ and NO_3^-, which are free in solution;
- Organic N held in the necromass (i.e. dead organic matter); and
- Organic N held in the microbial biomass (i.e. living organic matter).

Typically, exchangeable N accounts for 5% of the total N in soil, while dissolved N accounts for 1–2%, microbial N for 1–5% and necromass N for 90–95% of the total. Usually, the importance of N adsorbed to mineral surfaces, in terms of pool size, increases with depth in the soil profile as soil organic matter (SOM) decreases. Amino acids in a free or combined state (i.e. peptides and protein) usually account for half of the organic N, with the remainder largely present as amino sugars and humic substances (Brady, 1984). Table 2.1 summarises the different forms of N and their percentage in soil in different environments. In addition to solid forms, soil N also occurs in soluble

form in soil solution (e.g. NO_3^-, NH_4^+, amino acids, peptides, urea, etc.). Varying fractions of these N compound pools are available for plants to take up.

N is an essential nutrient for plants (Rentsch et al., 2007), as it is a component of proteins, nucleic acids, chlorophyll, diverse secondary compounds and many cellular structures. In plants, N is the fourth most abundant element and plays an important role in all plant growth and development processes such as cell division, transport and all biochemical reactions requiring enzymes. The plant availability of N is therefore closely linked to plant productivity in both natural and agricultural ecosystems (Vitousek et al., 1997; Galloway et al., 2008).

The many forms of N interact differently with the soil environment due to their varying physical and chemical properties (Näsholm et al., 2009). This can influence the competition between microorganisms and plants for N. Symbiosis with mycorrhizae and other adaptations improve a plant's ability to take up the N it requires (Rentsch et al., 2007; Näsholm et al., 2009). In addition, recent discoveries have confirmed that certain plants are able to compete with soil microbes for organic N. Increased knowledge of plant N uptake could result in the production of more efficient plants in agronomic and forestry production systems. This knowledge could be important for reducing the economic losses and environmental pollution from inorganic fertiliser whilst sustaining crop yield (Jan et al., 2009; Ge et al., 2011). Knowledge of plant and microbial competition for N would also be useful for an improved understanding of the breakdown of SOM, which has ramifications for both plant nutrition and climate change (Gärdenäs et al., 2011). A schematic of the plant-soil N cycle is shown in Fig 2.1, which highlights the role of DON in the N cycle.

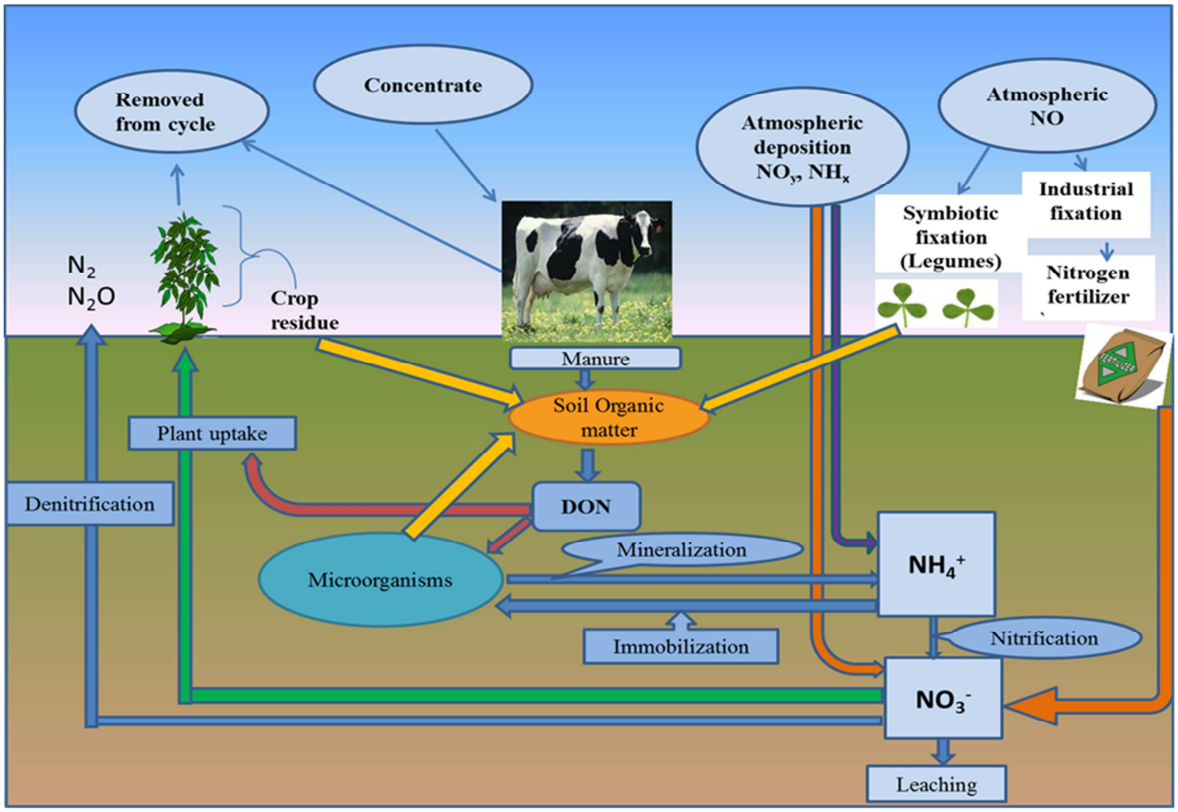

Fig. 2.1. Schematic representation of the terrestrial N cycle.

Table 2.1. Distribution of the N in representative surface of the world (Stevenson, 1982).

Table of Distribution of the N in representative surface soils of the world (from Stevenson,1982)							
Form of N,% Of total soil N							
Location	Acid insoluble	NH_3	Amino acid	Amino sugar	HUN	Amino acid N/Amino sugar N ratio	Reference
Africa							
Sierra Leone(8)	19.8-24.5	9.3-20.3	22.8-33.3	4.1-13.8	17.4-40.2	2.4-7.0	Amara & Stevenson+
Tanzania(4)	14.4-30.7	10.8-21.4	18.6-31.2	5.2-11.5	24.1-36.0	1.9-4.1	Singh et al.(1978)
Argentina							
Misc. group(4)	22.2-32.7	15.1-21.3	13.3-20.2	2.0-10.9	22.2-32.7	1.2-10.0	Rosell et al. (1978)
Canada							
Representative soils From nine soil orders							
Organic, LFH(6)	15.3±7.3	14.9±2.6	51.0±7.5	7.0±2.7	11.5avg.	7.3avg.	Sowden(1977)
Organic, TC(2)	17.3±1.6	14.4±5.7	52.0±2.3	6.4±0.5	9.2avg.	8.1avg.	Sowden(1977)
Mineral, Ah(14)	18.3±7.8	18.6±3.4	40.8±8.0	6.7±1.8	15.6avg.	6.1avg.	Sowden(1977)
Mineral, Ap (13)	13.4±4.1	21.6±3.2	37.6±4.8	6.7±1.3	20.7avg.	5.6avg.	Sowden(1977)
Mineral, Ae(5)	12.5±5.4	23.8±7.6	41.2±9.6	5.9±1.5	16.6avg.	7.0avg.	Sowden(1977)
Alberta Gray Wooded (12)	18.4-26.4	13.7-19.0	2.68-33.0	8.8-11.9	17.1-25.0	2.8-3.3	Khan & Sowden (1971)
Alberta Solonetizic (2)	24.8-25.4	20.7-21.2	34.6-35.8	4.8-5.2	12.9-13.9	6.7-7.5	Khan & Sowden (1971)
Quebec cultivated soils (20)	16.2-33.6	15.7-28.2	23.4-37.6	4.8-9.2	6.9-22.5	2.9-7.3	Kadirgamathaiyah&MacKenzie (1970)
Germany							
Misc. soils	15.3-18.3	26.4-32.2	22.1-31.2	3.8-5.0	20.3-29.1	4.9-8.2	Fleige & Baeumer (1969)
Japan							
Upland soils	9.0-28.0	12.0-24.0	28.0-38.0	5.0-11.0	19.0-29.0	--	Miki et al.(1966b)
Misc. group(3)	21.7-32.6	15.3-24.2	16.9-25.2	1,2-3.1	19.4-26.6	7.9-14.1	Kyuma et al.(1969)
Paddy soils(3)	11.4-16.8	22.3-22.8	38.8-43.3	7.5-8.8	20.3-23.4	4.4-5.7	Hayashi & Harada (1969)
United Kingdom							
Arable and Pasture(14)							
Forest(8)	14.0-34.0	16.0-29.0	20.0-39.0	4.0-12.0	13.0-34.0	5.0avg	Greenfield(1972)
	14.0-17.0	21.0-37.0	35.0-41.0	4.0-8.0	15.0-19.0	7.6avg	Greenfield(1972)
United States							
Illinois(4)	20.6-32.6	15.1-24.4	31.9-40.3	8.7-13.2	2.4-20.0	2.3-3.8	Amara & Stevenson
Iowa(20)	18.4-36.7	18.6-29.0	17.8-34.3	3.3-7.1	17.9-28.9	2.9-6.3	Keeney & Bremner (1964)
Nebraska(8)	18.0-22.0	18.0-28.0	31.0-46.0	5.0-9.0	6.0-17.0	4.2-8.0	Meints & Peterson (1977)
West Indies							
Volcanic(6)	7.6-14.0	19.5-34.3	37.2-49.6	5.6-7.6	7.6-15.3	5.9-7.0	Sowden et al.(1976)
Volcanic(4)	11.5-41.3	11.6-17.4	25.4-45.7	0.8-3.0	4.2-35.0	11.6-46.4	Dalal(1978a)
Nonvolcanic (3)	6.9-22.7	21.5-32.1	20.4-49.8	3.6-7.9	6.5-20.7	3.9-13.8	Dalal(1978a)

2.1.1. N fixation

Higher plants cannot directly using atmospheric N_2, however, they can harness the power of specific prokaryotic microorganisms which have the capacity to convert N_2 into compounds that can be assimilated either directly or indirectly by plants (LaRaue & Patterson, 1981). Essentially, inert N in the atmosphere may become usable to higher organisms by either of the two following processes:

- Spontaneous chemical reactions, such as those caused by lightning, forest fires, photochemical reactions, and volcanic activity. The N fixed in this way comprises about 10% of the total (Sprent et al., 1987); and
- Biological processes undertaken by microorganisms. These may be carried out non-symbiotically by free living bacteria or blue-green algae, or symbiotically by microorganisms in either a loose or structured relationship with a plant.

When N-fixing microorganisms have a close symbiotic relationship with a plant, the relationship is closely linked to photosynthesis, as this process supplies the C to drive fixation via the enzyme nitrogenase. The amount of N fixed in the soil by biological processes can be substantial, although it varies greatly according to plant and microbial species and can range from 0.1 to 100 kg N ha^{-1} y^{-1}. Usually, more N is fixed by symbiotic than by non-symbiotic organisms and the fixation rate tends to be repressed when N inputs from fertiliser or acid deposition are high (Lea & Azevedo, 2006; Tan, 2009). Soil N-fixing microorganisms are the main natural source of reactive N (Vitousek et al., 2013). Human activities currently contribute to half of N fixation (Fowler et al, 2013). N fixation and N deposition combined have increased since 1900 (155 Tg N/yr to 345 Tg N/yr in 2000), and according to a global distributed flow-path

model, are expected to increase still further (408–510 Tg N/yr) by 2050 (Bouwman et al., 2013).

2.1.2. N mineralization in soil

N mineralization is a process that continually replenishes the soluble N pool in soil and therefore directly supports plants. It represents a vitally important stage of the N cycle (Vitousek & Howarth, 1991). The term N mineralization pertains to the conversion of residues of organic matter derived from plants, animals or microorganisms to inorganic forms (i.e. NH_4^+ and NO_3^-), as shown in Fig 2.2. In soil, the process is largely driven by microbial activity with the aid of mesofauna which help to physically break up organic matter, thereby making it more available to the microbial community (Dessureault et al., 2010).

Heterotrophic microorganisms under aerobic conditions mostly control ammonification. Essentially, this process is driven by the microbes using the C in nitrogenous organic substances as an energy source, as most microbes in soil are C limited (Whitehead, 1995). When microorganisms consume substrates with a low C: N ratio, they often excrete the unwanted N into the soil as NH_4^+ (Clarholm, 1985). Organic N–containing materials (e.g. amino acids, proteins, amino sugars and urea) can also be converted outside the cell to NH_4^+ by enzymatic reactions (e.g. deaminases, urease). Various enzymes are involved in this because each N compound requires a special enzyme for its decomposition (Ladd & Jackson, 1982; Stevenson, 1982). For example, in proteins, the peptide bond is broken by proteases, but the cleavage of amino groups can involve transaminases and oxidases (Stevenson, 1982; Brady & Weil, 1996; Miller & Gardiner, 1998). Release of the amine group ($R\text{-}NH_3$) results in the release of

NH_3. This ammonia gas is in equilibrium with NH_4^+, as expressed in the following equation:

$$NH_3\,(g) + H_2O \leftrightarrow NH_4^+ + OH^-$$

The direction of this reaction is highly pH dependent, with volatilisation of NH_3 occurring when the soil pH rises above neutrality, as occurs in calcareous soils. In most acid soils, the reaction is largely driven in the direction of NH_4^+ formation (Vitousek & Howarth., 1991).

Plant N uptake from soil is directly affected by mineralization and nitrification (Yang et al, 2008), and therefore the intrinsic rate of N mineralization plays an important role in the environmental and economic utility of agricultural soils (Zebarth et al., 2009). Further, the mineralization process is also a major factor determining fertiliser N application rates and emissions of N_2O and NO_3^- leaching from soil (Jacinth et al., 2010). Recent increases in the price of inorganic fertilisers have made alternate N sources such as manure more appealing. Various authors have suggested that more work is required to study N mineralization rates in such N sources (Jones et al., 2005; Torbett & Watts, 2012).

A wide range of environmental and soil factors control the rate of organic N turnover in soil (Benbi & Richter, 2002). These include the C:N ratio of the soil (Dilly et al, 2003), soil temperature and moisture (Gilliam et al., 2001), aeration status and vegetation cover (Ross et al, 1996; Cote et al, 2000; Ross et al, 2004), agronomic regime and fertilisation (Gilliam et al., 2001). Tannins originating from leaf litter have also been suggested to decrease the rate of N mineralization via the inhibition of both the microbial community and extracellular enzymes (Torbett & Watts, 2012). Many studies from a diverse range of ecosystems have also provided evidence of temporal and

seasonal differences in mineralization (Pastor et al., 1984; Vitousek & Maston, 1985; Neil et al, 1997; Steltzer & Bowman, 1998). There also appear to be differences in mineralization between the growing season and the non-growing season, suggesting that the rhizosphere may exert a strong influence on SOM turnover (Van Wijnen et al., 1999; Groffman et al., 2001; Schimel et al., 2004; Zhu & Carreire, 2004). These studies also attribute increased net mineralization to the higher moisture and greater temperature during the growing season (Mladenoff, 1987; Uri et al., 2003). A few studies, however, have shown the opposite effect, with lower mineralization during the growing season than in the winter (Nadelhoffer et al., 1991; Schmidt et al, 1999). Very recently, Zhao (2010) demonstrated that N mineralization in grasslands plays a major role during the non-growing season, providing an N reservoir that will stimulate rapid plant growth in the upcoming spring.

Land use change can have an enormous effect on the net rate of N mineralization by altering both the biotic and a biotic propriety of a soil (Rhoades & Coleman, 1999; Mendham et al., 2004). For example, a recent study has shown that land use can affect the soil's total N content, pH and C: N ratio (Yang et al., 2008). Mineralization is also strongly influenced by precipitation, temperature and moisture (Zhang et al., 2008). Experimental warming across five contrasting ecosystems (tundra, grassland, forest, shrubland and cropland) has shown increased N mineralization by an average of 52.2% (Bai et al, 2013). This effect is suspected to be more pronounced in forests than grasslands, where it is thought that moisture limitation may be more important (Bai et al., 2013).

Some studies have indicated that in arctic regions, N mineralization in soil undergoes distinct seasonal patterns. Net N mineralization in arctic soils can be high,

low or even negative during the growing season depending upon the prevailing conditions (Giblin et al., 1991; Nadelhoffer et al., 1991; Jonasson et al., 1993; Hobbie & Chapin, 1996). When net mineralization is negative, this indicates the possibility of a strong microbial and plant sink for N (Schmidt et al., 1999). Another study in the arctic biome has shown that snow cover and freeze-thaw processes can greatly affect N mineralization (Schimel et al., 2004). At the start of snowfall in winter, together with low temperature, N mineralization is limited by microbial activity. However, when the snow is deep, this can increase soil temperature, thereby stimulating soil microbial activity and mineralization in the subnivean zone. The results of another study suggest that deeper snow affects microbial activity by increasing the nutrient pool and modifying the physiological characteristics of the microbe community during late winter (Buckeridge & Grogan, 2008). Such effects of cold on N mineralization have also been demonstrated in laboratory studies of different ecosystem (Schmidt et al., 1999; Cookson et al., 2002; Sulkava & Huhta, 2003).

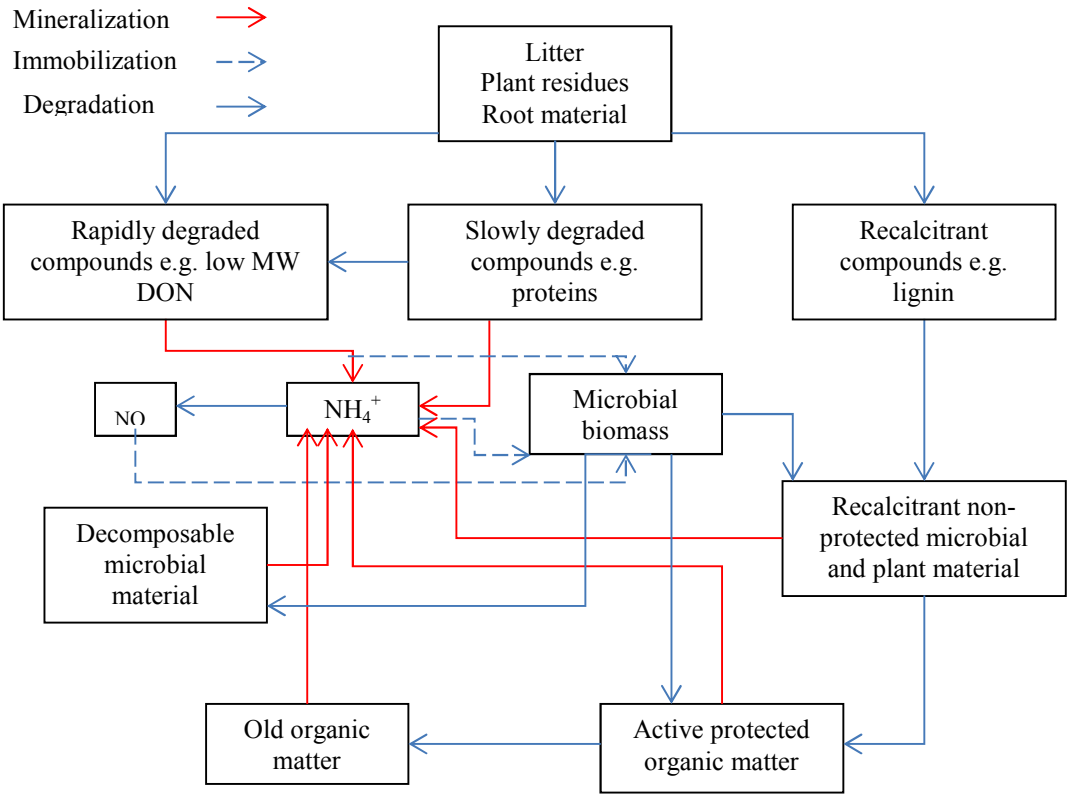

Fig.2.2. Soil N flow showing mineralization, immobilization and degradation. Adapted from Paul & Clark (1989)

2.1.3. N immobilization

N immobilization is the conversion of inorganic N into organic N by plants and microorganisms. Thus, the net balance between immobilization and mineralization largely governs the availability of soluble N in soil (Alexander & Wiley, 1961; Brady, 1984; Paul & Clark, 1989; Whitehead, 1995). Immobilization is essential in allowing mineralized N to be retained in an ecosystem, preventing its loss to fresh waters and back to the atmosphere. The effect of immobilization is not to increase or decrease the N content of soils, but rather to change its chemical form (Tan, 2009).

Conventionally, it is considered that immobilization directly and indirectly influences mineralization. Immobilization typically involves the incorporation of NH_4^+

into amino acids which are subsequently converted to other N forms within the organism. The rate of organism growth typically determines the rate of N immobilization, although some storage of N can occur in organisms even when they are not growing (e.g. to wait for a time when conditions become more favourable for growth, such as when C availability increases). Paul and Clark (1989) summarised the many possible fates of NH_4^+ in soil as follows:

- Plants can take up NH_4^+ from solution; however, the positive charge of NH_4^+ often leads to adsorption to the soil's solid phase and therefore reduced plant uptake. For this reason, plants take up NO_3^- and then reduce it to NH_4^+ within the plant (as NO_3^- is not sorbed to the soil's exchange phase and is therefore more plant available);
- NH_4^+ can be used by microbes for microbial growth. Much research has demonstrated that, in the reduced state, NH_4^+ is preferred over NO_3^- for incorporation into microbial metabolic pathways. Moreover, low levels of NH_4^+ can repress enzymes used in NO_3^- reduction;
- NH_4^+ can be held on cation exchange sites on clay particles and organic matter;
- As the NH_4^+ ion is roughly the same size as K^+, it can enter and become trapped in the interlayer of clays. The collapse of these layers, by drying, for instance, fixes the NH_4^+, making its removal difficult;
- The NH_4^+ can abiotically form R-NH_2 complexes by reacting with soil organic material (e.g. with humic substances);

- As NH_4^+ has a high partial pressure, when it is present and not adsorbed (e.g. in decomposing vegetation and manure), it may volatilise. Surface manure used as fertiliser can lose 50% of its N through volatilisation; and
- The NH_4^+ can be used as a source of energy by certain autotrophic organisms.

2.1.4. Controls on mineralization/immobilization

Both mineralization and immobilization are sensitive to changes in temperature and humidity, generally becoming slower in dry and/or cold conditions (Mikha et al., 2005). The balance between mineralization and immobilization can vary widely from soil to soil and from one time to another. The balance depends upon the availability, quality and quantity of decomposing organic matter (Wood & Hattey, 1995; Bruun et al., 2006). Mineralization and immobilization are highly correlated because each is related to microbial activity within the soil. A rise in the amount of inorganic N over time indicates net mineralization (Jones et al., 2004). However, a decrease in inorganic N does not necessarily indicate net immobilization. A decrease in inorganic N can be caused by plant uptake, denitrification, and volatilisation of ammonia and/or leaching. Similarly, a constant amount of inorganic N does not necessarily imply an absence of microbial activity. It could be that strong mineralization is balanced by immobilization by plants (Whitehead, 1995).

When temperature and moisture are high, there can be high rates of mineralization and immobilization. Here, one can distinguish between net and gross mineralization and immobilization. Gross N mineralization is the whole amount of soluble N created or produced by microorganisms, while gross N immobilization is the

amount of soluble N consumed. Net mineralization is the difference between gross mineralization and gross immobilization. The net balance between mineralization and immobilization is affected by the C: N ratio of the substrate and the organism. To promote net mineralization, it is important for the C: N ratio of the substrate to be lower than 30 and that the N is in a relatively labile form. When the C: N ratio is high (> 50:1), microbes can often easily obtain the C to derive energy, but there is insufficient N to support growth (Bruun et al., 2006). In such an event, there will be net immobilization (Sequi, 1989). Mineralization is predicted to increase with global temperatures, but immobilization is predicted to remain the same due to C limitation (Bai et al, 2013).

2.1.5. Nitrification

Nitrification converts NH_4^+ to NO_3^- via a two-step process undertaken by ammonia-oxidising bacteria (AOB). In the first step, NH_4^+ is oxidised to NO_2^- by the bacterium *Nitrosomonas*, whilst the second step sesoxidises NO_2^- to NO_3^- via the bacterium *Nitrobacter* as follows:

Step 1: $NH_4^+ + ½O_2 \rightarrow NH_2OH + H^+$

$NH_2OH + O_2 \rightarrow NO_2^- + H_2O + H^+$

Step 2: $NO_2^- + ½O_2 \rightarrow NO_3^-$

Ammonium-oxidising archaea (AOA), as well as bacteria, have also been shown to be important to nitrification in a range of environments. They appearing to contribute largely to nitrification in arctic soils, where the rate of nitrification appears to depend on the clade of AOA present (Alves et al., 2013).

Nitrification can subsequently lead to the production of gaseous NO, N_2O and N_2 via denitrification. Several environmental factors can affect nitrification rates in soil. Nitrification is particularly sensitive to pH with highly acidic (pH < 4.5) or alkaline (pH > 8) soils producing negligible quantities of NO_3^- (De Boer & Kowalchuk, 2001). The optimum pH for nitrification varies according to the soil, but is generally in the range of 6–8 (Strous et al., 1999; Dapena-Mora et al., 2007). It was originally thought that all organisms involved in nitrification depended upon the presence of O_2, which in turn depended on such factors as moisture content, temperature and soil texture. However, anaerobic ammonium oxidisers were discovered in 1999 (Strous et al, 1999). Nitrification is possible only if NH_4^+ is present; however, high levels of NH_4^+ can also repress nitrification (Brady, 1984).

The C: N ratio of the substrate is important, as high C: N ratios can lead to a lack of an available NH_4^+ substrate for nitrification to take place (Brady, 1984). Autotrophic nitrifying bacteria have been found which can fix dissolved inorganic C (DIC). However, this process has been shown to only produce low bacterial growth relative to the amount of nitrate produced (Veugar et al., 2012). Nitrification is slow at temperatures of 5°C and below and at temperatures of 40°C and above (Hoyle et al, 2006). Temperatures in the range of 25–35°C are generally considered to be optimal (Paul & Clark, 1989).

Nitrification in temperate ecosystems has been shown to increase by 32.2 % due to increased temperature (Bai et al., 2013). It is this temperature sensitivity of nitrification that leads to seasonal changes in N availability. In temperate areas, spring nitrification rates tend to be high and winter rates low (Dessureault et al, 2010). Soil moisture can indirectly affect NO_3^- production, as it affects soil aeration; however, it

also regulates NH_4^+ availability by controlling its diffusion rate in soil (Gleeson et al., 2010). Overall, water logging depresses nitrification, whilst low moisture retards the proliferation of bacteria. The optimum moisture level for nitrification differs according to the soil, but usually rests between matric potential levels of -0.1 to -1 MPa.

2.1.5.1. Different environmental effects on nitrification and mineralization

Environmental factors such as temperature (Hoyle et al., 2006), pH (De Boer & Kowalchuk, 2001) and water (Gleeson et al., 2010) generally affect nitrification activity more than N mineralization. This is because mineralization is carried out by many types of bacteria, and hence has a large degree of functional redundancy. Nitrification, by contrast, involves just two types of organisms – archaea and bacteria nitrifies. It had been indicated that the gross N flux, community organisation and abundance of AOA and AOB are affected by the water-filled pore space (WFPS) in semi-arid soils (Gleeson et al., 2010). Gross nitrification rates at 75–95 % WFPS were intrinsically low, however, due to the lack of NH_4^+ availability.

2.1.5.2. Heterotrophic nitrification

Many actinomycetes and heterotrophic bacteria are capable of creating traces of NO_2^- when grown in a culture media containing NH_4^+. Heterotrophic nitrification can be defined as the oxidation of reduced N compounds to generate NO_2^- and NO_3^-. Heterotrophic nitrification, unlike autotrophic nitrification, is not linked to cellular growth. Heterotrophic organisms employ C substrates as energy sources and their presence in soil has been known about for over 50 years. In a classic study, Eylar and Schmidt (1959) isolated 978 cultures of heterotrophic organisms from soil that could actively nitrify. Similarly, Remacle (1977 a, b) and Johnsrud (1978) identified many

fungi from coniferous forest soils that could nitrify, exemplifying the widespread nature of the trait. Various pathways have been postulated for nitrification, including the following one, which utilises inorganic NH_2OH (Aleem, 1965; Prosser, 1989):

$$NH_4^+ + NH_2OH \rightarrow NOH + NO_2^- + NO_3^-$$

A second possible pathway of heterotrophic nitrification uses organic N as a substrate; however, this appears to be limited to fungi. Organic pathways include oxidation of an amine or amide instead of hydroxylamine, with subsequent oxidation to a nitroso and then to a nitro-compound, as follows (Doxtader, 1965):

$$RNH_2 + RNHOH \rightarrow R\text{-}NO + R\text{-}NO_2 + NO_3^-$$

The amount of heterotrophic creation of NO_2^- or NO_3^- by nitrification is in general much lower than that of autotrophic nitrification (Prosser, 1989; Jetten et al., 1997).

2.1.5.3. Autotrophic nitrification

Autotrophic bacteria can synthesize carbohydrates, fats, proteins and other organic molecules from inorganic molecules. Autotrophs are of two sorts – those that use energy from light (photosynthesis) and those that use inorganic chemical reactions (chemosynthesis). The chemoautotrophic oxidation of NH_4^+ to NO_3^- is undertaken by Gram-negative bacteria (Bock et al., 1992; Schmidt & Belser, 1994). The sole energy source for these chemoautotrophic bacteria groups involves the oxidation of NH_4^+ to NO_2^- and then to NO_3^- (Wood, 1986). With the exception of some strains of *Nitrobacter*, nitrifying bacteria use CO_2 as their main C source (Bock, 1978; Matin, 1978). Some strains have the ability to grow mixotrophically, although the growth rate is relatively limited; this type of growth involves absorbing organic compounds (Matin, 1978; Krummel & Harms, 1982).

There have been no documented studies of bacteria directly converting NH_3 to NO_2^- (Hooper et al, 1997). Rather, the alteration of N in nitrification by autotrophic bacteria involves two steps (Haynes, 1986). The first comprises the conversion of NH_4^+ to NO_2^- with NH_2OH as an intermediary. The oxidation of NH_4^+ to NO_2^- is achieved through a number of reactions. The first is the conversion of NH_4^+ to NH_2OH, which involves NH_4^+, O_2 and reductant, as follows (Hollocker et al, 1981):

$$NH_4^+ + O_2 + H^+ + 2e^- \rightarrow NH_2OH + H_2O$$

$$NH_2OH + O_2 \rightarrow NO_2^- + H^+ + H_2O$$

$$NH_4^+ + 2e^- + 2O_2 \rightarrow NO_2^- + 2H_2O$$

The second step is to convert NH_2OH to NO_2^-. In this reaction, water contributes its oxygen atom to the NO_2^-, as follows (Andersson & Hooper, 1983):

$$E + H_2NHO \rightarrow E\text{–}NO^+ + 3H^+ + 4e^-$$

$$E\text{–}NO^+ + H_2O \rightarrow E + NO_2^- + 2H^+$$

Nitrite is generally produced in soils by oxidisers of ammonia, but does not accumulate, as it is oxidised rapidly to nitrate by nitrite-oxidising bacteria. *Nitrobacter* is the dominant organism involved in this process (Laanbroek & Woldendorp, 1995), which uses intermediates, and again, the extra atom of oxygen is derived from water as follows (Aleem et al., 1964, Kumar et al., 1983):

$$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^-$$

$$2H^+ + 2e^- + \tfrac{1}{2}O_2 \rightarrow H_2O$$

$$NO_2^- + \tfrac{1}{2}O_2 \rightarrow NO_3^-$$

2.1.6. Denitrification

Denitrification refers to the microbial reduction of NO_3^- to NO_2^- and then to gaseous NO, N_2O and N_2. Under field conditions, not all of the intermediate gaseous products are always converted to N_2 by one of the specific reductase enzymes involved, as portions of them escape to the atmosphere. Important determinants of the rate of denitrification in soil are the availability of NO_3^-, the supply of metabolizable C, soil moisture, soil aeration, soil pH and temperature (Brady, 1984). Most denitrification is accomplished by heterotrophic bacteria, and therefore the process is strongly dependent on C availability. Denitrification increased between 1900 and 2000 and is expected to increase again by 2050. Globally, the emissions of N_2O have increased correspondingly from 10 Tg N/yr in 1900 to 12 Tg N/yr in 2000 and it is predicted that they will reach 16 Tg N/yr by 2050 (Bouwman et al, 2013).

2.1.7. Recent amendments to the N cycle

Over the last century, our understanding of the N cycle has vastly improved, especially where it concerns plant nutrition. Traditionally, it was believed that higher plants used mostly dissolved inorganic N (DIN; Hill et al, 2011c), possibly because crops required large amounts of inorganic N (Näsholm et al., 2009). Furthermore, the high microbial use of organic N led to the belief that plants would be poor competitors for this substrate (Lipson & Näsholm, 2001). Therefore, it was thought that plants were limited by the microbial mineralization and oxidation of DON to ammonium and nitrate in systems with low N conditions (Chapin, 1995; Hill et al., 2011b). However, in some studies, the inorganic N supplied to plants was insufficient to meet the N requirement of the plants (Jones et al, 2005b; Näsholm et al., 2009). One explanation for this shortfall

could be that calculated rates of N mineralization from laboratory experiments are lower than what occurs in situ. Alternatively, plants could take up the organic N fraction (Jones et al., 2005b). The latter possibility was explored in the early 1900s (e.g. Hutchinson & Miller, 1912), and more recently, Hill et al (2011) demonstrated plants' ability to take up amino acids and peptides. Inselsbacher and Näsholm (2012) found that amino acids could make up a large proportion of plant available N in soil solution in flux terms. Thus, instead of mineralization to ammonium being the limiting step in the N supply to the plant, the rate of protein cleavage into peptides and amino acids appears to be more important (Hill et al, 2011b). Evidence that microbes also take up peptides directly indicates that plants and microbes could be competing for this N resource (Farrell et al, 2011; Hill et al., 2012; Farrell et al., 2013). The direct uptake of microbes by plant roots does not appear to be a very important process in the plant acquisition of N, despite reports involving sterile cultures which have suggested the opposite (Hill et al., 2013). The uptake of inorganic and organic N sources is shown schematically in Fig 2.3.

The importance of DON for plant nutrition is still uncertain. It has been argued that it is only important due to the presence of mycorrhizal symbionts, or for plants in low temperature or low pH environments (Gärdenäs et al., 2011). However, it has been shown that DON uptake also can occur in non-mycorrhizal plants and in a number ecosystems; whether this is important in comparison with inorganic N uptake requires further investigation (Jones et al., 2005; Näsholm et al., 2009; Gärdenäs et al., 2011; Kuzyakov & Xu, 2013).

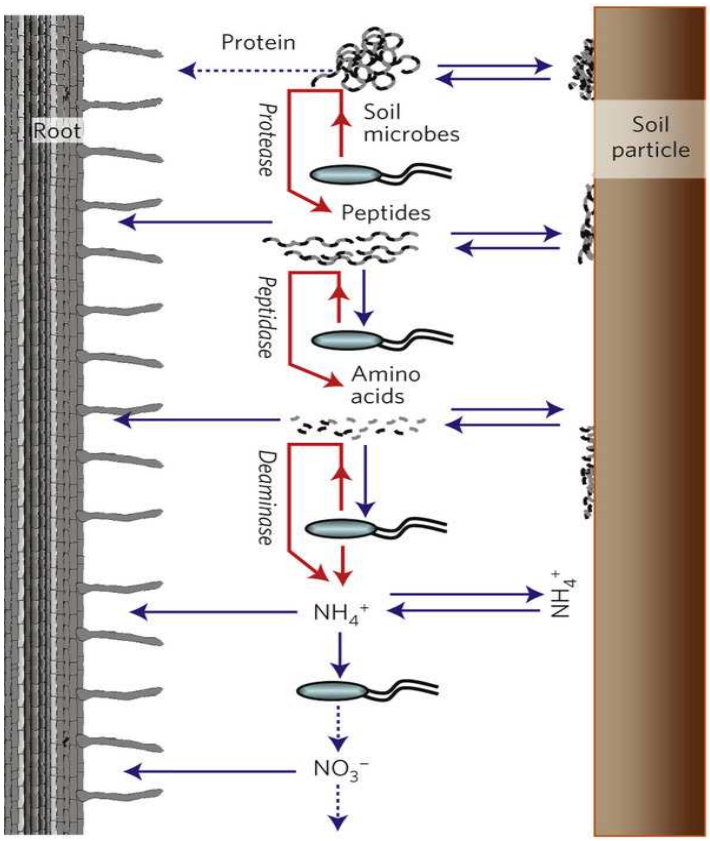

Fig. 2.3. Schematic representation of plant root uptake of inorganic and organic sources of with the interactions of soil microbes and soil particles. Source: Hill et al. (2011a).

2.2. Dissolved organic N

N often represents the main nutrient regulating plant productivity in terrestrial ecosystems (Lambers et al., 1998). As shown above, our understanding of the inorganic N cycle in soil is good (Jarvis et al., 1996; Murphy et al., 2003). In contrast, however, the processes preceding NH_4^+ production within the N cycle remain poorly understood. NH_4^+ is produced from DON, which is defined as the soluble fraction of a soil's organic N. In most soils and freshwater, DON represents a major pool of soluble N, it has also been hypothesised to play an important role in determining vegetation development, especially in pristine ecosystems (Chapin et al., 1993; Raab et al., 1996). DON can also be defined as organic N held in the exchangeable phase of soil, and this fraction is

typically termed extractable organic N (Murphy et al., 2000). Experimentally, this is normally measured in concentrated KCl and K_2SO_4 extracts of soil and quantification of the total amount of soluble organic N released. DON can contain many hundreds of different organic compounds which contain N. Of these, low–molecular weight (LMW) DON is thought to represent a significant source of N for microorganisms and some plants (Jones et al., 2005b).

With respect to controls on DON cycling, Jones et al (2004) argued that the conversion of insoluble organic N to LMW DON, but not of LMW DON to NH_4^+ or of NH_4^+ to NO_3^-, constitutes the main constraint on the supply of N to microbes in soil. The limiting step therefore appears to be the breakdown of protein into peptides and amino acids, which represent the main DON substrates for microbial uptake. Jones et al (2004) also showed that NO_3^- accumulates rapidly in grassland soils, while the concentrations of NH_4^+, LMW DON and free amino acid are maintained at low levels due to rapid microbial consumption. It follows that these neither determine nor control the rate of N mineralization in soil.

LMW DON represents a small portion of the overall DON in soil and it is the most available fraction for plant and microbial use (Jones, 1999; Jones et al., 2005b). The turnover rate of the LMW DON pool is rapid, whilst high-MW DON is less labile (Jones et al., 2004). This appears to be due to size rather than aromaticity, as it has been shown that the phenolic proportion of high-MW DON is not dissimilar to that of LMW DON (Jones et al., 2012). DON concentrations vary throughout the soil with areas of high concentration caused by the lysis of cells, high microbial activity or the addition of organic material from plants or animals (Jones et al., 2005b; Farrell et al., 2011). Plant roots can exploit these areas of high DON concentration.

DON and dissolved organic carbon (DOC) play important roles in many nutrient cycles within the soil and in pedogenic processes. DOC and DON derive from several sources, including wet and dry deposition, vegetation throughfall, root and microbial exudation, litter fall, urine and faeces, turnover of roots and microorganisms and organic fertiliser additions to soil (Kalbitz et al., 2000). Of these, plants provide the primary input of DON and DOC to the soil via root turnover and exudation (Jones & Darrah, 1994). Thus, plants can exert a substantial impact on the quantity and quality of DON and DOC cycling in soil; however, there seems to be little difference in the effects of individual grass species on total DON concentrations in soil (Khalid et al., 2007). DON comprises a wide range of different compounds ranging from LMW compounds such as amino sugars, amino acids, urea, and purines, to high-MW complexes such as proteins, DNA and chlorophyll (Antia et al., 1991; Jones et al., 2004). DON may also be created by abiotic fusions of chemicals in soil. Such secondary sources can generate a variety of high-MW recalcitrant polyphenolic materials (Stevenson, 1982). Although the ecosystem type can have a strong influence on DON concentrations in soil, studies have shown little effect of agricultural management style on DON fluxes (e.g. addition of fertiliser, drainage, crop type on turnover of amino acid; Jones et al., 2005b).

The size of the DON pool is simultaneously regulated both by adsorption-desorption processes and also by replenishment from external inputs and SOM breakdown (Kalbitz et al., 2000; Qualls, 2000; Gjettermann et al., 2008). Generally, DON is thought to be more susceptible to microbial attack if it is free in solution than if it is held on the soil's exchange phase due to the latter being both physically and chemically protected (Zsolnay, 2003; Jegajeevagen et al., 2013). From this perspective,

Ros et al (2009) argued that the EON and DON pools are neither analogous in size nor controlled by similar factors.

2.3. Introduction to peptides

2.3.1. Amino acids

A peptide can be defined as a polymer of amino acids linked by amide bonds between the carboxyl group and amino group of the next amino acid. Amino acids are molecules which comprise an amine group, a carboxylic acid group and a side chain. The side chain differs according to the type of amino acid. All amino acids contain H, C, O and N, but they may also contain other elements such as S. Twenty-two amino acids are readily incorporated into proteins (Creighton, 1993). These are termed standard amino acids; 20 of these are shown in Table 2.2. Amino acids may also be used to derive energy in times of C limitation (Sakami & Harrington, 1963). In addition to the 22 standard amino acids, there are numerous nonstandard amino acids. These are manufactured in organisms, and often have highly specific metabolic functions. Certain plants and microorganisms manufacture 3-aminopropanoic acid, for instance, which they then use to synthesise pantothenic acid (vitamin B_5; Coxon et al., 2005).

Table 2.2. Common amino acids found in proteins (Taiz and Zeiger 2006).

Amino acid	Charge / Polarity	Chemical formula
Glycine	Neutral / Non-polar	C_2H_3NO
Serine	Neutral / Polar	$C_3H_5NO_2$
Cysteine	Neutral / Slightly polar	C_3H_5NOS
Tyrosine	Neutral / Polar	$C_9H_9NO_2$
Phenylalanine	Neutral / Non-polar	C_9H_9NO
Tryptophan	Neutral / Slightly polar	$C_{11}H_{10}N_2O$
Alanine	Neutral / Non-polar	C_3H_5NO
Leucine	Neutral / Non-polar	$C_6H_{11}NO$
Valine	Neutral / Non-polar	C_5H_9NO
Asparagine	Neutral / Polar	$C_4H_6N_2O_2$
Aspartic acid	Acidic / Polar	$C_4H_5NO_3$
Lysine	Basic / Polar	$C_6H_{12}N_2O$
Methionine	Neutral / Non-polar	C_5H_9NOS
Threonine	Neutral / Polar	$C_4H_7NO_2$
Isoleucine	Neutral / Non-polar	$C_6H_{11}NO$
Proline	Neutral / Non-polar	C_5H_7NO
Glutamic acid	Acidic / Polar	$C_5H_7NO_3$
Glutamine	Neutral / Polar	$C_5H_8N_2O_2$
Arginine	Basic / Polar	$C_6H_{12}N_4O$
Histidine	Basic / Polar	$C_6H_7N_3O$

2.3.2. Peptides

Peptides are essentially short-chain proteins. Proteins may be defined as polypeptides comprising a chain of 50 or more amino acids, and peptides may be defined as comprising a chain of less than 50 amino acids. Nevertheless, the distinction between peptides and proteins is blurred: Short proteins (e.g. insulin) can be considered peptides, and long peptides (e.g. amyloid beta peptide) can be considered proteins. In any event, peptides may be sub-classified according to their length: Dipeptides contain two amino acids, tripeptides contain three, tetrapeptides contain four and so on up to decapeptides, which contain 10. In addition, oligopeptides are said to contain fewer than 30 amino acids (Anfinsen et al, 1972).

Peptides involve peptide bonds. A peptide bond links the carboxylic acid group of one amino acid to the amine group of its neighbour. Thus, peptides always comprise alternating sequences of carboxylic acid and amine groups. The bond itself uses oxygen, and in the process of its formation, the two amino acids shed one molecule of water. This is termed dehydration synthesis.

There are five broad classes of peptide depending on how the peptides are manufactured. So-called milk peptides are those that derive from the digestion or fermentation of milk. In the case of digestion, milk peptides arise from the enzyme breakdown of lacto-proteins. In the case of fermentation, they arise from the proteinases (enzymes) of the bacteria responsible for the fermentation.

Ribosomal peptides are synthesised by the translation of mRNA. In higher organisms, they operate as hormones or other signalling molecules. They are also implicated in the synthesis of venom and toxins. Plants and yeasts may also synthesise ribosomal peptides as antibiotics (Duquesne et al., 2007). In some cases, ribosomal peptides may be modified by mRNA translation. In general, like proteins, ribosomal peptides are linear; however, a few have a nonlinear (e.g. lariat) structure.

Non-ribosomal peptides are synthesised by specific enzymes (Shabanpoor et al., 2009). One common example is glutathione, which is important in aerobic organisms in the protection against oxidative stress. Many microorganisms use modular enzyme complexes to synthesise non-ribosomal peptides. Such complexes can be very sophisticated and can produce peptides of highly complex (i.e. nonlinear) structure.

Peptones are made from meat or milk digested by proteolytic digestion. Proteolytic digestion is digestion within the cell and this uses proteinases. In general, peptones are small. In addition, proteolytic digestion often gives rise to diverse by-

products, including fats, minerals, vitamins and salts. For this reason, peptones are often used as a nutrient medium for bacterial cultures.

So-called peptide fragments are fragments of protein degraded by enzymes. They can be used for the purposes of identifying source proteins during forensic analysis. They are sometimes produced in laboratories, but may also occur naturally (Tong et al., 2008).

The above discussion illustrates that peptides represent a diverse family of chemicals that are vital for life, perform many essential but diverse biological functions and may be produced in a variety of ways. Because of this, they have attracted a great deal of research attention. However, only recently has their role in soil chemistry and biochemistry become a focus of study. Their possible role from this perspective is discussed below.

2.3.3. Peptide structure and function

Peptides play a significant role in the life of humans and other organisms, including enzyme inhibitor/substrates, hormones, growth promoters, immunomodulating agents, antibiotics and neurotransmitters. Peptides are produced from 22 different α-amino acids; however, the inclusion of non-proteinaceous amino acids (e.g. selenocysteine, pyrrolysine) in peptides is common. This inclusion gives rise to heteromeric configurations (Sewald & Jakkubke, 2009). In soil, peptides can be formed from D- or L-isomeric amino acids, or mixtures of the two. Ribosomes mediate the production of peptides involving L-amino acids, but not peptides involving D-amino acids, cyclic peptides or other non-proteinaceous building blocks. Over 300 such peptides are known to occur; the latter are referred to as non-ribosomal peptide

synthesases (NRPSs). NRPSs are commonly found in marine and soil bacteria, or in lower-order eukaryotes such as filamentous fungi and marine sponges. Actinobacteria, ascomycetes, bacillus, cyanobacteria and myxobacteria produce the majority of non-ribosomal peptides (Howl & Jones, 2009).

A number of non-ribosomal peptide–producing microbes are parasites or epiphytes of plants (Bender et al., 1999; Audenaert et al., 2002). NRPSs occur particularly in Gram-positive bacteria. Because of the presence of a variety of proteins and proteases in soil, millions of peptides might be present in solution. The peptides may extend from 2–10 amino acids short-chain oligopeptides to 10–100 amino acid residues. The length of the peptide chain is limited by non-ribosomal protein template synthesis (Zuber et al., 1993). The peptide backbone can be a combination of linear, cyclic and cyclic branches, and can be turned over by acylation, glycosylation or heterocyclic ring information. Many short linear chains contain turns; these are defined by whether 3, 4, 5 or 6 amino acids are involved in making the turn. Large peptides contain a helix-and-sheet conformation; this is controlled by the different side chains of the amino acid component. The linking of the amino acid in peptides to or peptide bonds gives rise to different isomers and conformational shapes.

Peptides perform vital functions in almost all processes in the living cell. Cell concentrations of peptides can range from 10^{-9} to 10^{-3} M. Because of their complex structures and ability to specifically bind to receptors, many are involved in the complex network of chemical signalling required for cellular coordination. In human systems, several peptides have been documented (e.g. insulin, melanotropin). However, we know relatively little about the function of peptides in the plant-soil system. Of those that have been well documented, tripeptide glutathione, a biological redox agent, occurs in most

cells of plants and microorganisms. It is involved in metabolism, transport and cellular protection against oxidative stress. Senwo and Tabatabai (1998) showed that the peptide amino acid composition of the corresponding size fraction often differs between cultivated and uncultivated soils of the same series; they also showed that long-term cropping systems or cultivation may alter the quantitative and qualitative nature of the amino acid fraction in soils. Cropping systems involving legumes have been shown to increase the N content of the organic matter in soil.

Over 500 microbial produced toxic peptides have been identified (e.g. from *Bacillus* sp., *Actinomyces* sp. and phallotoxins and amatoxins produced by *Amanita* fungi; Wieland, 1986) and are used for defence purposes or in the struggle for limited resources. Many have been shown to be biologically active at nM concentrations. Fungi and bacteria produce many peptide antibiotics to kill or inhibit the growth of other microorganisms (e.g., bacitracins, polymyxins; Kleinkauf & Von Dohren, 1990; Jack et al., 1998). Antimicrobial defence peptides (27 to 84 amino acid residues in length) have also been documented in plants that are toxic to Gram-positive and negative bacteria, yeast and fungi (e.g. thionin; Montesinos, 2007). However, fewer plant defence–related peptides have been identified than are produced by microorganisms.

Many peptide antibiotics contain D-amino acids and non-proteinaceous amino acids, making them more resistant to proteolysis. In addition, many are cyclic and branched-cyclic peptidolactones and depsipeptides, structures not found in animal cells, again making them less prone to enzymatic cleavage and deactivation. Some are simple dipeptides (e.g. bacilysin and alaphosphin), while other peptides contain up to 20 amino acid residues (e.g. thiostrepton, alamethicin). The antibiotic peptides target various aspects of metabolism; these include nucleic acid and protein biosynthesis (e.g.

streptotogramins), lipid stability (e.g. thiostrepton, alamethicins), energy production (e.g. gramicidins), cell wall biosynthesis (e.g. bacteriocins, bacitracin A) and nutrient uptake (e.g. gramicidins; James et al., 1992). Gramicidin S is a cyclic decapeptide antibiotic which acts on Gram-positive bacteria. It comprises two repeating pentapeptide sequences.

In higher organisms, antimicrobial peptides (AMPs) are proteins of low MW. They exhibit broad spectrum antimicrobial activity against bacteria, viruses, and fungi. Most AMPs are positively charged and have both a hydrophobic and a hydrophilic side. This enables the molecule to be soluble in an aqueous environment, yet also enter lipid-rich membranes (Montesinos, 2007). Once in a target microbial membrane, the peptide uses a variety of mechanisms to kill target cells. Cathelicidins and defensins are major groups of epidermal cell targeting AMPs.

2.3.4. The interaction of N forms with soil

The interaction of amino acids and peptides with soil particles is more complicated than it is for inorganic N. The sorption of NH_4^+ to soil is strong, whilst that of NO_3^- is weak; this leads to NO_3^- being more available than $NH4^+$, as well as more prone to leaching. The adsorption of amino acids to the soil particles depends on the charge on the compound. Positively charged amino acids bind more strongly than neutral amino acids, which in turn are adsorbed more strongly than negatively charged ones (Jones & Hodge, 1999). The charge on amino acids and peptides depends on the pH of the soil solution, as they are amphoteric. They are neutral when the soil solution pH nears 7, whereas in acidic soils they become positively charged, as they are protonated; in basic soils, they are negatively charged (Tan, 2010). Adsorption can

occur due to Van der Waal's forces, dipole-dipole attraction, hydrogen bonds and covalent bonds (White, 2006); evidence for the latter was observed by Hsu and Hatcher (2005) between a pentapeptide and humic acids. A higher concentration of amino acid leads to greater sorption (Jones & Hodge, 1999). High-MW amino acids and peptides are more strongly adsorbed (Dashman & Stotzky, 1982, 1984). Although an increase in the chain length of peptides has not been shown to consistently increase sorption, a peptide with more functional groups has been shown to have higher sorption (Dashman & Stotzky, 1984). The clay content and composition of the soil is also an important factor determining adsorption potential (Dashman & Stotzky, 1982). The greater the MW of a compound, the more slowly it passes through the plasma membrane in plant roots (Kielland, 1994). The more strongly organic N binds to soil particles, the less available it will be to microbes and plants. Thus availability of amino acids and peptides depends on their concentration, structure and soil type (Kielland, 1994).

2.3.5. Peptide transport

2.3.5.1. Amino acid and peptide transporter

Peptide transport systems are varied and ubiquitous in nature and occur in prokaryotic bacteria, eukaryotic microorganisms, animals and plants. As peptides represent an important nutrient source, transport systems are required to move them into the cell where they can enter the metabolism. In certain cases, their importance in nutrition surpasses that of amino acids (Payne, 1980). Payne and Smith (1994) pointed out that researchers interested in the N cycle have largely ignored the role of microorganisms in the transportation of peptides. Most computer models describing terrestrial N cycling, for instance, do not consider the possibility of uptake of larger-MW N-containing compounds. It is therefore both timely and necessary to understand

the role of peptides in soil N cycling (Payne, 1980). Although many researchers have characterised amino acid transport systems in microorganisms, comparatively little work exists on peptide transport. However, as early as 1980, peptide transport systems were known to occur, not just in prokaryotic and eukaryotic microorganisms, but also in higher plant and animal cells. Current research suggests extensive conservation of structural and functional features within and between peptide transport systems and related transporters and channels (Payne & Smith, 1994).

The uptake of amino acids can occur in two ways. The first involves the direct uptake of individual amino acids via membrane transporters; the second comprises extracellular deamination followed by separate uptake of the amine and acid group (Barraclough, 1997). As occurs for amino acid utilisation, simple peptides are utilised by microorganisms, and again there are two ways in which the uptake can occur. In the first, the peptide is transported into the cell and subsequently cleaved into individual amino acid units. On the other hand, in the second, the peptides are split by extracellular enzymes (e.g. peptidases) and transported into the cell as free amino acids (Payne, 1980; see Fig 4). Peptide transport across membranes is energy dependent and is largely limited to small peptides of 2–6 residues long. Peptide transport systems can also transport a range of peptide derivatives. The molecular weight of peptides depend on the number of their amino acid and in our study the LMW was less than 1 KDa and the HMW more 1KDa.

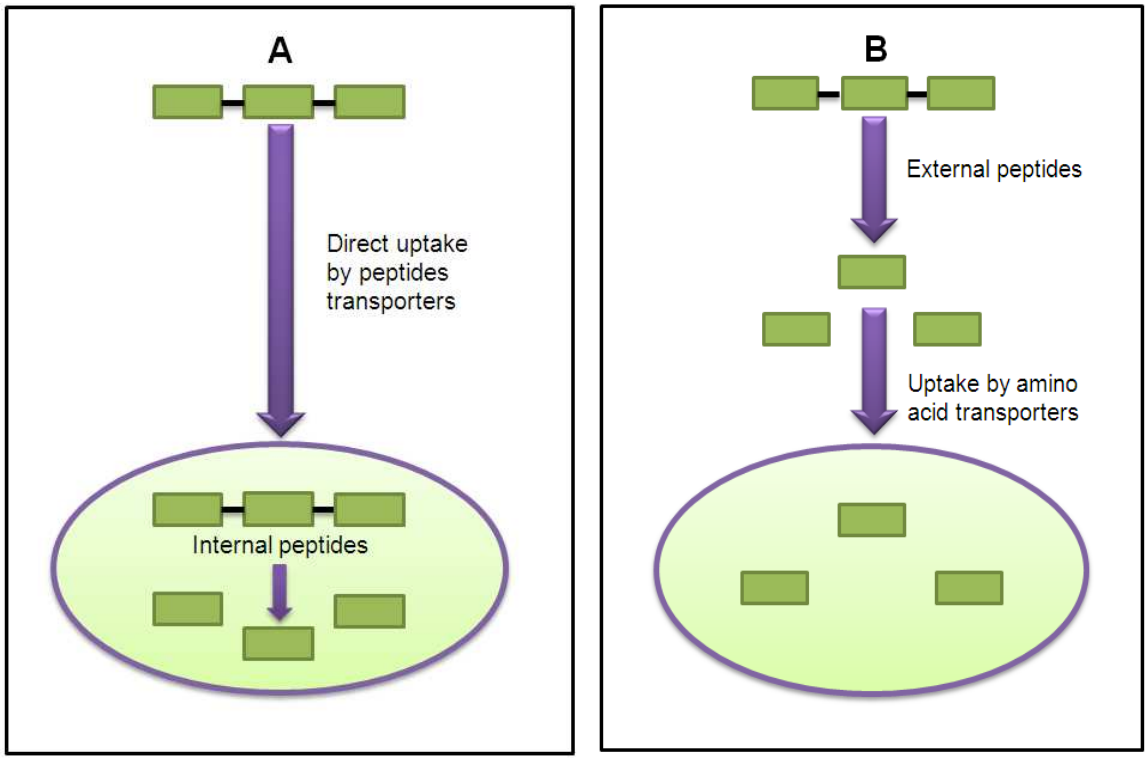

Fig. 2.4. Peptide transport (A) direct transport of peptides into the cell and (B) uptake with transporter after extracellular enzymatic cleavage to amino acid monomer units. (D.L. Jones, unpublished).

The transport system can be divided into two groups according to their energy source (Stacey et al., 2002) as follows:

- The ATP-binding cassette family ABC-type. This group uses ATP hydrolysis to drive uptake.
- The PTR peptide transporter and oligopeptide transporter (OPT) families. This group uses the proton motive force (PMF) to drive transport.

The ABC superfamily is the biggest family which has been identified. It is found in eukaryotes, prokaryotes and archaea. The ABC substrates are as various as the organisms in which they have been observed. The substrates range from small ions to large macromolecules, including peptides. Classification into the ABC family depends

on the presence of one or two ATP-binding domains (Stacey et al., 2002, 2006). In plants, many ABC transporters have been found; however, their roles are not well understood (Lu et al., 1998; Sanchez-Fernandez et al., 2001). PTR peptide transport, like ABC transport, is found in eukaryotes and prokaryotes and involves the transport a wide range of N-containing substrates, including peptides, amino acids and nitrate (Williams & Miller, 2001). Peptide transport by PTR is limited to dipeptides and tripeptides (Waterworth & Bray, 2006; Komarova et al., 2008). OPT transporters differ from PTR transporters in that the former recognise tetra- and pentapeptides, whereas the latter recognise only di- and tripeptides (Stacey et al., 2006).

Genes have been found in the plant *Arabidopsis thaliana* for the transportation of amino acids and peptides (Fischer et al., 1998; Arabidopsis Genome Initiative, 2000). The amino acid transporters are quite general (Senwo & Tabatabai, 1997). There are two types – one takes up neutral and acidic amino acids, whilst the other transports basic amino acids (Gärdenäs et al., 2011). Current research on the genome of *Arabidopsis* has shown peptide transporters that can transport peptides up to five amino acids in length using energy-dependent carriers (Steiner et al., 1994). PTR genes expressed in the peripheral cells of plant roots show the potential for peptide uptake and transport around plants (Waterworth & Bray 2006). A list of amino acid and peptide transporters is given in Table 2.3.

Table 2.3. Amino acid and peptide transporters found in plants (Tegeder and Rentsch 2010).

Family	Substrate
Amino acid transporter family	
LHT (lysine histidine transporter)	Neutral and acidic amino acids, lysine and histidine
ProT (proline transporter)	Proline, quaternary ammonium compounds
ANT1 (aromatic-neutral amino acid transporter)	Neutral and aromatic amino acids
GAT (aminobutryric acid transporter)	GABA and related compounds
APC amino acid – polyamine – choline	
CAT (cationic amino acid transporter)	Neutral and cationic amino acids
BAT (bidirectional amino acid transporter)	Alanine, arginine, glutamate and lysine
PRAT preprotein and amino acid transport	
OEP (plastid outer envelope protein)	Amino acids, amines, charged amino acids
MCF mitochondrial carrier family	
BAC (basic amino acid carrier)	Arginine, lysine, ornithine, histidine
DASS divalent anion: Na^+ symporter	
DiT (dicarboxylate transport)	Exchange glutamate/malate
PTR/NRT1 peptide transporter	
PTR1 (peptide transporter)	di- and tripeptides, histidine
OPT oligopeptide transporter	
OPT (oligopeptide transporter)	Tetra- and penta peptides

Peptides are potentially the first stage in the breakdown of protein, where plants and microbes can take up N. Therefore, this stage could be the most important in the competition between plants and microorganisms for this resource (Hill et al., 2011b). This is supported by the existence of different transporter groups. It has been shown that *Hakea actites* and *A. thaliana* can take up intact proteins (Paungfoo-Lonhienne et al., 2008). However, it has been suggested that this occurs too slowly to support plant growth. It has also been shown that roots can take up intact microbes, but again this does not seem to be a large enough N source to be relevant for plant growth (Hill et al., 2013). This has been proposed to be due to either root-derived proteases or root endocytosis rather than transporters. The 20 types of amino acid can produce 8000 different tripeptides, over 150,000 tetrapeptides and in excess of three million

pentapeptides. Thus, many possible substrates are available to be taken up by peptide transporters (Waterworth & Bray, 2006).

2.3.5.2. Peptide transport in bacteria

As indicated, peptides are important for all organisms. However, research suggests that in microorganisms, peptide transport is more direct and extensive than in other organisms. The research suggests that microorganisms show a growth response to a range of peptides; this helps to explain different aspects of peptide transport and utilisation by microorganisms (Sussman and Glivarge, 1971; Crampton, 1972; Barak & Gilvarge, 1975a; Meredith & Boyed, 2000). Peptide transport can occur independently from amino acid uptake (Cohen & Rickenburg, 1956). Gram-negative bacteria cell walls comprise a peptidoglycan layer and an outer membrane. The wall is a bilayer. The membrane helps to protect the cell, yet simultaneously allows passage of nutrient and waste products through it (Lugtenberg & Van Alphen, 1983; Nikaido & Vaara, 1985; Nakae, 1986). The region called the periplasm is located between the cytoplasm membrane and the outer membrane. Within the periplasm, there is a variety of proteins. These are important for many transport systems, including peptide permeases (Braun, 1975).

The Gram-positive bacteria cell wall is simpler than the Gram-negative bacteria cell wall. It comprises a cytoplasmic membrane overlaid by polymers. The polymers comprise peptidoglycan and teichuronic acid. There is no periplasm layer in Gram-positive bacteria. The transport system in microbes involves the uptake of exogenous peptides and other nutrients found within the cytoplasmic membrane (Payne & Smith, 1994).

Research on peptide size suggests that the overall length of a peptide determines whether it can be utilised. This appears to be true of *Escherichia coli* (Smith et al., 1970), *Pseudomonas putida* (Cascieri & Mallette, 1976a), *Streptococcus lactis* (Rice et al., 1978) and *Saccharomyces cerevisiae* (Naider et al., 1974; Marder et al., 1977). These studies suggest that the transport of small peptides is widespread in Gram-positive and negative bacteria, yeasts and fungi (Hagting et al., 1994; Saier, 2000). Research by Gilvarg and Levin (1972) on the toxic peptide triornithine has identified two distinct transport mechanisms for dipeptides and oligopeptides; the researchers also suggested that the carbonyl group is an important structural feature in dipeptide transport. It has been shown that trialanine can have faster uptake per unit N than dialanine (Farrell et al., 2013).

The peptide's structure is also important in determining its nutritional value of with the incorporation of amino acid analogues, or the presence of D-amino acids diminishing or even destroying its nutritional quality (Gilvarg & Katchalski, 1965; Losik & Gilvarg, 1966). Peptide utilisation for nutrition after the peptide has entered the cell is contingent on intracellular peptide activity. Peptidases, however, appear unessential for uptake (Kessel & Lubin, 1963).

Glivarge et al (1972) studied *E. coli* exposed to homologous oligopeptides compared to requisite amino acid using heteropeptides, and measured the subsequent bacterial growth response. Their result suggests that the transport is mediated by systems that exhibit properties such as competition and structural specificity. These appear similar to those involved in the transport of LMW molecules such as amino acids and sugars. There appear to be four main ways by which peptides can traverse the cytoplasm membrane of microorganisms:

1. *Passive diffusion*: The molecules move across the cell membrane by random motion. They do not to bind to any membrane component. Passive diffusion does not allow net movement against the gradient, but does allow net movement down a gradient. In general, passive diffusion plays a very minor to insignificant role in peptide uptake, although small, neutral peptides may use it.
2. *Facilitated diffusion*: This is achieved by specific carriers binding with the molecules to make the movement easier during the passage across the membrane. Diverse models have been suggested for the carriers, including pores lined with ligands to achieve substrate specificity. Facilitated diffusion has no direct requirement for energy. It does not allow for net movement against a gradient;
3. *Group translocation*: This comprises catalysis of the modification of a substrate that crosses the membrane. Group translocation allows net movement against a gradient. An example of group translocation is the accumulation of phosphorylated sugar by certain bacteria by means of a phosphoenolpyruvate for particular sugars (Roseman, 1972). Group translocation, however, requires energy. There have been no reported cases of peptides being transported by group translocation in microorganisms.
4. *Active transport*: This is similar to facilitated diffusion, except that it is coupled with metabolic energy. It increases the concentration of a substrate against an electrochemical gradient. The present study indicates that peptides are taken up by bacteria by active transport.

Recent studies have shown that peptides are rapidly taken up from soil solution and that this process is largely biotic (Ge et al., 2013). L-peptides have been shown to

have a half-life in soil solution of less than 1 min (Hill et al., 2012). D-peptides are more slowly removed than L-peptides, but their uptake is still rapid, between 10 and 36 min (Hill et al., 2013). Half-life measured using mineralization are slower than this due to a delay between uptake and mineralization. Such half-lives have been found to be around 1 h for trialanine in soils from around the world, whilst glutamate-phenylalanine has a half-life of 6.7 ± 1.5 h and valine-proline-proline has a half-life of 9.4 ± 2.2 h in Chinese cultivated soils (Farrell et al., 2011; Ge et al., 2013; Farrell et al, 2013). Other types of peptides may have different half-lives.

Whether soil microbe's uptake peptide in preference to amino acids is unclear. Farrell et al (2011) found no effect of increased peptide concentrations on the alanine mineralization rate constant, whilst Hill et al (2012) found decreased alanine uptake at high peptide levels. Peptide mineralization to NH_4^+ was found to occur at a similar rate to that of amino acids (half-life = 30.3 ± 4.2 days compared to 28.9 ± 5 days) and this was faster than for proteins (Jones & Kielland, 2012). The uptake rate of N appears to be greater for longer peptide chain lengths (Farrell et al., 2011; Hill et al., 2012). It has been suggested that the uptake of peptides may be partly controlled by soil organic carbon (Farrell et al., 2013). More research is needed to get a clearer picture of the fluxes of the many potential soil peptides and what soil characteristics affect them.

2.3.5.3. Plant uptake of amino acids

More research has been undertaken on amino acid uptake than peptide uptake. Plants from many ecosystems, including the arctic tundra and salt marshes, have been examined for amino acid uptake (Kielland, 1994; Schimel & Chapin, 1996; Henry & Jefferies, 2003). It was found that 31 boreal plants had the ability to take up amino acids (Persson & Näsholm, 2001). Glycine, a commonly occurring amino acid in soil, can be

taken up by temperate grasses (Streeter et al., 2000). Moreover, glycine uptake from hydroponic solution has been shown for *Lupinus albus* and *Leucadendron laureolum* which have adapted to soils with the low N content in the Fynbos biome (Hawkins et al., 2005). Although most studies on amino acid uptake have focussed on plants from low N soils, agricultural and temperate grassland plants have also been shown to take up amino acids.

Examples of agricultural species include a plant-soil microcosm study by Ge et al (2011) which showed that a tomato cultivar was able to take up 21% of N in the form of dual-labelled (^{13}C, ^{15}N) glycine from a 100 μM solution. Wheat was found to capture 6% of a 100 μM solution of lysine, glycine and glutamate, while the rest was captured by the microbial community in the microcosms (Owen & Jones, 2001). There is simple evidence to suggest that amino acid uptake is a widespread ability among plants and can occur with or without mycorrhizal associations. How important amino acid uptake is for plant nutrition remains a debated issue; one important aspect is whether or not there is significant flux through the amino acid pool. Jones et al (2005b) hypothesised that amino acid uptake by plants may mainly occur for the recapture of root exudation; competition by microbes, which are intensely active in the rhizosphere, may also result in lower rates of amino acid uptake by plants in the field. Variation in the demand for individual amino acids by microbes exists within the soil. For example, Lipson et al (1999) demonstrated that glycine is less demanded by the microbial community, which relates to increased availability and consequent uptake, for the alpine sedge *Kobresia myosuroides*. Plant roots are more long lived than microbes, which may convey an advantage over longer timescales for the capture of amino acids.

2.3.5.4. Peptide transport by plants

Peptide transport in plants involves the translocation of peptides 2–6 residues in length across the cellular membrane and is an energy-dependent process. Although not well studied until recently, one of the first demonstrations of peptide transport in plants was by Higgins and Payne (1981), who established the presence of a millimolar concentration of small peptides in the endosperm of germinating cereal grain and their ability to be taken up into cells. More recent research has also indicated that the peptides are important in the control of plant cell differentiation and organogenesis, where they appear to be important nutrients (Stacey et al., 2002).

As indicated in the above discussion on peptide transport in microorganisms, research suggests the existence of two families of peptide transporter (OPTs and PTRs) in plant growth and development (Waterworth & Bray, 2006). Higgins (1992) also added to these two families the ATP-binding ABC superfamily. The ABC family of peptide transporters includes the recently identified Arabidopsis GSH s-conjugate transporters, AtMRP1 to AtMRP4 (Lu et al., 1997, 1998; Sanchez-Fernandez et al., 1998). Higgins and Payne (1982) argued that transporting peptides is more efficient for N distribution than transporting individual amino acids. Higgins and Payne (1982) also argued that this is especially true of long-distance transportation during the bulk movement of protein-degradation products (e.g. during leaf senescence, seed germination, etc.). Because they contain N, peptides might also protect amino acids from catabolism by enzymes in the phloem during transport within the plant (Higgins & Payne, 1980; see Figure 2.5).

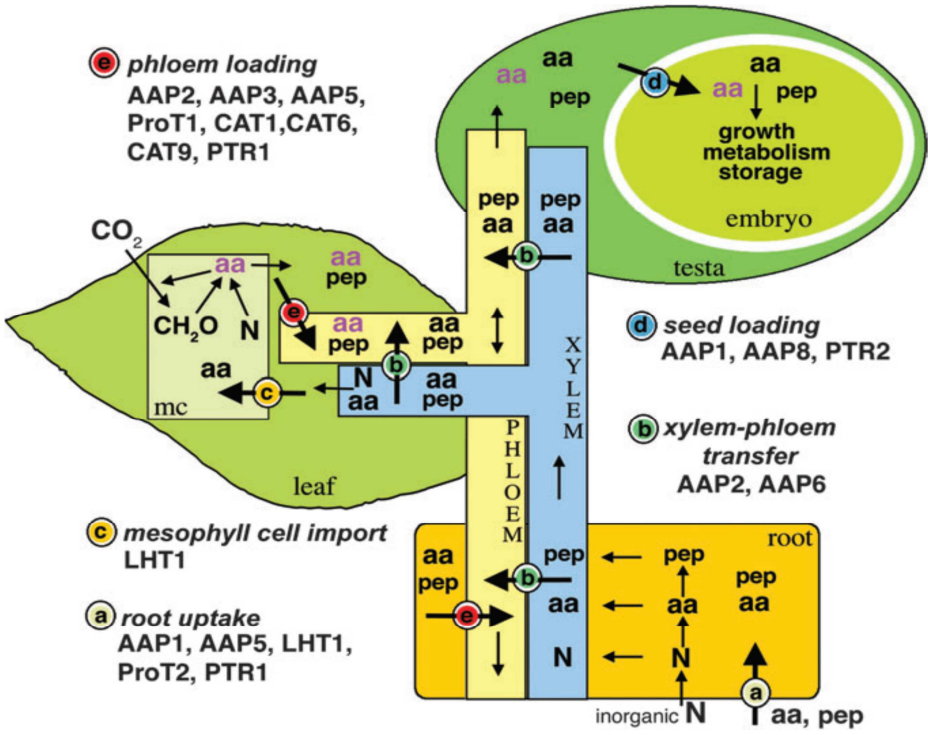

Fig. 2.5. Function of amino acid and peptide transporters in *A. thaliana* (Tegeder and Rentsch, 2010) (A) Root uptake. (B) Xylem–phloem transfer. (C) Import into mesophyll cells (mc). (D) Seed loading. (E) Function of transporters in phloem.

Research confirms that peptides have a role in within-plant signalling. They are important in cell differentiation, plant development and plant defence (Schaller, 1999; Pearce et al., 2001; Matabyashi et al., 2002; Karim et al., 2007; Gray et al., 2008; Haruta et al., 2008). This is in addition to the role peptides play in providing plants with N. Research also suggests the possibility that communication between bacteroids and plants in the root nodules of legumes occurs via peptides; this last possibility, however, has not been confirmed (Mergaert et al., 2003).

Carnivorous plants can utilise organic compounds as a source of N. It was demonstrated by Darwin in 1875 that *Utricularia* and *Drosera* exhibit enhanced growth and reproductive capacity when supplied with animal proteins. The peptide transportation generated from insect digestion is implicated in the N nutrition in

carnivorous plants such as *Nepenthes* (Schulze et al., 1999), and carnivorous behaviour is an important means of obtaining N (Lloyd, 1942). However, a digested insect may also supply P, S and other elements to the growing plant (Chandler & Anderson, 1976a).

In *Arabidopsis* seedlings, root growth is inhibited by toxic dipeptides due to their uptake by root PTRs (Steiner et al., 1994). There are many recent reports of PTRs in roots, and a mutant *Arabidopsis* not expressing PTRs has been found to have reduced growth and N content relative to controls when grown on dipeptides as a sole N source (Komarova et al., 2008; Paungfoo-Lonhienne et al., 2009).

2.3.6. Amino acid and peptide exudation from cells

If material, including peptides and amino acids, can enter cells by passive diffusion, then in theory at least, it can also leave it by passive diffusion. Indeed, early work on the subject suggested that at least amino acids may leave the cell in this manner (e.g. Payne, 1980). How significant amino acid and peptide efflux is in soil, and particularly from microorganisms, is uncertain, and currently no research has demonstrated its occurrence in soils. Nevertheless, this remains a possibility. Thus, it should be noted that it is the net influx (i.e. the balance between influx and efflux) which should be considered when investigating transport processes.

2.3.7. Methods of quantifying peptides in soil

The isolation and quantification of peptides from soil samples is fraught with difficulty, and there are no accepted standards for performing these tasks. Peptides can easily and quickly be destroyed by enzymes such as proteases, peptidases and deaminases, which are co-extracted with the peptides. Consequently, heat-treated or

untreated biological samples are often extracted with cold HCl (ca. 0.1 to 0.5 M) to minimise this degradation. The peptides are then purified and a range of techniques is applied to determine their MW and amino acid sequence. Peptides can also be hydrolysed to determine the amount of each amino acid present (Stevenson, 1982). Another approach is to use ultrafiltration equipped with specific MW cut-off membranes. This assumes that oligopeptides have a maximum size of ca. 1000 MW and polypeptides have a maximum size of 10,000 MW.

2.3.8. Peptide sorption

Research has shown that some peptides and proteins can become associated with mineral surfaces in soil and with polyphenols. These bound N forms may persist in soil, such that they are subject neither to uptake nor decomposition (Knicker et al., 2000; Kleber et al., 2007). Information about the sorption behaviour of peptides in soils, however, is sparse. In contrast, much research has been carried out on the adsorption of NH_4^+ and amino acids by clay minerals. The results of such research suggest that the degree of adsorption is a function of the peptides' basicity and MW, with basicity being more important. The type of clay is also relevant. Montmorillonite adsorbs higher levels of peptide than does kaolinite or illite (Greenland et al., 1961).

Recent research on poly-amino acid indicates that polymers of basic, positively charged amino acids (e.g. poly-lysine) bind more strongly to negative charged minerals such as montmorillonite. Conversely, polymers of acidic, negatively charged amino acids (e.g. poly-asparagine) interact more strongly with minerals with positively charged surfaces such as goethite (Ding & Henrichs, 2002). Thus, peptide sorption in soil appears to be a function of both the mineral surface and the peptide functional

groups available for binding. Some studies have reported that peptide size influences adsorption, with greater size being associated with greater adsorption. These researchers also found little difference between mineral and organic soils (Greenland et al., 1961, 1965b). However, Dashman and Stotzky (1984) found a negative correlation between peptide size and adsorption, where small peptides were adsorbed more readily. Similarly, Bujdak et al (2006) suggest a negative correlation between peptide adsorption in soil, but only of small peptides of the same isomer (e.g. L-Ala-L-Ala compared to L-Ala-D-Ala) onto clay particles in suspension. Research on this last point is lacking, however, and it is clearly an area for further investigation.

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Chapter 3

Microbial activity differentially regulates the vertical mobility of nitrogen compounds in soil

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ABSTRACT

Alongside nitrate, dissolved organic nitrogen (DON) represents a significant N loss pathway in many agroecosystem. To better understand the factors controlling DON leaching in soil we followed the vertical movement of ^{15}N-labeled NO_3^-, NH_4^+, alanine and tri-alanine in soil columns in response to a simulated rainfall event. We show that in sterile soil where sorption is assumed to be the dominant regulating factor, leaching followed the series NO_3^- > tri-alanine > alanine > NH_4^+. In the non-sterile soil columns, the rapid rate of NO_3^- leaching was unaffected whilst the movement of the amino acid, peptide and NH_4^+ was almost completely prevented due to microbial immobilization. Our results support the view that (1) DON loss from agricultural soils occurs mainly in the form of high molecular weight (MW) recalcitrant compounds rather than in the form of labile low MW DON, and (2) that although nitrate was bioavailable, it was not a preferred N form for the C-limited microbial biomass.

Keywords: Dissolved organic carbon, Grassland, Hydrological pathway, Mass flow

Introduction, methods, result and discussion

The enhancement of nitrogen (N) use efficiency and a reduction in N leaching are central goals in the development of sustainable agricultural systems. To achieve these goals requires that we have a very good understanding of N processes and cycling rates in plant-soil-water systems. While our knowledge of the factors regulating inorganic N cycling are well understood (i.e. NO_3^-, NH_4^+, N_2O), the behaviour of dissolved organic N (DON) remains poorly characterised (Gardenas et al., 2011). To a large extent this has arisen due to the difficulties in the chemical characterisation of DON alongside the general view that strategies to optimize N management are largely targeted towards inorganic N fertilizers (Jones et al., 2005). Recent evidence, however, has suggested that DON also (1) constitutes a major loss pathway from agroecosystem (via leaching; Heathwaite and Johnes, 1996; Willett et al., 2004; van Kessel et al., 2009), (2) plays a crucial role in soil C sequestration (Knicker, 2012), and (3) represents an important direct source of N for crop plants (Hill et al., 2011ab; Soper et al., 2011).

While key putative roles of DON in ecosystem functioning have been identified, our ability to properly quantify these roles has been hampered by the inherent complexity of DON behaviour in soil. For example, amino acids and peptides can undergo a range of fates in soil including: (1) sorption to the soil's solid phase (Dashman and Stotzky, 1982), (2) uptake by soil microorganisms (Fischer et al., 2010; Hill et al., 20012), (3) mineralization or stabilization by soil minerals (Mantion et al., 2008), (4) abiotic fusion and precipitation with soil organic matter (Knicker, 2012), (5) uptake by plant roots (Hill et al., 2011ab), (6) cleavage by extracellular enzymes (e.g. deaminases, peptidases; DeBusk and Ogilvie, 1984), (7) complexation with metals in solution (Jones et al., 2004), and (8) movement via mass flow and diffusion. To

improve N cycling models requires that we understand the relative importance of these processes. The aim of this study was therefore to focus on the influence of microbial activity and chemical form on the potential for organic and inorganic N vertical transport in soil.

Table 3.1. Background soil characteristics.

Parameter	
Water content (%)	29.1 ± 1.0
Total soil C (g kg^{-1})	34 ± 3.0
Total soil N (g kg^{-1})	3.4 ± 0.3
Microbial C (g kg^{-1})	3.9 ± 0.5
Microbial N (g kg^{-1})	0.54 ± 0.08
Dissolved organic C (mg C l^{-1})	57.9 ± 3.2
Dissolved organic N (mg N l^{-1})	2.41 ± 0.77
NH_4^+ (mg N l^{-1})	1.65 ± 0.32
NO_3^- (mg N l^{-1})	6.07 ± 1.04
Soil respiration (mg C kg^{-1} h^{-1})	1.02 ± 0.06

Values represent means ± SEM, n = 3.

Replicate samples of an aerobic sandy clay loam textured soil (Eutric Cambisol; n = 3) were collected from a *Lolium perenne* L. dominated agricultural sheep-grazed grassland located at Abergwyngregyn, Gwynedd, UK (53°14'N, 4°01'W). Prior to use, the field-moist soil was sieved to pass 2 mm to remove roots and stones. Characteristics of the crumb structured soil are shown in Table 3.1. Microbial C and N content were determined by CHCl3 fumigation-extraction according to Joergensen (1996) and soluble C and N determined with a TCN-V analyzer (Shimadzu Corp., Kyoto, Japan). Soil moisture content was determined by drying at 105 ºC overnight. Soil solution was extracted by the centrifugal drainage procedure of Giesler and Lundström (1993) and dissolved organic C and total dissolved N determined using a TCN-V analyzer. Soil solution NO_3^-, and NH_4^+ were determined using the colorimetric methods of Mulvaney

(1996) and Miranda et al. (2001) whilst free amino acids were determined fluorometrically according to Jones et al. (2002).Soil respiration was determined in the laboratory at 20 ºC using an SR1 automated soil respirometer (PP Systems Ltd, Hitchin, UK). Total C and N were determined with a CHN2000 analyzer (LecoCorp., St Joseph, MI).

To conduct the leaching experiments, plastic tubes (9 mm diameter, 30 cm long) were packed with soil to a bulk density of 1 g cm^{-3}. Different ^{15}N-labeled N forms (50 µl; 1 mM, 98 atom %) were then added to the soil surface. Immediately, rainfall (locally-collected rain water) was added to the soil surface via a peristaltic pump at rate of 20 µl min^{-1} to simulate a heavy rainfall event and to facilitate downward movement of ^{15}N. Prior to rainfall application the packed soil columns were deemed aerobic (soil moisture ¼ 291 g kg^{-1} soil dry weight). At the end of the leaching period the soil moisture had increased to 484 ± 6 g kg^{-1} which represented 74.4 ± 1% of the soil's water holding capacity. Although we did not measure denitrification losses of 15N, based on previous results from this free draining soil, the aerobic nature of the soil and the timescales involved, we hypothesize that this is a very minor loss pathway (Jones et al., 2012).

The different N forms added to the soil were $^{15}NH_4Cl$, $K^{15}NO_3^-$, ^{15}N-L-alanine and ^{15}N-L-trialanine was chosen as it represents a dominant free amino acid in this soil (Jones et al.,2005b) whilst trialanine represents a major component of peptidoglycan (Komagata and Suzuki, 1987). Alanine and trialanine possess no net charge. After a leaching time of 30 min the leaching front had penetrated to 10 cm depth. At this point the plastic tubes were sectioned into 10 × 1 cm pieces, dried (80°C, 24 h), weighed and ground for ^{15}N analysis. The leaching experiments were performed on either sterile

(autoclaved for 30 min, 121°C immediately prior to use) or non-sterile soil. Sterilization was predicted to both inhibit both microbial and extracellular enzyme (e.g. peptidase) activity and has been shown to be an effective sterilant in this soil previously (Hill et al., 2008). Isotopic analysis was undertaken at the UC Davis Stable Isotope Facility using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). Across all treatments, the amount of ^{15}N recovered from the soil columns at the end of the experiment was 90 ± 3% of the total ^{15}N added at the start. Small losses of ^{15}N may have occurred during sample processing (e.g. subsampling) or if a small amount of leachate passed below 10 cm. Significant differences between treatments were identified by ANOVA with Tukey post hoc test (SPSS v14; SPSS Inc., Chicago IL.).

The vertical transport of the different forms of N down the soil columns is shown in (Figure. 3.1). Overall, both sterility and chemical form had a significant impact on the redistribution of ^{15}N in the soil ($P < 0.001$). Under sterile conditions the rate of movement followed the series NO_3^- > tri-alanine > alanine > NH_4^+. With the exception of NO_3^-, which was transported almost in entirely to the base of the soil columns, the other three N forms could be recovered in significant quantities throughout the soil columns. In contrast, under non-sterile conditions the movement of NH_4^+, alanine and tri-alanine was significantly repressed presumably due to microbial uptake, with most ^{15}N recovered in the top 1 cm of the soil columns ($P < 0.001$). Overall, the amount of transport followed a similar series to that of the sterile columns: NO_3^- > tri-alanine > alanine = NH_4^+.

Our results clearly demonstrate the strong influence of microbial activity and electrostatic interactions on the movement of different N forms in soil. As expected,

NO_3^- was readily leached in this soil and was not a preferred form of N for microorganisms. This is consistent with the high nitrification potential of this soil, the fact that the microbial biomass is more C than N limited, and that energetically the metabolism of NO_3^- is not favoured over NH_4^+ (Jones et al., 2004; Hill et al., 2011a). In contrast, the ^{15}N-labeled NH_4^+, amino acid and peptide were all rapidly assimilated by the soil microbial biomass limiting their potential to be transported by mass flow. This finding is consistent with other reports showing rapid immobilization of low MW DON. These previous studies, however, have tended to focus on the C contained within DON from which we know that typically 30-40% of the C is used directly in respiration and that excess N generated during catabolism induces NH_4^+ excretion back into the soil (Roberts et al., 2009). While this could partially explain the greater movement of DON relative to NH_4^+, it is more likely that NH_4^+ sorption to cation exchange sites co-limited its movement relative to the net neutrally charged peptide and amino acid. This is supported by the calculated solution-to-solid phase partition coefficient (K_d) values for the N concentration employed here (K_d = 29 for NH_4^+ and 0.35 for neutral amino acids; Jones and Hodge, 1999; Jones et al., 2012). In conclusion, our experiments provide direct evidence that microbial immobilization rather than sorption is the key factor limiting the movement of neutrally charged amino acids and peptides in soil. It also supports the view that DON loss from agricultural soils occurs mainly in the form of high MW recalcitrant compounds rather than labile low MW DON. One caveat to this is in soils where preferential flow pathways (i.e. larges cracks not present in our packed columns) may facilitate rapid bypass flow of water down the soil profile. If plants had been included in our packed soil columns we predict that root uptake of soluble N would further repress the downward movement of all N forms. Recent studies, however,

suggest that similar to microorganisms, crop plants often also prefer peptides, amino acids and NH_4^+ over NO_3^- (Hill et al., 2011b).

Although we only used alanine and trialanine as model substrates, we feel that numerous previous studies on this soil with a range of amino acids and peptides (including alanine and trialanine) suggest that that our model compounds broadly reflect the use of other amino acids and oligopeptides. Exceptions to this may include charged peptides (e.g. containing multiple repeats of charged amino acids such as Lys, His, Glu, Asp, Arg) where sorption may predominantly limit leaching rather than microbial consumption. We also acknowledge that this study was performed in only one soil at one initial moisture content. However, based on the amino acid and peptide mineralization profiles from soil types across the world (Jones et al., 2009; McFarland et al., 2010; Hill et al., 2012), we believe that the findings reported are likely to be directly relevant to many other ecosystems.

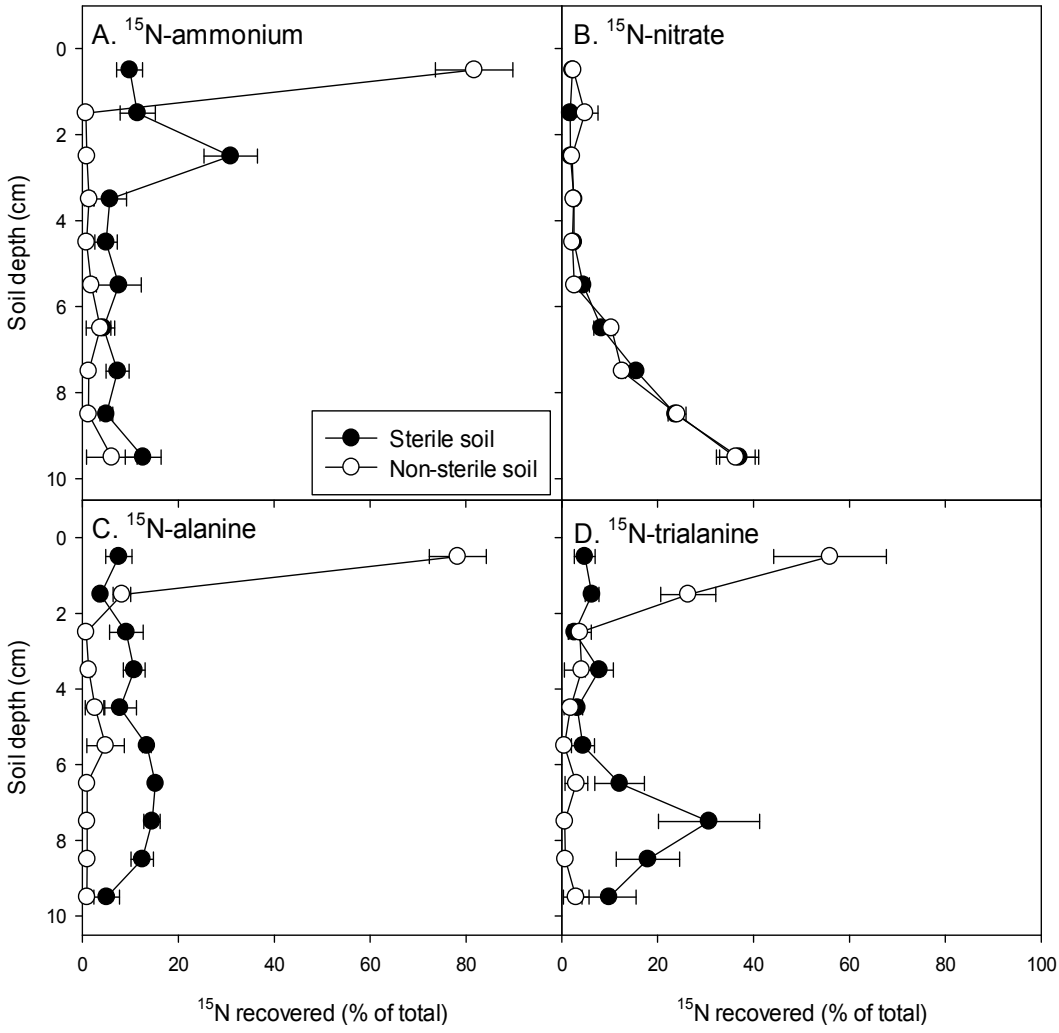

Fig. 3.1. Vertical leaching of ^{15}N-labeled forms of inorganic (NH_4^+, NO_3^-) or organic N (alanine, trialanine) under sterile or non-sterile soil conditions in response to a simulated rainfall event. Values represent mean ± SEM (n = 3). The legend is the same for all panels.

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Chapter 4

Nutritional competition between plants and soil microorganisms for different nitrogen forms as influenced by peptides and vice versa

Abstract

Nitrogen (N) is a key element that regulates ecosystem functioning, providing the basic building blocks for both plants and soil microorganisms. Recent studies have shown that both plants and soil microbes can directly utilize short-chain peptides, without the need for prior cleavage by extracellular peptidases. In addition, the availability of amino acids and peptides in soil has gained considerable attention as a potential source of organic N. Our knowledge of the interaction and potential competition between organic and inorganic N forms with respect to plant root N acquisition, however, remains unclear under both sterile and non-sterile growth conditions. Similarly, the microbial preference for different N forms in soil is not well characterized. The results presented here clearly show that soil microbes prefer organic N forms to inorganic N forms, probably due to the community being C-limited rather than N-limited. Wheat plants grown under sterile conditions demonstrated the ability to rapidly take up peptides, yet NH_4^+ showed the greatest rate of uptake. In sterile culture, the availability of the different N forms was reduced by the presence of an alternative (competing) N form, with the exception of NO_3^-, which was not affected by trialanine. This effect was less apparent when the plants were grown in soil and in competition with a rhizosphere microbial community. These results support the theory that plants have the potential to acquire peptides from soil under a wide range of N conditions, but that they are poor competitors for organic N in comparison to soil microorganisms.

4.1. Introduction

Nitrogen (N) provides a basic building block for both plant and soil microbial growth and its availability is a key factor in regulating primary productivity in many ecosystems (Vitousek and Howarth, 1991). In managed agroecosystems, the addition of inorganic fertilizers and the mineralization of soil organic matter (SOM) generally provide sufficient inorganic N to microbial populations and plants, with excess N readily leached and lost from the system (Ryden et al., 1984). In this scenario, growth of the soil microbial community is typically limited by the availability of carbon (C) and therefore little competition for soil N may occur between microbes and plants. When fertilizer is not applied, either due to environmental concerns or for socioeconomic reasons, there is typically insufficient N to meet plant demand. Under these conditions, it has been shown that plants may use a wide range of organic N forms to satisfy their N shortfall (e.g. peptides, amino acids, and urea) (Hill et al., 2011a, c; Warren, 2013). As these organic N compounds are rich in both C and N, plant roots are in direct competition with microorganisms for this limited resource (Lipson and Monson, 1998; Owen and Jones, 2001). However, the competitive balance between plants and microorganisms depends on myriad abiotic and biotic factors (e.g. plant and microbial community structure, presence of mycorrhizas, prevailing climate, type of N form, plant ontology, soil physicochemical properties, etc.) and the key regulators remain poorly understood (Vinolas et al., 2001; Bardgett et al., 2003; Bajwa and Read, 1985; Benjdia et al., 2006; Schimel and Bennett, 2004).

In flux terms, proteins represent the dominant organic N pool in soil, and their breakdown by proteases, peptidases and deaminases leads to the release of peptides, amino acids and NH_4^+ into solution. As most protein in soil is either insoluble or held

electrostatically on the soil's solid phase, the release of these soluble products is critical for movement towards and uptake by roots and microbes. As peptides represent the primary cleavage product, it has been hypothesised that peptides may be more important than amino acids and NH_4^+ as a source of N for microbes (Matthews and Payne, 1980; Hill et al., 2012). This is supported by recent studies showing that short peptides constitute a higher proportion of the soil dissolved organic Nitrogen (DON) pool compared to free amino acids (Farrell et al., 2011a; Hill et al., 2011ab). The definition of short peptides varies, in this study we consider them to be of a size that can be directly taken up by microbes i.e. less than 650 Da which corresponds to around 6 amino acids in length. These short peptides are thought to be utilised by both plants and soil microorganisms without further cleavage by extracellular peptidases (Komarova et al., 2008; Farrell et al., 2011b; Hill et al., 2011c). Energetically, it is also more favourable to take up oligopeptides in comparison to free amino acids (Payne, 1980; Kirchman and Hodson, 1984; Carlsson, 1997). Together these findings indicate that the most intense inter- plant and plant-microbial competition in N-limiting ecosystems will be for peptides (Hill et al., 2011c).

Despite abundant evidence suggesting that organic N is important for plant growth, the quantitative significance of this flux remains very uncertain. This is mainly due to difficulties in translating hydroponic experiments to field conditions, the interpretation of ^{15}N tracer studies, numerous N feedback loops and poor methods for quantifying soil N (Inselsbacher and Näsholm, 2012; Jones et al., 2005, 2013).

Soil solution contains a cocktail of NH_4^+, NO_3^- and thousands of different organic N compounds; however, most experiments have focussed on the uptake and utilisation of single forms of inorganic and organic N. Based on the interaction between

NO_3^- and NH_4^+ uptake in plants (Kong et al., 2013), we hypothesise that interactive effects may occur between amino acids, peptides and inorganic N in terms of root transport activity. This study therefore aims to: 1) Determine whether peptides are taken up intact by plants growing under sterile conditions; 2) Evaluate how much N can be taken up as peptides by plants grown in soil; 3) Assess which forms of N soil microorganisms prefer; and (4) Determine the effect of inorganic N and amino acid availability on peptide uptake, and the effect of peptides on the uptake of these other N forms.

4.2. Materials and methods

4.2.1. Plant uptake of peptides of different chain length

Seeds of wheat (*Triticum aestivum* L. var Claire) were sterilised in *ca*. 14% sodium hypochlorite with 1 drop of Tween for 20 min, followed by placement in 90% ethanol for 2 min. Subsequently, the seeds were washed thoroughly 3 times in sterile distilled water. Sterilised seeds were placed on the surface of 10% Murashige and Skoog agar and sealed in Phytatrays® (Sigma Aldrich, Gillingham, UK). Trays were placed in a climate-controlled cabinet with light intensity of 500 µmol m^{-2} s^{-1} PAR, temperature of 25 °C and relative humidity of 70%. At the third leaf stage, the plants were removed from the agar and placed in KCl (0.5 mM) over night to remove adhering agar and nutrients. Intact root systems of individual plants (n = 3) were then placed in 15 ml vials containing 12 ml of a sterile (0.2 µm-filtered) solution of either ^{13}C-^{15}N-labelled L-dialanine, L-trialanine or L-tetraalanine (all 50 µM, 98 atom %). After 5 h, plants were removed from solutions and washed in deionised water (for 1.5 min), followed by $CaCl_2$ (100 mM) for 1.5 min, followed by a final rinse with deionised

water. This washing was designed to remove any N present in the apoplast and charged solutes adhering to the roots.

After washing, the plants were placed in paper bags and oven dried (80 °C, 24 h) after which, root and shoot biomass were recorded. The roots and shoots of the plants were ground together; the ground material was weighed into tin foil cups and prepared for analysis by mass spectrometry. Background plants for each treatment were prepared following the same procedure, except they were not exposed to ^{15}N or ^{13}C. Total C and N in the root and shoot was determined using a TruSpec® CN analyzer (Leco Corp., St Joseph, MI). The samples were sent to UC Davis Stable Isotope Facility, California, for ^{15}N and ^{13}C analysis.

4.2.2. Phosphor image

Replicate sterile wheat plants were removed from agar and placed in KCl (0.5 mM) over night to remove adhering agar and nutrients. The plants were then placed in 4 ml of either 10 μM ^{14}C-trialanine or 10 μM ^{14}C-alanine solution (2 kBq ml^{-1}) for 30 min. Plants were then removed from the solution, washed in deionised water (1.5 min), and the roots and leaves were subsequently oven dried (24 h, 80 °C). To confirm amino acid and peptide uptake, plants were placed flat on phosphor imaging plates and scanned by Cyclone plus storage phosphor system (Perkin Elmer, Waltham, USA) after exposure to the plates for 48 h.

4.2.3. Uptake of nitrogen from solution by sterile grown plants

Wheat plants were grown in sterile culture on 10% Murashige and Skoog agar as described above. When the plants were at the third leaf stage, they were removed from the agar and placed in KCl (0.5 mM) overnight. To determine how peptide uptake was

affected by the availability of other N forms, roots of single plants (n = 3) were placed for 5 h in 15 ml of a sterile (0.2 μm-filtered) solution of dual-labelled ^{13}C-^{15}N L-trialanine (50 μM) alongside other forms of unlabelled N (either NO_3^-, NH_4^+ or L-alanine). Next, KNO_3 and L-alanine were added at either 0 μM (control), 150 μM, (same amount of N as trialanine), 750 μM (5 times as much N as trialanine) and 7.5 mM (50 times as much N as trialanine). Then, NH_4Cl was added at 0, 150 μM, 750 μM and 1.5 mM to avoid toxic effects of high concentrations of exogenous NH_4^+.

In the converse experiment, the uptake of NO_3^-, NH_4^+ and L-alanine were measured in the presence of increasing concentrations of peptide. Individual plants were placed in a sterile solution (15 ml) of either $K^{15}NO_3$, $^{15}NH_4Cl$ or ^{13}C-^{15}N-alanine (50 μM), with unlabelled trialanine added at either 0 μM (control), 16.7 μM (same amount of N as NO_3^-, NH_4^+ or alanine), 83.5 μM (5 times as much N as NO_3^-, NH_4^+ or alanine), 835 μM (50 times as much N as NO_3^-, NH_4^+ or alanine). After 5 h, plants were removed from solutions, washed and prepared for mass spectrometry as described previously.

4.2.4. Uptake of nitrogen by plants growing in soil

Soil samples were collected from agricultural soil at Bangor University, Henfaes Research Station Abergwyngregyn, Gwynedd, UK (53°14'24"N, 04°00'58"W). The soil is classified as a Eutric Cambisol. The mean annual soil temperature at 10 cm is 11 °C and the mean annual rainfall is 1250 mm. The soil had a total C content of 34 g kg^{-1} DW, a total N content of 3.4 g kg^{-1} DW, a microbial biomass C of 3.9 g kg^{-1} and a microbial biomass N of 0.54 g kg^{-1}. The soil was collected from the top 0–10 cm Ahp horizon and stored in CO_2-permeable polythene bags. Upon return to the laboratory, the soil was sieved to pass 2 mm, removing any vegetation, stones and earthworms.

Rhizotubes (15 cm long, 0.5 cm diameter; Jones et al., 2009) were filled with 12.5 g FW of sieved soil. Pre-germinated wheat seeds were planted in the top of these tubes and then the rhizotubes were placed in a climate-controlled cabinet as described above. The plants were watered daily to maintain the soil close to field capacity.

To measure how peptide uptake was affected by the availability of other N forms, ^{15}N-^{13}C L-trialanine (100 μM; 98 atom %) was mixed with different forms of unlabelled N. At the third leaf stage, rhizotubes containing a single plant (n = 3) were injected with 100 μM ^{15}N-^{13}C L-trialanine in the presence of either 0, 300, 1500 μM, and 15 mM unlabelled L-alanine or KNO_3, or 0, 300, 1500 μM, and 3 mM unlabelled NH_4Cl. Then, 1 ml of solution was injected in four 0.25 ml portions at 2 cm intervals down the rhizotubes to ensure even spread of the substrate through the soil. After 5 h, the roots were removed from the rhizotubes and washed as described above. The plants were then dried, weighed and ground before preparation for mass spectrometer analysis. Roots and shoots were ground and analysed separately.

To determine how the uptake of NO_3^-, NH_4^+ and L-alanine was affected by the availability of peptide, we added 100 μM of either $K^{15}NO_3$, $^{15}NH_4Cl$ or ^{13}C-^{15}N-L-alanine with either 0, 33.4, 167 or 1670 μM of unlabelled L-trialanine. The plants were then labelled and harvested as described above for the labelled trialanine experiment.

The presence of arbuscular mycorrhizas (AM) within the soil-grown plant roots was determined following the method of Ruzin (1999). Briefly, washed roots were fixed in 50% ethanol (24 h), rinsed 3 times in deionised water, cleared in 10% KOH (20 °C, 16 h), rinsed 3 times in deionised water, acidified in 2% HCl (2 min), stained (0.05% w/v Trypan blue), destained (50% glycerin, 24 h), mounted on glass slides in 50%

glycerin and examined under the microscope. The presence of diagnostic AM features (vesicles, hyphae, and arbuscules) was then recorded.

4.2.5. Nitrogen uptake by soil microbes

Peptide and amino acid uptake by the soil microbial community was determined by the snap-elution method of Hill et al. (2008). Briefly, microcentrifuge tubes with a hole pierced in the bottom were each filled with 1.2 g FW of sieved soil (soil properties as described in Hill et al., 2008). A solution of uniformly ^{14}C-labelled L-alanine or L-trialanine (0.3 ml, 50 μM, 4 kBq ml^{-1}) or $^{15}NH_4Cl$ or $K^{15}NO_3$ (0.3 ml, 50 μM, 98 atom %) was added to the surface of soil in each tube. Each tube was then placed into another intact microcentrifuge tube and the pair was centrifuged (4000 *g*, 1 min, 20 ^{o}C) after 1, 5, 10, 20, 40 or 60 min from the addition of the solution. During centrifugation, the soil solution passed into the lower tube, leaving the soil in the upper tube. Activity of ^{14}C in the collected solution was determined by liquid scintillation counting in a Wallac 1404 scintillation counter (Perkin Elmer Life Sciences, Boston, MA) after mixing with Scintisafe3 scintillation cocktail (Fisher Scientific, Loughborough, UK). Microbial uptake of inorganic N was determined according to Hill et al. (2011a), where the residual soil was oven dried (80 °C, 24 h), weighed and ground for mass spectrometer ^{15}N analysis as above.

To distinguish N loss from solution due to microbial uptake from that associated with abiotic processes (e.g. sorption to the solid phase), the experiments were run either on field-moist soil or on soil that had been autoclaved (121 °C, 30 min) prior to use.

4.2.6. Soil solution characteristics under wheat

At the same site where the soil was collected for the rhizotube experiments, soil solution was collected in situ using Rhizon® soil water samplers (Rhizosphere Research Products, Wageningen, Netherlands). At the time of collection, the soil supported a crop of winter wheat (*Triticum aestivum* var Granary) at growth stage Z50 (Zadoks et al., 1974). Total amino acid-N in soil solution was determined fluorometrically according to Jones et al. (2002). The NO_3^- and NH_4^+ were analysed colorimetrically using the vanadate method of Miranda et al. (2001) and the salicylic acid procedure of Mulvaney (1996), respectively. To isolate the oligopeptide fraction, soil solution was also passed through a 1 kDa ultrafiltration membrane using an Amicon Stirred Cell pressurised under N_2 (Millipore, Billerica, MA, USA). The concentration of oligopeptides in the < 1 kDa fraction was subsequently determined by measuring the total free amino acid concentration before and after hydrolysis (6 M HCl, 16 h, 105 ºC under N_2) (Amelung et al., 2006).

4.2.7. Plant respiration

Six sterile plants were removed from agar and placed in KCl (0.5 mM) over night to remove adhering agar and nutrients. The plants were then placed in 5 ml polypropylene vials containing (50 µM, 1.9 kBq ml^{-1}, 2.3 kBq ml^{-1}) ^{14}C-alanine or ^{14}C-trialanine. Each vial was put into an airtight container through which air was pumped at a rate of *ca.* 600 ml min^{-1}. After passing through the chamber housing plants, air was bubbled through Oxosol scintillant (National Diagnostics, Atlanta, GA, USA) to capture respired $^{14}CO_2$. Oxosol traps were changed after 5, 10, 20, 40 and 60 min. Captured ^{14}C was measured by liquid scintillation counting. There were 3 replicates for both alanine and trialanine.

4.2.8. Statistical and data analysis

Statistical analysis of the results was carried out by ANOVA, followed by LSD post hoc test using SPSS v14 (IBM UK Ltd., Hampshire, UK) with $P < 0.05$ used as the cut-off for statistical significance.

A first order exponential decay function was used to describe N uptake by soil microbes after addition of ^{14}C-labelled L-alanine or L-trialanine or $^{15}NH_4Cl$ or $K^{15}NO_3$ to soil:

$$Y = (a_{1 \times \exp}{}^{-k_1 t}) \qquad \text{[Eq. 1]}$$

where Y represents the amount of ^{14}C remaining in the soil, a describes the size of the pool, k is the rate constant describing the rate of turnover of pools, a_1 and t are time after substrate addition (Boddy et al., 2008; Jones et al., 2009). The half-life $t_{1/2}$ of the substrate pool (a_1) was calculated as follows:

$$t_{1/2} = \ln (2) / k_1 \qquad \text{[Eq. 2]}$$

4.3. Results

4.3.1. Available forms of nitrogen in soil solution under wheat

The pool of soil solution N under the wheat crop was dominated by NO_3^- (Table 4.1). The pool of NH_4^+, amino acids and short chain (< 1 kDa) peptides were considerably smaller, at < 3% and < 0.2% that of NO_3^-, respectively. Concentrations of amino acid and peptide N were not significantly different from each other.

4.3.2. Phosphor imaging of amino acid and peptide uptake by plants

The phosphor image of sterile plant roots after the addition of ^{14}C-labelled alanine and trialanine showed the plant's ability to take up and translocate the amino

acids and peptides (Fig. 4.1). Dark areas within the phosphor image of the sterile roots show incorporation of ^{14}C-labelled alanine and trialanine into the roots. It can be seen that all parts of the roots are labelled, suggesting that the transporters are present throughout the root system.

Table 4.1. Soil solution characteristics. Value are mean ± SEM (*n=3*).

Parameter	Concentration
NH_4^+-N (mg N l^{-1})	5.8 ± 1.1
NO_3^--N (mg N l^{-1})	195.0 ± 1.4
DON (mg N l^{-1})	32.2 ± 1.0
DON <1 kDa (mg N l^{-1})	30.2 ± 8.3
Amino acid-N	0.38 ± 0.06
Peptide <1 kDa	0.37 ± 0.1
TOC (mg C l^{-1})	15.99 ± 2.07
TOC <1 kDa (mg C l^{-1})	8.28 ± 0.94

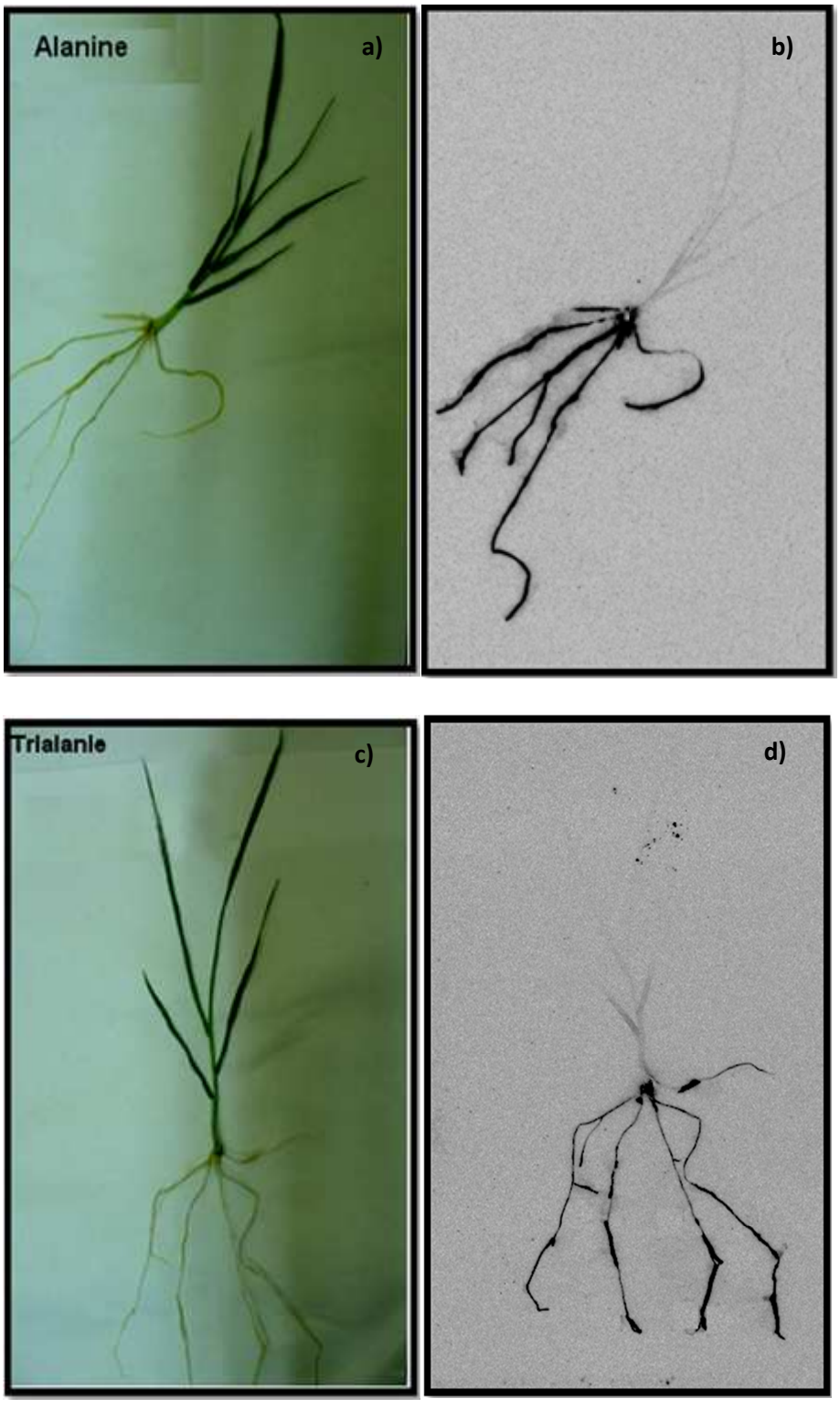

Fig. 4.1. Visualisation of the uptake of ^{14}C-alanine and ^{14}C-trialanine by sterile wheat plants. Panels b &d are phosphor images whilst panels a & c are photos taken with a normal camera of the dry wheat plant.

4.3.3. Uptake of peptides of different chain lengths

The plants showed the ability to take up the oligopeptides of all the chain lengths tested. Five hours after addition to sterile plants, the total uptake of ^{15}N supplied as dialanine was significantly lower (105 ± 20 nmol N DW plant g^{-1} h^{-1}) than that of ^{15}N delivered as both trialanine and tetraalanine ($P < 0.02$). Trialanine uptake was not significantly different from that of tetraalanine (156 ± 36.5; 142 ± 12.7 nmol N DW plant g^{-1} h^{-1}) ($P > 0.05$).

4.3.4. Nitrogen uptake by plants grown in sterile culture

The uptake rate of trialanine by wheat plants grown in sterile culture was 0.65 ± 0.04 μmol N g^{-1} DW root h^{-1}. However, the rate of trialanine uptake decreased significantly ($P < 0.05$) when it was added in the presence of other N forms (0.32 ± 0.03, 0.25 ± 0.02, 0.20 ± 0.03 μmol N g^{-1} DW root h^{-1}, when added with 150 μM N NO_3^-, NH_4^+ and alanine, respectively; Fig. 4.2). Increasing the concentration of the other N forms did not cause further change, nor was there a significant difference due to the type of N form added. The N uptake rate for other N forms alone was 0.033 ± 0.01, 5.60 ± 0.69, 0.88 ± 0.06 μmol N g^{-1} DW root h^{-1} for NO_3^-, NH_4^+ and alanine, respectively. This suggests that sterile plants show a preference for NH_4^+ over alanine and trialanine, whilst the uptake of NO_3^- was the lowest. Simultaneous addition of trialanine significantly decreased the uptake rate of NH_4^+ and alanine, whilst the rate of NO_3^- was unaffected. The concentration of trialanine was not a significant factor (Fig. 4.2).

The ratio of ^{13}C recovery to ^{15}N recovery in plants did not differ between alanine (0.64 ± 0.03) and trialanine (0.65 ± 0.08).

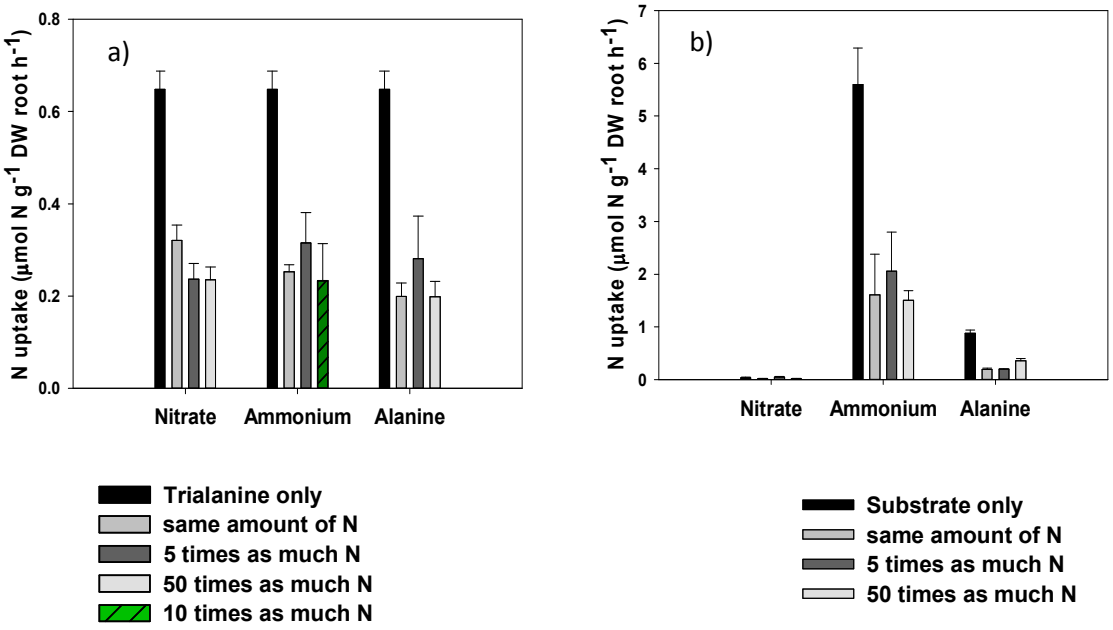

Fig. 4.2. Graphs showing the uptake of peptides by sterile roots wheat plants (a) explaining how the peptide uptake was affected by availability of other forms of nitrogen $^{15}N^{13}C$ L-trialanine with unlabelled L-alanine or KNO_3 and NH_4Cl, (b) showing how uptake of other forms of N ($K^{15}NO_3$, $^{15}NH_4Cl$ or ^{13}C-^{15}N-L-alanine) was affected by availability of unlabelled L-trialanine. Values are means ± SEM, *n*=3

4.3.5. Nitrogen uptake by plants grown in soil

The rate of trialanine uptake by the plants was 67.5 ± 8.6 nmol N g^{-1}DW root h^{-1} when grown in soil. The uptake rate of trialanine decreased significantly ($P < 0.05$) when it was added alongside high concentrations of the other N forms (44.1 ± 5.4; 36.1 ± 4.1; 46.3 ± 0.8 nmol N g^{-1}DW root h^{-1}; 15 mM of NO_3^- and alanine, 3 mM of NH_4^+, respectively). The addition of alanine at 1.5 mM also significantly decreased the uptake rate of trialanine to 44.7 ± 5.1 nmol N g^{-1}DW root h^{-1} ($P < 0.05$). The uptake rates for NO_3^-, NH_4^+ and alanine were 145.0 ± 13.6; 102.6 ± 0.35; 88.2 ± 10.3 nmol N g^{-1}DW root h^{-1}, respectively, when they were added individually, with the uptake of NO_3^- being significantly greater ($P < 0.05$) than all the others, and the uptake of trialanine being significantly lower ($P < 0.05$) than uptake of the other N forms. The uptake of

alanine and NO_3^- were not significantly affected by adding trialanine at any concentration. The uptake of ammonium was significantly reduced by addition of the two highest trialanine concentrations 27.9 ± 9.1; 44.1 ± 5.4 nmol N g^{-1} DW root h^{-1}, 167 μM, 1670 μM, respectively (Fig. 4.3).

The root allocation of ^{15}N added as nitrate, ammonium, alanine and trialanine was higher than that allocated to the shoots. The ratio of ^{15}N recovery in the roots to ^{15}N recovery in the shoots was significantly greater for trialanine (38.5 ± 10.9) than for NO_3^- (5.73 ± 1.96), NH_4^+ (2.61 ± 0.72) or alanine (3.12 ± 0.87). The addition of alanine with trialanine significantly decreased the ^{15}N root/shoot for trialanine ($P < 0.05$), but there was no effect with increased concentration (10.3 ± 1.2, 9.3 ± 2.8 and 12.0 ± 1.1 for 0.3 mM, 1.5 mM and 15 mM alanine, respectively). The two lowest NO_3^- concentrations also significantly lowered the ^{15}N root/shoot of labelled trialanine to 10.4 ± 1.2 and 12.6 ± 2.4 for 0.3 mM, 1.5 mM NO_3^- respectively. The highest NO_3^- concentration did not produce a ^{15}N root/shoot (28.07 ± 3.86) that was significantly different from trialanine only, but it was significantly higher than that of the lower NO_3^- concentrations. When NH_4^+ was added with ^{15}N trialanine, only the 1.5 mM caused a significant change in the ^{15}N root/shoot, decreasing it to 11.6 ± 2.4. The root/shoot ^{15}N for NO_3^- and alanine was not significantly changed by addition with any concentration of trialanine. When NH_4^+, was added with 33.4 μM and 1670 μM trialanine the root/shoot ^{15}N ratio increased significantly to 9.5 ± 2.0 and 8.0 ± 2.1, respectively, whilst 167 μM trialanine caused no significant difference (3.3 ± 1.6). Testing for the presence of arbuscular mycorrhizas within the roots revealed no signs of colonisation or infection.

The $^{13}C/^{15}N$ recovery ratios for plants grown in soil were very variable. In the whole plant after the addition of dual-labelled trialanine, $^{13}C/^{15}N$ was 0.36 ± 0. 39. The

mean value of the $^{13}C/^{15}N$ ratio was negative due to some samples having slightly higher ^{13}C content than backgrounds. We attribute these results to the addition of amino acid and peptide ^{13}C to a pool of plant C > 10,000 times larger, with a variable ^{13}C content. These data should consequently be interpreted with caution.

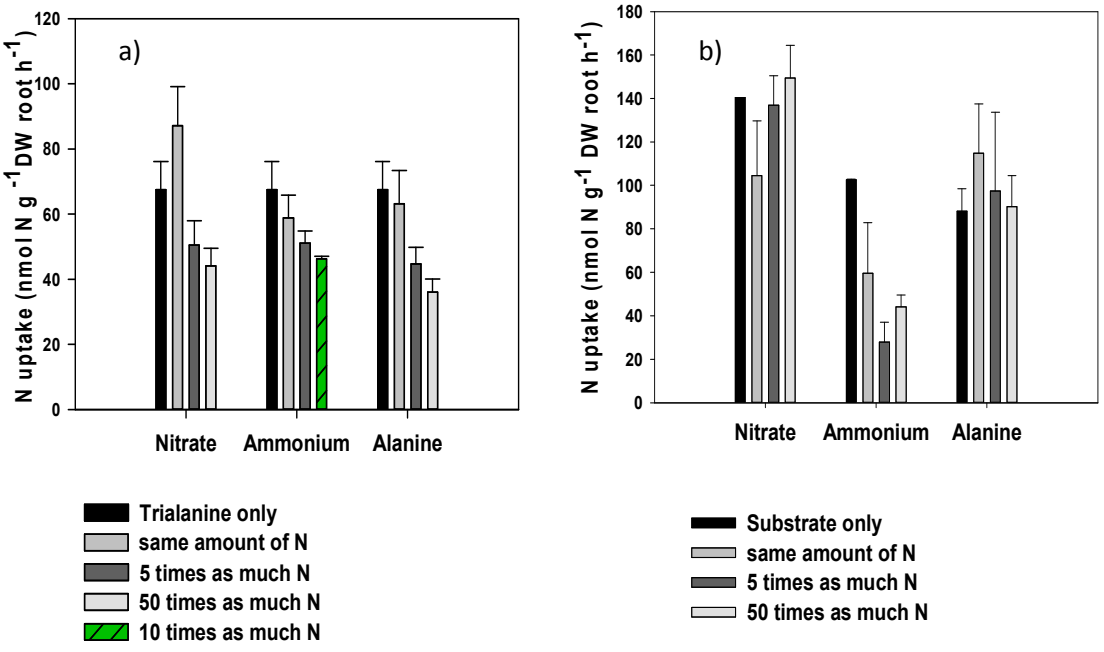

Fig.4.3. Graphs showing the uptake of peptides by roots of wheat plants growing in soil (a) explaining how the peptide uptake was affected by availability of other forms of nitrogen ^{15}N-^{13}C L-trialanine with unlabelled L-alanine or KNO_3 and NH_4Cl, (b) showing how uptake of other forms of N ($K^{15}NO_3$, $^{15}NH_4Cl$ or ^{13}C-^{15}N-L-alanine) was affected by availability of unlabelled L-trialanine. Values are means ± SEM, *n*=3

4.3.6. Nitrogen uptake by soil microbes

Sterilisation of soil was expected to inhibit both microbial and extracellular enzyme (e.g. peptidase) activity. Therefore, the concentration of the N forms in soil solution is controlled by mixing and sorption to soil particles. Under sterile conditions, the N retained in the first minute was 18.7 ± 0.8 (nmol N g^{-1} DW) in the soil which had NH_4^+ added, and 20.6 ± 1.15 (nmol N g^{-1} DW) at 60 min. For the soil that had NO_3^-

added, the N retention for the first minute was 8.3 ± 1 (nmol N g^{-1} DW) and after 60 min, it was 14.4 ± 2 (nmol N g^{-1} DW). The retention rates for trialanine and alanine in the first minute were 20.8 ± 0.5, 20.0 ± 0.2 (nmol N g^{-1} DW), respectively, and after 60 min they were 21.1 ± 0.3 and 21.7 ± 0.2 (nmol N g^{-1} DW), respectively. Assuming that NO_3^- was not bound to soil particles and remained mostly in solution, it can be estimated that around 10% of the labelled substrate added remains in the soil due to incomplete extraction of soil solution.

In non-sterile soils, the half-life ($t_{1/2}$) for microbial removal of alanine from soil solution (0.68 ± 0.12 min) was lower than that of trialanine (1.76 ± 0.46 min), but this was only significant at the $P < 0.1$ level. The rate of uptake of N by soil microbes was significantly greater for the organic N forms (17.7 ± 5.9 and 13.7 ± 2.8 nmol g^{-1} FW min^{-1} for trialanine and alanine, respectively), whilst the rate of NH_4^+ uptake (3.2 ± 0.87 nmol g^{-1} FW min^{-1}) was faster than for NO_3^- (0.75 ± 0.26 nmol g^{-1} FW min^{-1}). This suggests that soil microbes take up more amino acids and peptides than NH_4^+. Microbes did not take up NO_3^- to any great extent (Fig. 4.4).

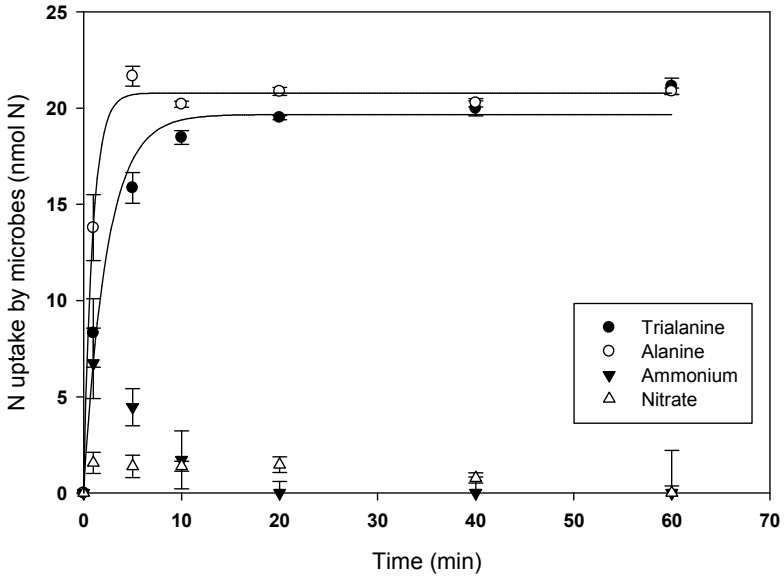

Fig. 4.4. N uptake by microbial after addition of ^{14}C-labelled L-alanine or L-trialanine or $^{15}NH_4Cl$ or $K^{15}NO_3$. The lines are fits of first -order exponential model. Values are means ± SEM, n=3.

4.3.7. Respiration of organic forms of nitrogen

Sterile wheat plants rapidly released $^{14}CO_2$ when they were supplied with either ^{14}C-labelled alanine or trialanine, showing that both these forms of N were metabolised in the plant (Fig. 4.5). Almost twice as much C was respired from plants supplied with trialanine ($P < 0.04$). However, when normalised by the C content of alanine and trialanine, the proportion of available C respired was not significantly different between the two organic forms of N.

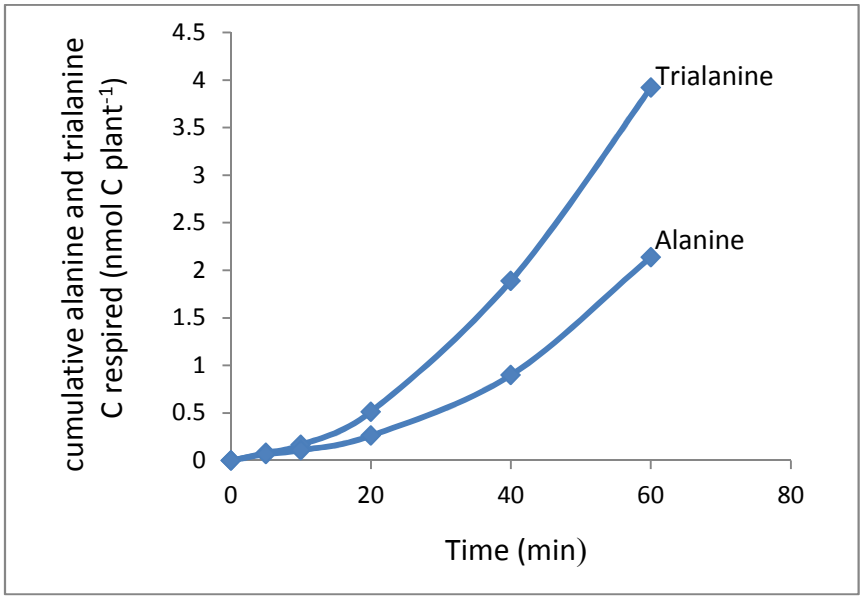

Fig.4.5 Respiration of organic N forms by sterile plants, C respired after addition of ^{14}C-alanine or ^{14}C-trialanine. Values are means ± SEM, (*n=3*).

4.4. Discussion

4.4.1. Phosphor imaging of plant peptide and amino acid acquisition

Phosphor images of plants supplied with ^{14}C L-alanine and L-trialanine clearly show that both the amino acid monomer and its tripeptide were taken up by wheat roots. Although it is not known whether translocation of intact molecules took place, it is clear that ^{14}C delivered to roots as both alanine and trialanine was translocated from roots to shoots.

4.4.2. Uptake of different nitrogen forms by sterile plants

Plants with sterile roots showed an apparent preference for NH_4^+, and the uptake of NO_3^- was significantly slower than the other N forms. The same results were found by Hill et al. (2011a), and they found similar root uptake for both of the L- forms of alanine and trialanine. They also showed that peptides were taken up rapidly even in the presence of other N forms. Here, this was further investigated.

The availability of different N forms (NO_3^-, NH_4^+ and alanine) decreased peptide uptake, relative to trialanine only. The availability of peptide had no significant effect on NO_3^- uptake. Ammonium uptake was significantly lower when trialanine was added, and alanine uptake was also significantly lower when trialanine was applied. It appears that, with the exception of trialanine on NO_3^-, an increase in N, regardless of form, decreased the uptake of the labelled form. This suggests that plants were getting more N than they required at the higher N concentrations. It is not clear why NO_3^- uptake was not down-regulated in the same way as other N forms. This may relate in some way to the fact that NO_3^- uptake was always lower than that of other forms of N, even after their down regulation.

It is clear from the capture of $^{14}CO_2$ in respiration from sterile plants that both the amino acid and the monomer were rapidly metabolised in the plant. The ratio of ^{15}N to ^{13}C recovered in plants suggests that around 35 % of the alanine and trialanine C taken up was lost in respiration during the experiment.

4.4.3. Uptake of peptides by the soil microbial community

In sterile soil, the biotic retention of NH_4^+, trialanine and alanine were not significantly different and were significantly higher than that of NO_3^-. Whilst there is no net charge for trialanine and alanine at the pH of the experimental soil (pH 6), these results still suggests that they were adsorbed to particle surfaces as NH_4^+ was adsorbed to cation exchange sites.

In non-sterile soil, the microbes rapidly took up the alanine and trialanine, and the rate of uptake of N by soil microbes was significantly greater for the organic N forms, supporting the findings of Hill et al. (2011a). This implies that the amino acids

and peptides were all rapidly assimilated by the soil microbial community, which is consistent with past reports showing rapid microbial immobilization of amino acids and peptides. Microbial uptake of NH_4^+ was low, and microbes did not uptake NO_3^-. This probably relates to the fact that, energetically, the metabolism of NH_4^+ is favoured over NO_3^- (Jones et al., 2004; Hill et al., 2011a).

4.4.4. The uptake of nitrogen by plants grown in soil

The N uptake by sterile plants was much higher than by those grown in soil. This was in complete contrast to the results for sterile roots, where NO_3^- was taken up by plants more than the other forms of N. Some previous studies have suggested that microbes frequently compete better for organic N than plants (Jones et al., 2005; Kuzyakov and Xingliang, 2013). Our results appear to support this conclusion. Also, sorption to soil particles can reduce N availability to plants and microbes. As our results for both plant uptake and retention in sterile soil indicate, this is especially true of cationic forms of N, such as NH_4^+. In contrast, NO_3^- is more mobile in soil and is also less attractive to soil microbes (Abaas et al., 2012). The lower microbial uptake of NO_3^- relative to amino acids and peptides means that $^{15}NO_3^-$ remains in the soil solution pool much longer than amino acid forms of ^{15}N. In contrast, removal of amino acids and peptides from the soil solution occurred within minutes. Consequently, it may be that even relatively short pulse-chase periods of a few hours lead to higher apparent uptake of NO_3^- than occurs when there is a constant release of amino acid forms of N into the soil solution. Thus, although it is clear that, relative to amino acids and peptides, NO_3^- uptake is much greater in soil than it is in sterile solutions, some caution must be

exercised when interpreting the results of pulse-chase experiments using forms of N with different residence times in soil solution.

It took greater concentrations of other N forms to decrease the uptake rate of peptides in soil than it did in sterile solutions. This could be due to greater N demand because of uptake by microbes in relation to alanine, and perhaps NH_4^+, but seems unlikely in the case of NO_3^-. Higher peptide concentrations decreased NH_4^+ uptake, but not alanine uptake as it did in sterile solution. Again, this may relate to rapid removal of the peptide from the soil solution by soil microbes. The inconclusive $^{13}C/^{15}N$ ratio in plants grown in soil may also suggest that alanine and trialanine were mineralized prior to plant uptake and that their influence on uptake of other forms of N was not necessarily due only to their organic form.

4.5. Conclusions

We found that the microbes prefer organic N forms more than inorganic forms. Sterile plants can uptake intact peptides without first requiring extracellular cleavage and metabolize them. This finding shows that plants have the ability to compete with microbes for organic N sources, like peptides, at an early stage in protein decomposition. The uptake of trialanine by plants was affected by the availability of other N forms, and peptide availability also reduced plant uptake of all forms of N other than NO_3^-. However, in soil this did not appear to take place at realistic soil solution concentrations of any forms of N. The uptake of trialanine N by plants in soil was less than the inorganic forms (53.5 % less than NO_3^-), but it was still not inconsequential. However, whether it was taken up intact and is ultimately of ecological significance remains unclear.

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Chapter 5

Ability of plants to directly acquire protein and potential competition with microorganisms

5.1. Introduction

Nitrogen (N) is an essential element for plant growth and is acquired directly from soil or via symbiosis with N_2-fixing microorganisms. There are a number of chemical forms of N that can be taken up by plants. For example, it has been shown that plants can take up NO_3^-, NH_4^+, urea and amino acids (Read, 1991; Lipson& Näsholm, 2001; Näsholm & Persson, 2001; Neff et al., 2003; Schimel & Bennett, 2004; Rentsch et al., 2007). In chapter 2 of this thesis it was confirmed that plants have the ability to directly take up intact peptides from outside the root. This evidence shows that plants at least have the ability to compete with microbes for N at the early stages of protein decomposition. However, exactly at which stage of protein decomposition plants can take up N is still unclear. Protein is the main form of N in plants, comprising about 2-20% of a plants dry weight, making it one of the main inputs of N to the soil upon plant tissue senescence (Jones et al., 2005; Schulten and Schnitzer, 1998). If a plant had the ability to take up protein directly it would provide a competitive advantage when in competition with soil microbes many of which are incapable of taking up molecules of this size (Kuzyakov and Xingliang, 2013). However, there has been only limited study the potential for the direct uptake of proteins by plant roots, very few of which have been undertaken in soil (Paungfoo-Lonhienne et al., 2008).

The aim of this study was to find out whether plants can directly acquire proteins intact from both soil and sterile culture. This outcome of this work is to provide evidence for the ability of plants to compete with microorganisms at the earliest possible stage in the soil N cycle.

5.2. Material and methods

5.2.1. Seed sterilization

Seeds of wheat (*Triticum aestivum* L. cv Atlas) were sterilized in ca.14% sodium hypochlorite with 1 drop of Tween for 20 min followed by 2 min in 90% ethanol. Seeds were washed thoroughly in sterile water 3 times. Sterilized seeds were placed on the surface of 10% Murashige and Skoog (MS) agar (Murashige and Skoog, 1962) and sealed in Phytatrays® (Sigma Aldrich, Gillingham, UK). After adding the seeds, the Phytatrays were placed in a growth cabinet with light intensity of 500 µmol m^{-2} s^{-1} PAR, temperature of 25°C and relative humidity of 70%.

5.2.2. Plant growth media

Four types of plant growth media were prepared based on that produced by Murashige and Skoog (1962), but amended with N in the following forms:

1. MS media without N (negative control)
2. MS media with inorganic N (positive control; 10 mM)
3. MS media with hydrolysed casein protein (10 mM)
4. MS media with unhydrolysed casein protein (10 mM)

The total volume for each media was 320 ml. Each protein was filtered before addition using a sterile 0.2 µm syringe filter. The concentration of the protein stock solution was 50 mM. Unhydrolysed casein protein was passed through a 1 kDa ultrafiltration membrane (Millipore, Billerica, Massachusetts, USA), to take out any impurities. After filtering greater than half of the solution, the fraction which > 1 kDa was used for the plant growth experiment. In addition, this protein fraction was mixed with α-Cyano-4-hydroxy cinnamic acid (4 h at 20 ºC) and analysed by matrix-assisted laser desorption/ionization-time of flight mass spectrometer (Bruker Dattonics, Billerica,

MA, USA) after mixing with TiO_2 and a protein internal standard to account for differing ionization efficiency.

At the 5 leaf stage, plants were removed from agar and washed in deionized water (1.5 min), followed by $CaCl_2$ (100 mM) for 1.5 min, followed by rinsing with deionized water. All the plants were put in paper bags and dried for 24 hours at 80 ºC, followed by weighing and recording the dry weight of the root and shoot biomass.

5.3. Results

The results for this experiment were poor and are therefore not presented. The following results or problems were encountered:

1. The mass spectrometer peak for the > 1 kDa fraction of unhydrolysed casein protein solution showed that there was no low MW contamination presents (Fig. 5.1).
2. There were many traces of microbial contamination from different types of bacteria and fungi, especially in the hydrolysis and unhydrolysed protein treatments.
3. Some seeds did not grow very well. That was in all the treatments.

5.4. Potential Contamination Sources

Whilst the incomplete sterilisation of seeds may have been a potential cause of contamination, previous use of this method has produced sterile plants. Therefore, it seems unlikely that this step introduced contamination. All the equipment was sterilised prior to syringe filtering the protein and this process was conducted in a laminar flow cabinet. However, there is a possibility that during the process some contamination may have occurred. The ultrafiltration process was not conducted in a laminar flow

cabinet so this process could be the cause of contamination. Conducting this process in strictly sterile conditions may improve the method and eliminate contamination.

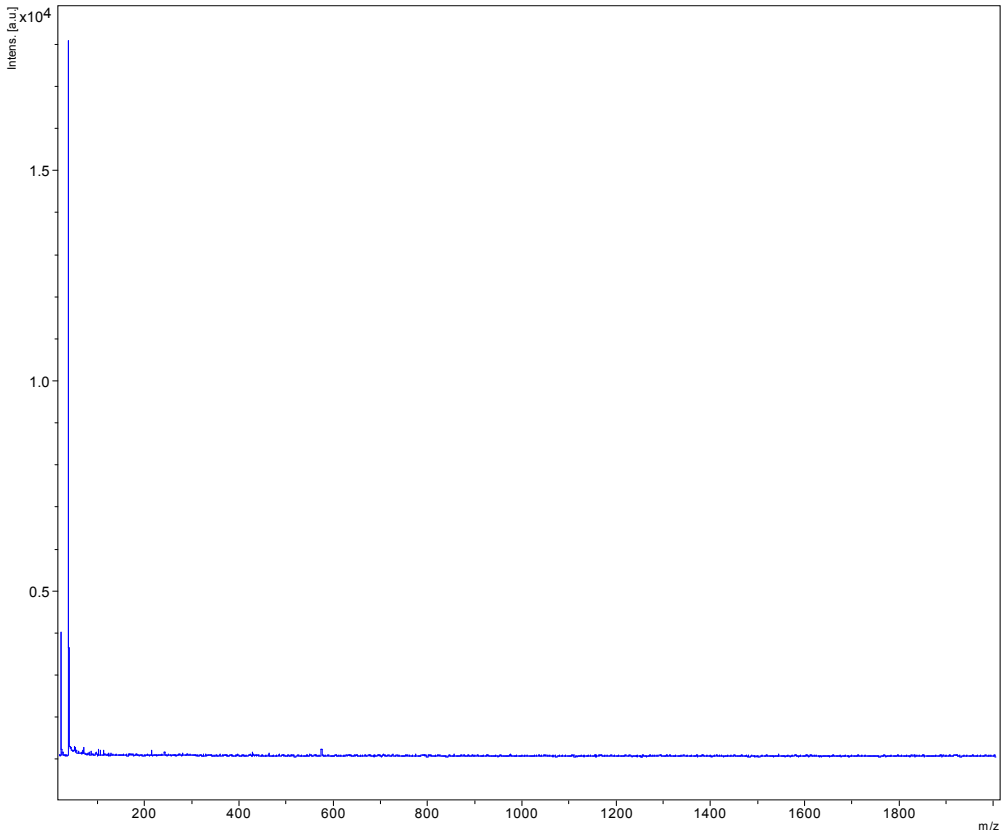

Fig. 5.1. Mass spectrometer peak for unhydrolysed Casein protein showing no low MW amino acids and peptides present.

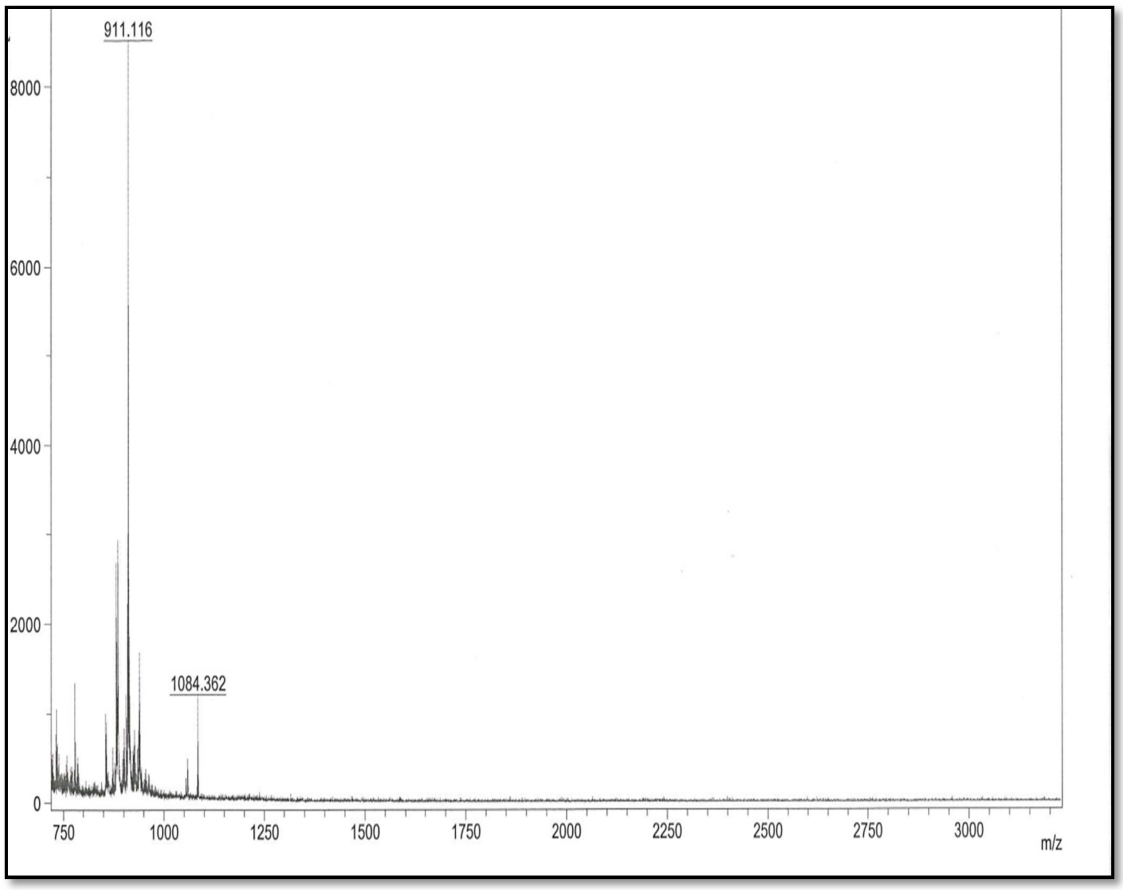

Fig. 5.2. Mass spectrometer peak for unhydrolysed Casein protein showing the low MW amino acids and peptides present in the < 1 kDa fraction.

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Chapter 6

Nitrogen availability down the soil profile in a wheat cropping system: Relationships with root density, soil N reserves and microbial activity

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Keywords: Ammonium; Dissolved organic nitrogen; Nitrate; Plant-microbial competition; Rhizosphere

Abstract

Most studies on plant nutrition tend to focus on the topsoil (plough layer) and frequently neglect subsoil processes. However, plant roots can potentially acquire nutrients such as organic and inorganic nitrogen (N) from deep in the soil profile. Greater knowledge of N cycling and water availability in subsoil is therefore important to capitalise on the deep rooting ability of a number of cereals. This study aimed to evaluate the relationship between root distribution, organic and inorganic N availability and potential N supply in a sandy textured Eutric Cambisol at the critical growth period during the wheat cropping cycle. Significant microbial potential activity was evident in the subsoil. The rate of plant residue turnover and the mineralization of low molecular weight compounds (e.g. glucose, amino acids and peptides) declined with increasing

soil depth; however, these rates were not correlated with basal soil respiration. This suggests that the microbial population in subsoil is highly C limited but that its activity can be readily stimulated upon addition of a C substrate. Significant N turnover can also occur at depth, although N mineralization again appears to be substrate limited. Root density declined in an exponentially pattern down the soil profile, with few roots present past 50 cm, suggesting that this is the major factor limiting C recharge of soil organic matter and microbial activity in subsoil. Vertical soil moisture profiles suggest that these deep roots are proportionally more important in water uptake than are those at the soil surface. Our hypothesis that subsoil provides an additional N reservoir when topsoil dry out was not supported. Greater root proliferation at depth could allow greater recapture of N lost by leaching; however, our results suggest that plant-microbial competition for N will be as intense in subsoil as it is in topsoil.

6.1. Introduction

Nitrogen (N) is typically the primary regulator of agroecosystem productivity, so that food security depends on maintaining an adequate supply of N over successive cropping cycles (Jones, 2009). However, N cycling is highly dynamic, with the relative abundance of N known to vary both spatially and temporally within the soil profile in response to a myriad of factors (Aber and Driscoll, 1997; Butterbach-Bahl et al., 2011). Agricultural systems therefore need to carefully balance the supply of plant N while minimising N losses to the wider environment. While this goal is simple in concept, achieving it has proved difficult, despite many decades of research (Raun and Johnson, 1999; Cassman et al., 2002).

In high input agricultural systems, N availability is largely controlled by fertiliser events and the subsequent transformation and redistribution of N within the soil (Van Egmond et al., 2002). Many countries, however, are aiming to reduce the reliance on mineral fertilisers and to use added and intrinsic soil N reserves more efficiently. Ultimately, the goal is to reduce economic costs as well as simultaneously lower the losses due to leaching (NO_3^-), denitrification (N_2/N_2O) and volatilisation (NH_3). Increases in N efficiency can potentially be achieved using a range of plant-based strategies (e.g. changes in root architecture combined with deeper rooting, release of nitrification inhibitors, use of N-fixers; Di et al., 2008; Zehang and Wang, 2013) as well as changes in agronomic practices (e.g. improvements in fertiliser timing, formulation, placement; Sartain and Obrezai, 2010). Some of these scenarios suggest that plants will likely have to take up and utilise a wider range of organic and inorganic N forms (e.g. amino acids, peptides and polyamines). Further, we predict that increased competition with the soil microbial community will occur to both mineralise N contained in SOM (via the direct release of root proteases or stimulation of SOM priming) and the capture of any N released (Bardgett et al., 2003; Farrell et al., 2012).

Soils frequently become progressively drier during the growing season, resulting in a reduced root capture of water and nutrients from the topsoil and the growth of roots to depths in excess of 1.5 m. This suggests that subsoil may play a significant role in N supply later in the season, especially under reduced fertiliser input regimes. Plant and microbial N cycling, however, have received much less attention in deeper soil horizons than in surface soils. If we are to capitalize on the deep rooting phenomenon of most cereals and our recent potential to manipulate root architecture, it is important that we understand water and nutrient availability in

deeper soil layers as well as the microbial processes that control them. The capability of plants to utilise different forms of N is also unclear, a situation that needs to be addressed (Cardona et al., 2013).

Agronomic estimates of N supply to plants are typically predicted from the amount of inorganic N released during the laboratory incubation of soils collected from within the plough layer (0-30 cm). These mineralization rates are unlikely to be representative of deeper soil layers, so that ways of integrating potential N supply from subsoil are therefore needed. The amount and turnover of N in subsoil will largely depend on the subsoil's exchange capacity, structure, organic material availability and microbial activity. Significant microbial activity is well established to occur at depth (Doran, 1987; Hadas et al., 1989; Soudi et al., 1990), albeit at much lower levels and with a different community structure than occurs in topsoil (Federle et al., 1986; Qualls and Haines, 1992). Physical parameters such as water availability, temperature and oxygen availability are also important factors that differ in subsoil and that can readily affect microbial activity. The quantity and quality of organic inputs to subsoil may also differ from those of the soil surface due to lower rates of root and microbial turnover and the lack of leaf litter and crop residue inputs. Subsoil C has also been suggested to be older and more recalcitrant than C held in subsoil (Schrumpf et al., 2013). While this may favour C sequestration, it may conversely limit N supply to the plant.

Root length density (RLD) has been used as a proxy to predict water and nutrient uptake by plants (Taylor and Klepper, 1975; Herkelrath et al., 1977). This relationship can work well when soil moisture is adequate; however, it lacks precision when surface soils become dry. The root systems of mature wheat plants typically extend to depths greater than 120 cm by the end of the growing season. However, the

time at which maximal crop N demand and subsoil exploitation coincide earlier in the season (i.e. GS31-71; HGCA, 2010). This study therefore aimed to evaluate the relationship between root distribution, organic and inorganic N availability and potential N supply at this critical period during the wheat growing cycle.

6.2. Materials and methods

6.2.1. Site characteristics

Soil was obtained from a replicated wheat field trial site located in Abergwyngregyn, Gwynedd, North Wales (53°14'29"N, 4°01'15"W). The soil is classified as a Eutric Cambisol, with a sandy loam texture and a single grain structure. The climate at the site is classed as temperate-oceanic with a mean annual soil temperature of 11°C at 10 cm depth and a mean annual rainfall of 1250 mm yr^{-1}. The field trial consisted of six replicated plots (12.5 × 3 m), which were ploughed (0-30 cm) and planted with spring wheat (*Triticum aestivum* L. cv. Granary) in May 2013. Fertiliser was added after crop emergence (60 kg N ha^{-1} as ammonium nitrate, 80 kg K ha^{-1}, 28 kg P ha^{-1}) and dicot herbicides applied following standard agronomic practices.

Soil samples were collected from the plots in July, 2013, when the plants had reached late stem extension (Feekes growth stage 9, Zadoks growth stage 39; Large, 1954; Zadoks et al., 1974). Root density was estimated from intact soil cores taken to a maximum depth of 80 cm using a Cobra-TT percussion hammer corer (Eijkelkamp Agrisearch Equipment, 6987 EM Giesbeek, The Netherlands). After removal from the soil, the intact cores were split into 10 cm sections, the samples transferred to plastic bags and placed at 4°C to await root recovery. Few roots were present in the 60-80 cm layer, so soils were only sampled to 60 cm for the microbial N cycling and N pool size

estimates. Briefly, samples were collected at 10 cm intervals to a depth of 60 cm and the samples were stored in CO_2 permeable polythene bags. Upon return to the laboratory, the soils were immediately sieved to pass 2 mm, removing any vegetation, stones and earthworms.

Crop height and biomass were determined weekly by destructive sampling throughout the growing season. Briefly, in six replicate plots, all the crop biomass was removed within a sub-plot (0.5 m × 0.5 m), the samples placed in paper bags and the harvested biomass dried at 80°C for 7 d to determine its dry weight. At the same time, crop height was recorded at 5 points (1 m apart) within each of the 6 plots.

6.2.2. Quantification of root biomass

Roots were washed from the soil by a combination of mechanical shaking and flotation, using a 1 mm mesh to capture roots. The roots were then placed on 20 × 20 cm clear plastic plates and root length determined with WinRhizo® (Regent Instruments Inc., Canada).

6.2.3. Water content

Soil water content was determined weekly by destructive sampling throughout the growing season. Briefly, a spade was used to obtain topsoil (0-30 cm) and subsoil (30-60 cm) from six replicate plots. The samples were then sieved to pass < 2 mm and a subsample used to determine moisture content by drying at 105°C overnight. The gravimetric moisture contents were corrected for stone-corrected bulk density to allow expression of water content on a volumetric basis.

6.2.4. Soil solution extraction and soil chemical analysis

Soil N availability was estimated according to Jones and Willett (2006). Briefly, 5 g of field-moist soil was extracted with 25 ml of 0.5 M K_2SO_4 on a reciprocating shaker (Edmund Bühler GmbH, SM-30, Germany; 200 rev min^{-1}) for 60 min. After shaking, the samples were centrifuged (10 min; 1699 *g*) and the supernatant recovered and stored at -20 °C until analysis.

Soil solution samples were analysed for dissolved organic C (DOC) and total dissolved N (TDN) using a Multi N/C 21005 (Analytik-Jena AG, Jena, Germany). Total amino acid-N was determined fluorometrically using the *o*-phthalaldehyde-β-mercaptoethanol procedure of Jones et al. (2002). NO_3^- and NH_4^+ were analysed colourimetrically using the methods of Miranda et al. (2001) and Mulvaney (1996). Soil solution was also passed through a 1 kDa ultrafiltration membrane (Millipore, Billerica, MA, USA). The concentration of small (<1 kDa) peptides was subsequently determined by measuring total amino acid before and after hydrolysis in 6 M HCl for 16 h at 105 ºC (Amelung et al., 2006).

Total C and N of soils were determined using a Truspec® CN analyser (Leco Corp., St Joseph, MI, USA). Soil pH and electrical conductivity (EC) were determined in soil: distilled water extracts (1:5 v/v) with standard electrodes, while moisture content was determined by drying at 80°C. Basal soil respiration was determined in the laboratory at 20°C over 24 h using an SR1 automated multichannel soil respirometer (PP Systems Ltd, Hitchin, UK),

6.2.5. Net nitrification

Net nitrification was determined according to Hart et al. (1994). Briefly, 5 g of field-moist soil from each soil layer was placed in a 50 cm^3 polypropylene tube and 0.5

ml of NH_4Cl (100 mg l^{-1}) added to the soil surface. The tubes were then loosely sealed and the samples incubated in the dark at 20°C. After 1 or 7 d, the soil was subsequently extracted with 0.5 M K_2SO_4 and NO_3^-, determined as described above. Net nitrification was calculated as the amount of NO_3^- in the sample after a known incubation time minus that present at the start of the experiment (i.e. NO_3^- extracted on day zero).

6.2.6. Net N mineralization

Net N mineralization was determined by anaerobic incubation according to Waring and Bremner (1964) and Kresoivć et al. (2005). Briefly, 10 g of field-moist soil was placed in 50 cm^3 polypropylene tubes and anaerobic conditions imposed by filling the tubes with distilled water and then sealing the tubes. The soil samples were then incubated for 7 d in the dark at 40ºC. Subsequently, solid KCl was added to achieve a final concentration of 1 M KCl and the samples were extracted by shaking for 60 min (200 rev min^{-1}). The extracts were then centrifuged (1699 *g* 10 min) and NH_4^+ and NO_3^- content determined as described previously. Net ammonification was calculated as the amount or NH_4^+ present after 7 d minus that present at the start of the experiment (i.e. NH_4^+ extracted on day zero).

6.2.7. Amino acid, peptide and glucose turnover

The rates of turnover of low molecular weight (MW) DON were estimated by determining the mineralization of amino acids and peptides. For comparison, the turnover of glucose was also used as a general reporter of soil microbial activity (Coody et al., 1986). Briefly, field-moist soil (5 g) was placed in 50 cm^3 polypropylene containers and 0.5 ml of either ^{14}C-labelled glucose (25 mM, 1.85 kBq ml^{-1}), amino acids (10 mM, 1.55 kBq ml^{-1}) or peptides (25 mM, 1 kBq ml^{-1}) was added to the soil

surface (Farrell et al., 2011). After the addition of each ^{14}C-substrate to the soil, a $^{14}CO_2$ trap containing 1 ml of 1 M NaOH was placed above the soil and the tubes were sealed. The tubes were then incubated at 20°C for 30 min, after which the NaOH traps were removed to determine the amount of substrate mineralization. The ^{14}C content of the NaOH traps was determined with a Wallac 1404 liquid scintillation counter (Wallac EG&G, Milton Keynes, UK) after mixing with Scintisafe3 scintillation cocktail (Fisher Scientific, Loughborough, UK).The amino acids consisted of an equimolar mix of 20 different L-amino acids (L-glycine, L-isoleucine, L-arginine, L-glutamine, L-phenylalanine, L-histidine, L-asparagine, L-valine, L-threonine, L-leucine, L-alanine, L-methionine, L-cysteine, L-lysine, L-tryptophan, L-serine, L-proline, L-glutamate, L-aspartate acid, L-ornithine) (Jones, 1999) while the L-peptides consisted of a mixture of equimolar L-dialanine and L-trialanine.

The rate of arginine mineralization was determined by adding 0.5 ml of a ^{14}C-labelled L-arginine solution (25 mM; 2.17 kBq ml^{-1}; Amersham Biosciences UK Ltd, Chalfont St Giles, Bucks, UK) to 5 g of field-moist soil and measuring the rate of $^{14}CO_2$ evolution over a 48 h period, as described in Kemmitt et al. (2006). After 48 h, the net amount of NH_4^+ and NO_3^- produced from the added arginine was determined by extracting the soil with 25 ml 0.5 M K_2SO_4 and subsequent analysis as described previously.

6.2.8. Mineralization of plant-derived Carbon

The microbial turnover of complex, plant-derived C across the different soil depths was evaluated according to Glanville et al. (2012). Briefly, high MW plant material was prepared by heating 2.5 g of ^{14}C-labeled *Lolium perenne* L. shoots (Hill et al., 2007) in distilled water (25 ml, 80°C) for 2 h. The extract was then centrifuged

(1118 *g*, 5 min) and the soluble fraction removed. The pellet was then resuspended in distilled water and the heating and washing procedure repeated twice more until > 95% of the water soluble fraction had been removed. The remaining pellet was dried overnight at 80ºC and ground to a fine powder.

The mineralization dynamics of the high MW plant material was determined by mixing 100 mg of ^{14}C-labelled plant material with 5 g of field-moist soil. The production of $^{14}CO_2$ was monitored as described above for the low MW substrates but the duration of the treatment was 40 d. To ensure that moisture was not limiting, the experiment was also repeated but after the simultaneous addition of distilled water (to reach field capacity) and the ^{14}C-labelled plant material.

6.2.9. Molecular analysis of microbial communities

DNA was extracted from the soil following the instructions of the MO-BIO power Lyzer power Soil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad).

6.2.10. Statistical and data analysis

Statistical analysis of the results was carried out by ANOVA followed by LSD post hoc test using SPSS v14 (IBM UK Ltd, Hampshire, UK) with $P < 0.05$ used as the cut-off for statistical significance.

6.3. Results

6.3.1. Site characteristics

At the time of the main sampling event, soil moisture content was significantly lower at soil depths below 30 cm than above ($P < 0.05$), except between 0-10 cm, which was not significantly different with any depth. The pH did not vary significantly with

depth, while the EC decreased significantly with increasing depth. Soil total organic C, total N, C: N ratio and soil respiration also decreased with increasing depth (Table 6. 1, Fig.6.1). Amino acid concentrations were greatest in the surface layer and at 20-30 cm depth than in deeper layers, while peptide concentrations did not vary significantly with depth. Both DOC and DON concentrations decrease significantly with depth.

As expected, crop height showed a sigmoidal extension pattern over the growing season, with full stem extension evident after 8 weeks. Crop biomass also showed a sigmoidal growth pattern; however, above-ground biomass continued to increase up until week 13 due (Fig 6.2).

Table 6.1. Soil properties at different soil depths in an agricultural wheat cropping soil. a, b, c and d indicates where significant differences occur between the different depths ($P < 0.05$). Values represent mean ± SEM (n=4)

Depth (cm)	Moisture Content (%)	pH	$EC_{1:1}$ (µS cm^{-1})	C:N ratio	Total N (g kg^{-1})	Total C (g kg^{-1})	Soil respiration (µmol CO_2 kg^{1})
0-10	8.8 ± 1.0 ab	6.1 ± 0.2a	34.9 ± 6.7a	8.2 ± 0.1 a	2.4 ± 0.2a	19.4 ± 1.5a	11 ± 1.9 a
10-20	10.6 ± 0.8a	6.3 ± 0.2a	27.9 ± 2.9 ab	8.1 ± 0.2 ab	2.0 ± 0.3ab	6.3 ± 2.4ab	11.8 ± 3.4a
20-30	9.6 ± 0.7 a	6.3 ± 0.1a	20.4 ± 1.7 bc	6.9 ± 0.6 bc	1.6 ± 0.2bc	11.6 ±2.2bc	4.6 ± 0.8 b
30-40	7.1 ± 0.7 b	6.3 ± 0.1a	16.9 ± 2.1 c	4.6 ± 0.2 cd	0.8 ± 0.1d	3.9 ± 0.6 d	1.8 ± 0.5 b
40-50	6.9 ± 0.6 b	6.4 ± 0.1a	15.6 ± 1.3 c	4.5 ± 0.8 d	1.1 ± 0.2d	5.3 ± 1.7 d	1.3 ± 0.2 b
50-60	6.7 ± 0.6 b	6.4 ± 0.1a	15.6 ± 0.8 c	3.4 ± 0.5 d	0.8 ± 0.1d	3.0 ± 0.8 d	1.2 ± 0.2 b

Depths (cm)	NH_4^+ (mg N kg^{-1})	NO_3^- (mg N kg^{-1})	Amino acids (mg N kg^{-1})	Peptides (mg N kg^{1})	DON (mg/ kg)	DOC (mg/ kg)
0-10	2.60 ± 1.0 a	5.0 ± 1.8 a	7.25 ± 2.13 a	2.8 ± 0.4 a	14.35 ± 3.79 a	85.13 ± 16.47 a
10-20	0.95 ±0.25b	1.9 ± 0.4b	4.16 ± 0.69 b	2.8 ± 1.7 a	10.69 ± 1.38 ab	68.50 ± 9.72 ab
20-30	0.57 ±0.17b	2.2 ± 0.5b	5.14 ± 1.05abc	2.3 ± 0.8 a	6.99 ± 0.87 abc	54.12 ± 9.11 bc
30-40	0.57 ±0.23b	1.8 ± 0.3b	2.29 ± 0.46bcd	2.7 ± 0.6 a	5.85 ± 1.65 bc	49.79 ± 8.93 bc
40-50	0.60 ±0.25b	1.7 ± 0.5b	1.25 ± 0.41 bd	3.4 ± 0.7 a	1.05 ± 0.45 cd	34.08 ± 3.73 c
50-60	0.56 ±0.09b	2.5 ± 0.5b	1.04 ± 0.16 d	3.4 ± 0.6 a	1.65 ± 0.45 d	36.35 ± 6.79 c

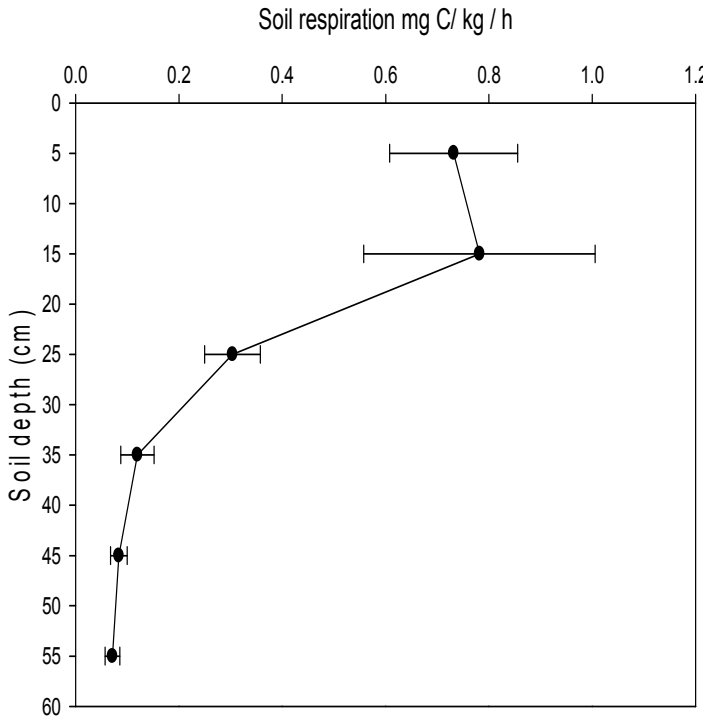

Fig. 6.1. Basal soil respiration at different depths in an agricultural wheat cropping soil. Values are means ± SEM (*n*=5).

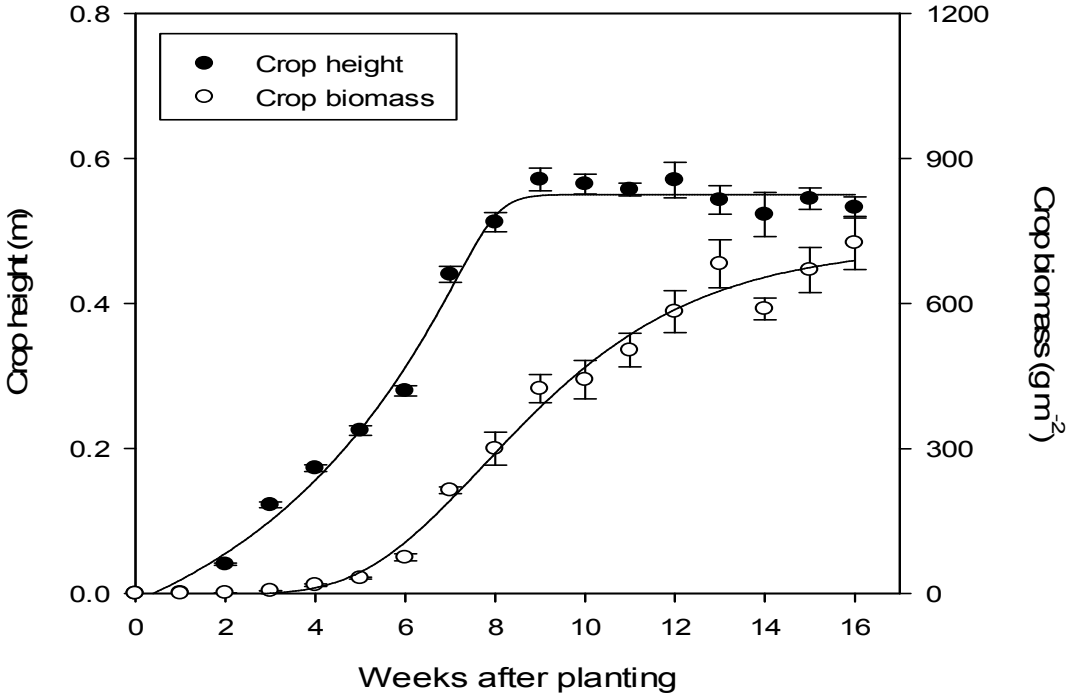

Fig. 6. 2. Crop biomass and stem extension over the wheat cropping cycle from planting to harvest. Values represent mean ± SEM (*n* = 6).

6.3.2. Soil water content

Overall, no significant differences were observed in soil water content between topsoil and subsoil layers at the start of the season (0-4 weeks). After this time (weeks 5-8), and corresponding with the period of maximum crop growth and low rainfall, soil water content declined dramatically so that the amount of water consistently lower in the subsoil than in the topsoil ($P < 0.05$). At week 9, significant amounts of rainfall caused recharge of the soil profile, with significantly more water retained in the topsoil than in the subsoil ($P < 0.01$; Fig. 6.3).

6.3.3. Soil net ammonification

Exchangeable and free NH_4^+ concentrations in the field-collected samples were significantly higher in the surface layer ($P < 0.05$) than in the deeper soil horizons. Below the surface (0-10 cm) layer, the NH_4^+ concentrations were not significantly different. The concentration of NH_4^+ after 30 days of aerobic incubation only increased significantly in the 10-20 cm soil layer. After 30 days, the NH_4^+ content within the 0-20 cm layer of the soil profile was significantly different from the bottom 40-60 cm ($P < 0.05$). In contrast to the aerobic incubation, the anaerobically incubated soils showed large increases in NH_4^+ concentration at all depths, with the largest increase occurring in the surface soil layer (Fig. 6.4).

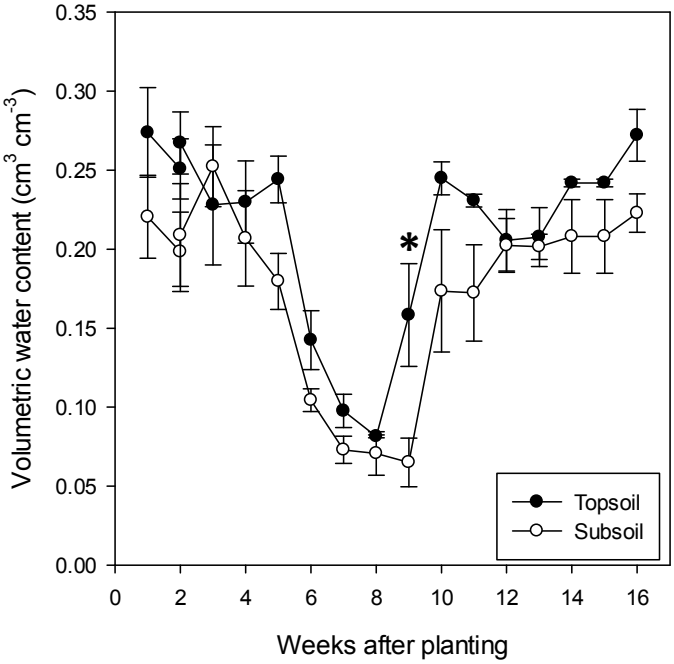

Fig. 6.3. Soil water content during the wheat cropping cycle from planting to harvest in the topsoil (0-30 cm) and subsoil (30-60 cm) layers. Values represent means ± SEM (*n* = 6). This symbol * denotes a significant difference.

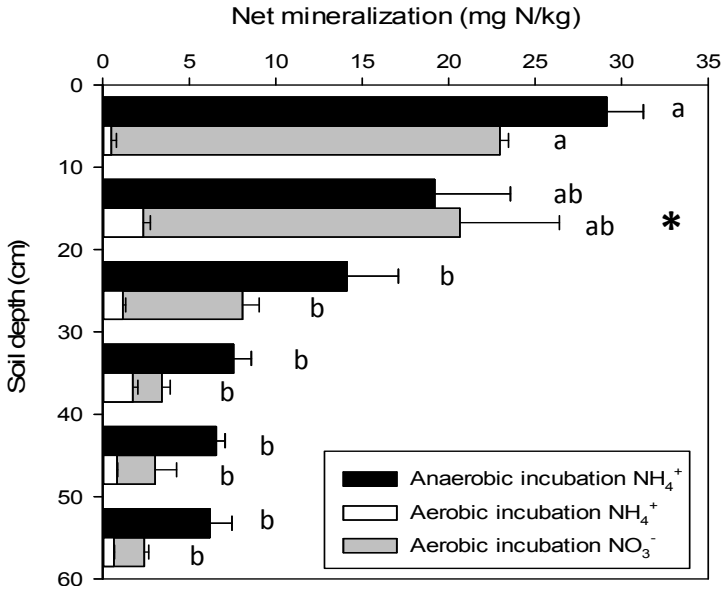

Fig. 6.4. Net mineralization and nitrification after incubation for 7 days under anaerobic conditions and net mineralization in aerobic conditions after incubation for 30 days at different soil depths in an agricultural wheat cropping soil. Values represent means ± SEM (*n* = 4). The letters a and b indicate significant difference between soil depths whilst * indicates an increase in NH_4^+ after aerobic incubation.

6.3.4. Soil net nitrification

The NO_3^- concentrations in the field-collected samples were significantly greater in the surface layer than at all other depths, except 50-60 cm (Table 6.1). The addition of an NH_4Cl spike and incubation of the soils for 24 h resulted in a significant increase in the concentration of NO_3^- for all depths except the 30-40 and 50-60 cm soil layers (data not presented). Incubation for 7 d led to further increases in the NO_3^- concentration in the soil surface layers (0- 30 cm) while the deeper soils did not increase significantly. Overall, the N mineralization in the aerobic and anaerobic incubations showed a similar decreasing exponential pattern down the soil profile (Fig. 6.4).

6.3.5. Low MW carbon substrate mineralization

The mineralization of glucose, amino acids and peptides was significantly greater above 30 cm soil depth than below 30 cm, as shown in Fig 6.5. Amino acids and peptides mineralised significantly more slowly in the 20-30 cm layer than in the overlying soil layers, while glucose mineralization in the 20-30 cm layer was significantly different from the surface layer but not from the10-20 cm layer. The top two soil layers did not differ from each other. The mineralization rate for glucose was lower than amino acids and peptides at all soil depths.

6.3.6. ^{14}C-arginine mineralization

The initial (0-6 h) arginine mineralization rate decreased with soil depth (Table 6.2). However, by 48 h, the amount of arginine mineralization was statistically similar at all soil depths. The rate of mineralization was linear in the topsoil; however, a significant lag phase in mineralization was observed in the subsoil horizons (Fig. 6.6).

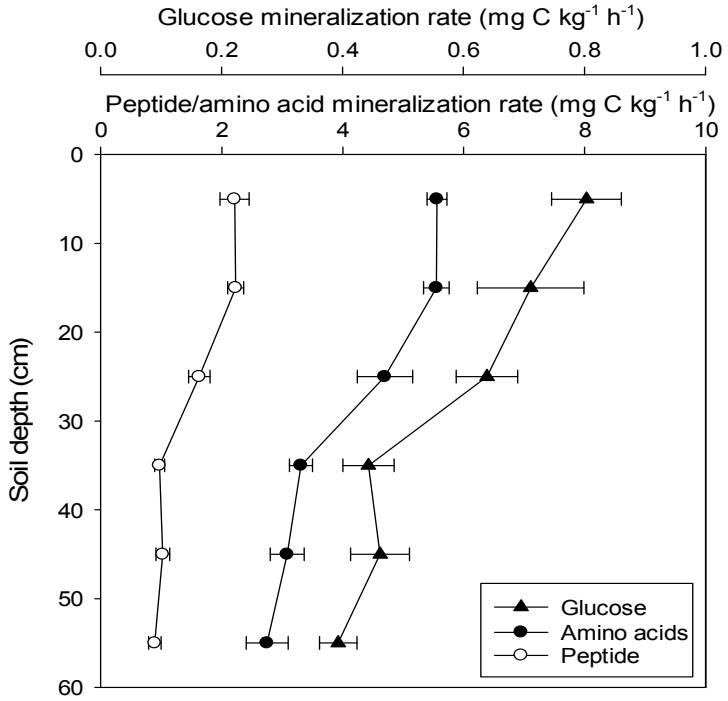

Fig. 6.5. Mineralization of ^{14}C-glucose, ^{14}C-amino acids and ^{14}C-peptides at different soil depths in an agricultural wheat cropping soil. Values represent means ± SEM (n = 4).

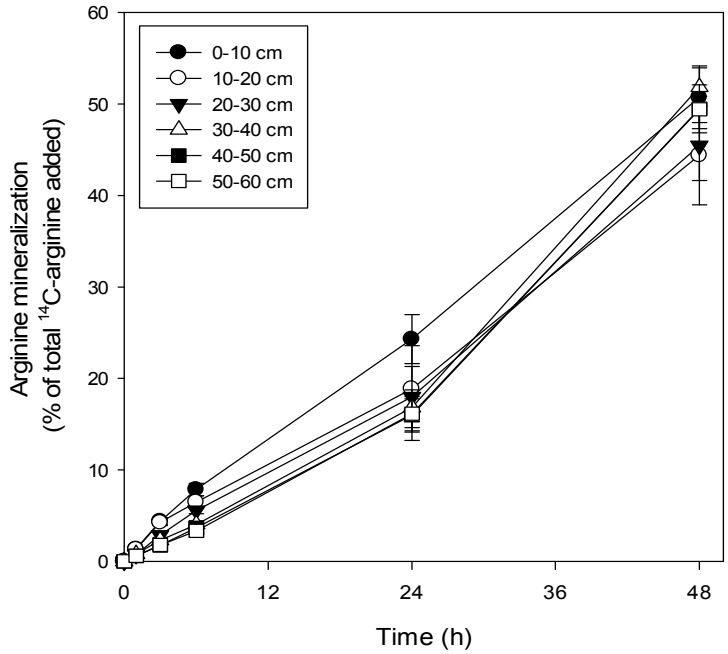

Fig. 6.6. Cumulative percentage of ^{14}C- arginine mineralization at different soil depths in an agricultural wheat cropping soil. Values represent means ± SEM (n = 4).

The net amount of NH_4^+ produced from added arginine significantly increased with soil depth. In contrast, however, the net amount of NO_3^- decreased significantly with increasing soil depth (Table 6.3). Overall, the ratio of C mineralization to N immobilization was highest in the topsoil than in the subsoil.

Table 6.2. Cumulative amount of $^{14}CO_2$ released from soil by the mineralisation of ^{14}C-labelled arginine as a function of soil depth in an agricultural wheat cropping soil. Values are percentage of the total arginine added. Values represent means ± SEM (n = 4).

Depth (cm)	Time after ^{14}C -arginine addition				
	1 h	3 h	6 h	24 h	48 h
0-10	1.4 ± 0.1	4.4 ± 0.4	7.8 ± 0.6	24.3 ± 2.6	50.7 ± 3.4
10-20	1.3 ± 0.1	4.2 ± 0.2	6.4 ± 0.7	18.8 ± 4.7	44.3 ± 5.4
20-30	1.0 ± 0.1	3.1 ± 0.2	5.8 ± 0.4	18.2 ± 3.4	45.6 ± 3.8
30-40	0.8 ± 0.1	2.3 ± 0.2	4.0 ± 0.4	16.8 ± 0.9	51.8 ± 2.1
40-50	0.6 ± 0.1	1.7 ± 0.4	3.6 ± 0.7	15.9 ± 2.7	49.4 ± 2.6
50-60	0.6 ± 0.1	1.7 ± 0.2	3.3 ± 0.6	16.1 ± 1.8	49.4 ± 1.4

Table 6.3. Net amount of NH_4^+ and NO_3^- released as a function of soil depth after the addition of arginine and incubation for 48 h. Values represent means ± SEM (n = 4).

Depth (cm)	NH_4^+ after 48 h (mg N/kg)	NO_3^- after 48 h (mg N/kg)
0-10	72.2 ± 1.9	18.3 ± 2.1
10-20	72.1 ± 2.7	12.4 ± 1.4
20-30	76.4 ± 6.7	7.8 ± 0.5
30-40	93.0 ± 3.8	4.2 ± 0.5
40-50	95.7 ± 3.2	4.5 ± 1.2
50-60	124.2 ± 2.8	5.2 ± 0.9

6.3.7. Plant residue mineralization

The amount of plant residue mineralized to $^{14}CO_2$ increased over time for soils in the two contrasting moisture states; however, no significant difference was noted for different soil depths ($P > 0.05$). The total amount of plant residue mineralized was lower in the dry soil than in the surface layer of the wetter soil but did not differ at the other depths (Figs. 6.7-6.8).

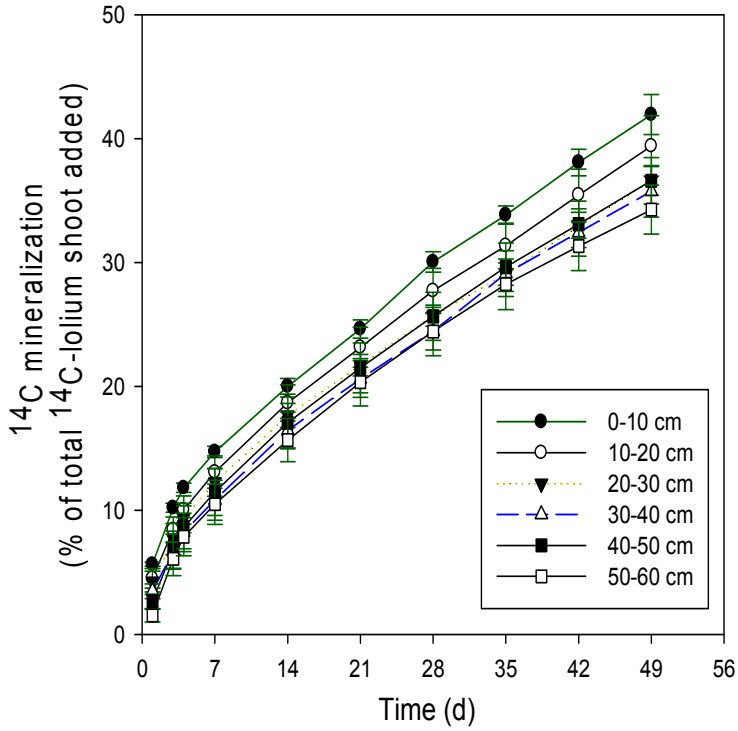

Fig. 6.7. Cumulative percentage of ^{14}C-lolium shoots mineralization after addition to soil at field capacity. Values represent means ± SEM (n = 4).

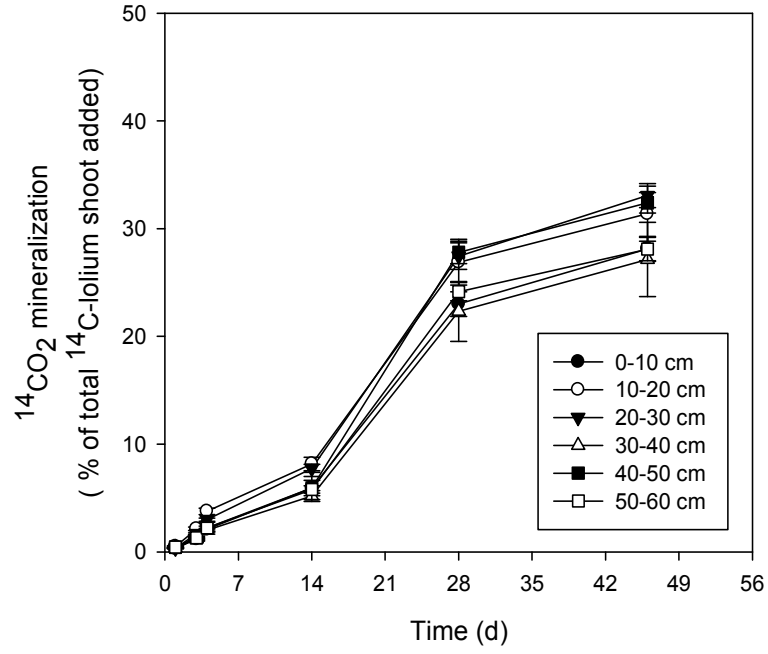

Fig. 6.8. Cumulative percentage ^{14}C -lolium shoot mineralization after incubation in a dry soil from different depths within a wheat cropping field. Values are mean ± SEM (n=4).

6.3.8. Root length density

Total root length decreased in a non-linear pattern down the soil profile. A significant decrease in lateral root length was noted between each successive 10 cm soil layer until a depth of 40 cm. Below 40 cm, the presence of root material is relatively low and no significant differences were noted between soil layers (Fig. 6.9).

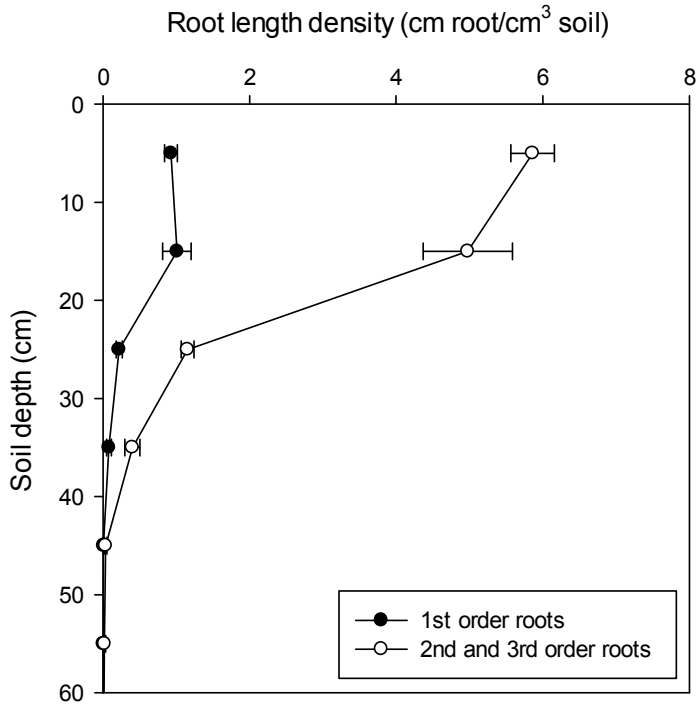

Fig. 6.9. Density of primary (first order) and lateral (second and third order) roots at different soil depths in an agricultural wheat cropping soil. Values represent means ± SEM (n = 4).

6.3.9. DNA concentration

The DNA concentration was highest in the surface soil layer and decreased approximately 6-fold down the soil profile. No significant difference was observed between the two top layers of soil and the two deeper layers ($P > 0.05$), while the DNA

concentration was significantly different at 20-30 cm compared to all other depths ($P < 0.05$), except for 30-40 cm layer (Table 6.4, Fig. 6.10).

Table 6.4. DNA concentration at different soil depths. Values represent mean ± SEM (n=4).

Depth (cm)	DNA content (ng μl^{-1})
0-10	46.8 ± 4.9
10-20	42.1 ± 4.3
20-30	21.3 ± 2.8
30-40	15.1 ± 1.7
40-50	9.9 ± 1.5
50-60	8.4 ± 1.1

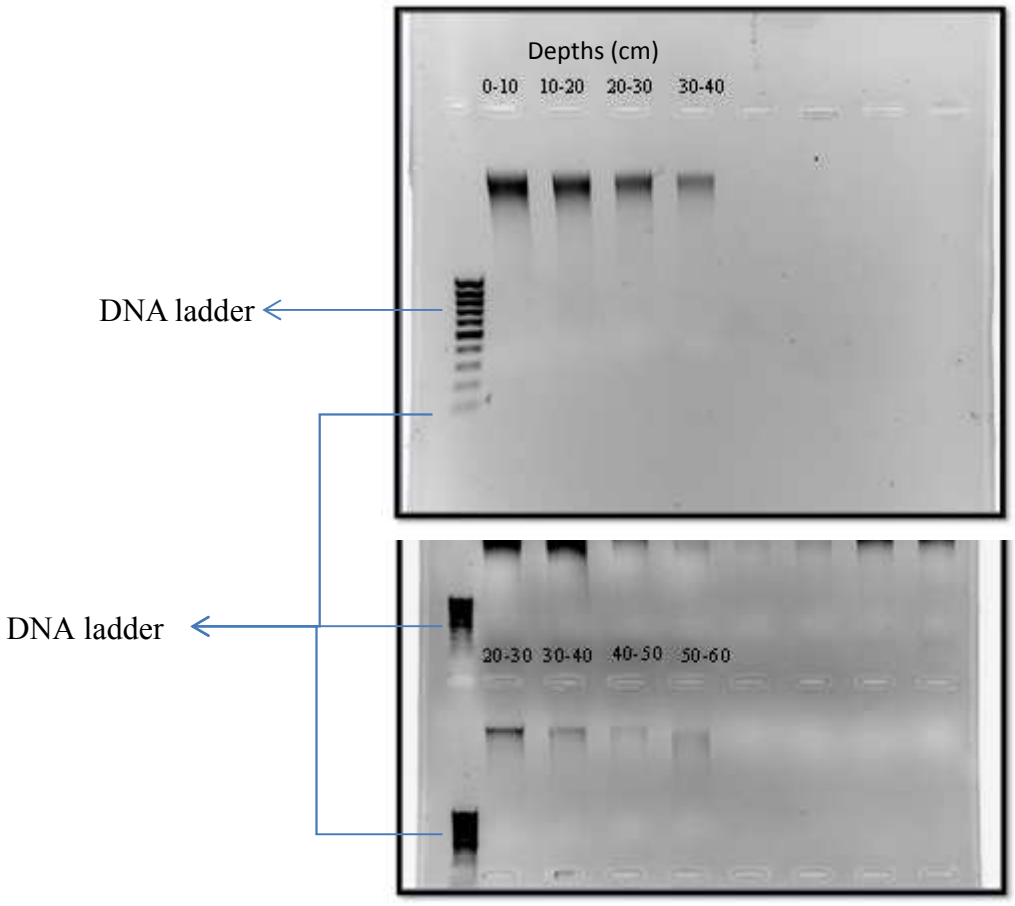

Fig. 6.10. DNA concentration at different soil depths. Bands represent individual replicate core.

6.4. Discussion

6.4.1 Changes in microbial activity with depth

Our results show clear evidence of microbial activity in the subsoil, with significant amino acid, glucose, peptide, plant residue and N mineralization occurring at high rates. Other studies have also shown microbial activity at depth causing pesticide and atrazine degradation at depths up to 1 m (Ajwa et al., 1998; Phelps et al., 1989; Kruger et al., 1993; Issa and Wood, 1999; Holden and Fierer, 2005; Charnay et al., 2005). However, microbial processes have a tendency to be greatest in the surface layers; decreased microbial activity at depth has also been observed previously. For example, Charnay et al. (2005) found little microbial activity and organic C below 2.5 m and Phelps et al. (1989) found that the microbial degradation of acetate and glucose at 6 m was a factor of 100 less than the rate observed at the surface.

Numerous factors could contribute to lower microbial activity in deep soils. The results obtained here show much lower root biomass at depth, so it is likely that less low MW DOC or fresh particulate C is being delivered to the subsoil. Fontaine et al. (2007) also found this to be the case. Microbes at depth experience C limitation, which is backed up by the decrease in C-to-N ratio with depth that was observed here. Past research has also shown that the microbial population changes and decreases with increasing soil depth (Kautz et al., 2013; Vinther et al., 1999; Taylor et al., 2002). Greater soil bulk density has also been observed at depth, which can cause physical protection of potential carbon sources by separating them from the bacteria (Kinyangi et al., 2006). The N concentrations also decreased with depth. While C limitation appears to be more important, the N may be less available to microbes and less mobile, as indicated by the decrease in the concentrations of amino acids, NH_4^+ and NO_3^-.

Reduced microbial activity at depth has implications for C storage in subsoil. However, while low MW compound mineralization decreased with depth, no difference in plant residue mineralization was observed with increasing depth (Charnay et al., 2005). The amount of plant residue mineralized in the dry soil is lower in the surface layer than under wet soil conditions, indicating that moisture deficit in topsoils may limit nutrient recycling. The plant residues degraded faster in the moist soil than in the dry soil; this is because dry conditions, such as those that occur during drought, limit the movement of substrate and enzymes.

6.4.2 Variations in the soil nitrogen cycling with depth

Protein is a major input of organic N to soil systems. Therefore, the mineralization of peptides and amino acids is an important part of the N cycle and supplies the substrate for inorganic N production. The breakdown of proteins to peptides has been suggested as the main rate limiting step in the soil N cycle. Peptide mineralization rate decreased with soil depth, although the concentration in soil solution remained constant. The amino acid mineralization rate decreased with depth, as predicted by Murphy et al. (1998). However, this was not apparent for arginine, which has a surprisingly long turnover time at all soil depths. Alef et al. (1988) found that most microbes could take up arginine. Potentially, more of the positively charged arginine could have been adsorbed to soil particle surfaces compared to the amino acid mixture. Amino acid concentrations decreased with soil depth. Root exudates and microbial lyses in the surface soil could contribute to the observed higher amino acid concentration. Deeper soils are more reliant on the breakdown of peptides for amino

acids. Immediate use of peptides could prevent the production of amino acids in deeper soil.

Arginine addition caused the mineralization of amino acid N to NH_4^+ at all soil depths. However, our soil incubation results showed that NH_4^+ only increased under anaerobic conditions, where C degradation and nitrification are oxygen limited. Rapid ammonium oxidation occurs under aerobic conditions (Kresovic et al., 2005). Potentially, the addition of arginine could cause the formation of anaerobic pockets or could serve as a sufficiently large N source that not all N is immobilised (Rey et al., 2008). The NH_4^+ concentration is lower in the surface soil than in the subsoil. One reason might be that the microbes at the surface need more N than at the depth and are more efficient at recycling. Arginine is also both a C and N source: Where C is more limited than N, the use of arginine as a C source would produce NH_4^+ as a waste product (Kemmitt et al., 2008). Our results also reveal a greater nitrification potential in surface soils than at depth; potentially due to a larger active community of nitrifiers. Therefore, more NH_4^+ would be transformed to NO_3^- in the surface soil and potentially more could be lost as N_2O or N_2.

6.4.3 Implications of N cycle variations with depth for root uptake

Our results show that root length density decreased with depth. This result was predicted and is similar to that reported by Kautz et al. (2013). Soil structure can have a large influence on root length; for example, greater soil density at depth could impede root growth by limiting oxygen and water access and by physical obstruction (Whalley et al., 1995; da Silva et al., 1997; Bengough et al., 2006). Watt et al. (2006) found that

the growth of plant roots in the subsoil was largely limited to macropores. This is not likely, however, in our poorly structured soil.

We hypothesized that a greater involvement in nutrient uptake would be found for roots at the surface than for those at depth. The higher root length observed in this study corresponds with the areas of higher microbial activity, N concentrations and turnover rates. Therefore, more N likely is taken up by surface roots than by those at depth. The surface soil is also likely the area where N demand is greatest. Greater root length would allow for greater competition with microbes. The adverse soil structure and bulk density at depth can make accessing nutrients more difficult, thereby limiting root activity. Our results are supported by those of Salome et al. (2010), who found that the low root length at depth meant that only a small proportion of the subsoil was accessible to roots; consequently, it did not contribute much to plant nutrition.

Water uptake is an equally important function of plant roots as nutrient uptake. The uptake rate of water is often proportional to root length density (Hinsinger et al., 2009; Hodge et al., 2009). During drought, soil surfaces dry, thereby limiting water and, potentially, nutrient uptake in roots near the surface. This can lead to death of near-surface roots and greater root growth at depth (Smucker et al., 1991). In dry conditions, deeper roots could be vital for plant nutrient uptake. Roots are able to take up a wide range of N forms including peptides, which are an important source of N for plants during drought. However, more roots at depth could lead to a greater input of exudates. As shown here, an addition of arginine resulted in N mineralization to NH_4^+ at depth, so NH_4^+ could also be a source of N for deeper roots during drought.

Contrary to our expectations, less water was apparently retained in the subsoil than in the topsoil. We ascribe this to the subsoil's lower soil organic matter content,

which is known to aid water retention and promote soil structure. Further, the subsoil dried out and rewet at a similar rate to the topsoil. This does not support the hypothesis that soil moisture becomes proportionally more available in subsoil as the soil progressively dries out due to evapotranspiration losses. Our results suggest that, irrespective of root length density, water is removed evenly throughout the soil profileto balance plant demand – or less likely, that plant-mediated hydraulic lift is redistributing water from deeper soil layers to the surface. This suggests that drying out of the soil profile does not induce spatial niche partitioning in N availability.

6.4.4 Study limitations and suggestions for future work

Only one ecosystem was studied here; therefore, others should be investigated (e.g. forests and grasslands) to gain a clearer picture of how topsoil and subsoil differ. Roots of different plants can reach greater depths than those studied here, so depths below 60 cm should be explored. More studies about how microbes and roots compete for N resources in deeper soils should also be investigated. The role of mycorrhizae was not considered in this work, yet they can greatly influence plant nutrient uptake and increase the area for uptake. This area should be considered further.

6.4.5. Conclusions

This study aimed to evaluate the relationship between root distribution, organic and inorganic N availability and potential N supply. Our study showed evidence of microbial activity in the subsoil, with arginine and plant residue mineralization and anaerobic N mineralization occurring. The mineralization of HMW and LMW C substrates decreased with increasing soil depth. Root length density and microbial

activity also decreased with increasing soil depth. This all suggests that microbes are substrate limited at depth, due to lower inputs from root exudates and senescent plant material, but that given an input of material; the microbes respond by mineralizing both C and N. Root length corresponds with the soil locations where microbial activity stimulates the N cycle, suggesting that surface roots are more important for nutrient uptake than are those at depth. Preferential uptake of water by deep roots does not appear to occur, as water retention is similar in both the subsoil and the topsoil.

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Chapter 7

Long-term nutrient enrichment drives soil and vegetation community development in Arctic habitats

Abstract

Polar regions are expected to experience an increase in N deposition in future years, which is in turn expected to have an effect on ecosystem functioning. Studying areas of high N concentration in the arctic could therefore give insight into potential future conditions. Seabirds create large nutrient gradients in the tundra, which impact nutrient cycling and the functioning of microbial communities. This study investigated the turnover of high and low molecular weight (MW) organic compounds along two contrasting nutrient enrichment gradients in the high Arctic. It also investigated N mineralization along the same transects. The mineralization was approximately 6-fold faster for a low MW root exudate mixture than for ^{14}C-plant litter within the first 7 d and the mineralization of root exudates was faster in the control soil than in the orthinogenic soil 0.5 m from the cliff. The mineralization of the ^{14}C-labelled amino acids was marginally faster in the orthinogenic treatments. Comparisons of the half-life between the orthinogenic treatment and control for each substrate separately showed no significant differences for amino acids, peptides and root exudates. Plant litter showed some significant differences in half-life between the orthinogenic treatment and the control, but only within 2 m of the cliff. Nitrification was significantly greater at the orthinogenic site than at the control 2 m from the cliff. The soil respiration was significantly different ($P < 0.05$) between the orthinogenic treatment and the control. Soil respiration was significantly higher for the first 20 m along the orthinogenic transect than in the control transect. The high MW organic material in the Arctic would

therefore appear to be more sensitive to microbial mineralization in response to increasing N deposition, which may then lead to reduced carbon storage in Arctic soils. This could have serious implications for positive climate change feedback.

7.1. Introduction

Net primary production in the terrestrial Arctic is typically limited by short growing seasons, cold temperatures, frequent and strong winds and low nutrient supply (Bobbink et al., 2010). Consequently, the projected rapid increase in temperature, changes in precipitation patterns and enhanced atmospheric nitrogen (N) deposition (Geng et al., 2010; Førland et al., 2011;) are predicted to have profound effects on the functioning of polar ecosystems (White et al., 2000; Myers-Smith et al., 2011). One of the primary impacts of these anthropogenically mediated changes is the projected nutrient-induced shifts in plant community composition, which will impact directly on a range of ecosystem services including water quality, soil carbon storage and food availability (grazer biodiversity) (Post and Pedersen, 2008; Natali et al., 2012). Understanding and predicting the long-term resilience and potential feedback in response to environmental changes therefore remain central goals in polar ecosystem science.

Short-term (ca. 5–10 y) studies in Arctic tundra have frequently reported that nutrient enrichment (e.g. N and P) causes relatively rapid changes in above and below-ground diversity (Gordon et al., 2001; Madan et al., 2005; Campbell et al., 2010). These studies, however, have often simulated enrichment by pulse additions of high levels of inorganic nutrients, which can lead to bias in environments where spatial and temporal niche separation for nutrient and water acquisition occurs (Clarkson et al., 2009; Glanville et al., 2012). While these studies have proved extremely useful in

demonstrating the importance of plant-soil-grazer feedback, a number of other studies have also concluded that they may not accurately reflect long-term responses to nutrient addition (Dormann and Woodin, 2002; Ellis and Rochefort, 2004).

An alternative strategy to artificial nutrient manipulation is to investigate ecosystem responses at sites that have experienced natural enrichment over much longer time periods (ca. 1000-10,000 y) and that are assumed to be at a quasi-steady state (Holloway et al., 1998; Osada et al., 2010). Unlike most small-scale experimental plot additions (< 10 m^2), the natural long-term addition of nutrients typically occurs in a spatially heterogeneous pattern that often leads to the formation of large scale (>1000 m^2) nutrient enrichment gradients. Further, differential grazing across these expansive gradients over long time periods can induce genuine feedbacks in vegetation and soil ecology, which are difficult to capture in small scale plots. The disadvantages of using these sites, however, are that their full chronology can only be partially resolved from either biomarkers (e.g. pollen records) or geomarkers (e.g. ^{14}C dating, $\delta^{15}N$, $\delta^{13}C$) and that the nutrients are rarely deposited singly (Rozema et al., 2009).

Large scale variation in nutrient enrichment in tundra environments typically occurs due to differences in underlying parent material, hydrology, anthropogenic pollution and homoeothermic animal abundance (Löffler and Pape, 2008; Naito and Cairns, 2011). Seabirds are responsible for the creation of some of the largest tundra nutrient gradients (Stempniewicz, 2005). This arises due to their continual foraging for food at sea, with subsequent roosting and breeding on land, which leads to transfer of nutrients from a highly productive marine habitat to an intrinsically nutrient poor terrestrial habitat. This is exemplified by estimates of annual guano deposition of up to 60 t km^{-2} y^{-1} by Little Auks (*Alle alle*), which commonly exist in dense land colonies

(>1000 pairs site^{-1}) often located kilometres from the coast (Stempniewicz et al., 2006). Faecal deposition tends to be concentrated close to the colony, but also occurs during bird flight, leading to a progressive decline in nutrients from the nesting site towards the coast.

Changes in vegetation and herbivore populations in response to orthinogenic nutrient enrichment are well documented (Zmudczynska et al., 2008; Jakubas et al., 2008). In contrast, the knowledge of seabird impacts on soil development, microbial community functioning and nutrient cycling remains fragmentary (Solheim et al., 1996). The aim of this study was therefore to investigate the turnover of high and low molecular weight (MW) organic compounds along two contrasting nutrient enrichment gradients in the high Arctic.

7.2. Materials and methods

7.2.1. Site characteristics

The experimental site was located at Scoresbygrotta on Blomstrandhalvøya Island located in Kongsfjorden close to Ny Ålesund, Svalbard (78°59'10"N, 11°59'12"E). The site has a mean annual air temperature of -6.5°C (minimum -24.8°C, max. 12.1°C) and an annual precipitation of 197 mm, with approximately 65% falling as snow. The site consists of a cliff face approximately 600 m in length running parallel to the coast (500 m from the shoreline). Significant vegetation and soil development has occurred beneath the cliff in response to the presence of seabirds. The cliff is composed of Precambrian Upper Proterozoic limestone 25 m in height and is characterized by a middle section approximately 50 m in length that is heavily inhabited by seabirds (black-legged kittiwakes, *Rissa tridactyla*; Black guillemots, *Cepphus grill*; Glaucous

gulls, *Larus hyperboreus*) (Fig. 7.1). Areas with very few seabirds are found adjacent to this central area due to a lack of nesting sites; these were used as the control sites. Water movement down the cliff face was evident across all areas due to snowmelt and runoff from land directly above the cliff. At the three sampling times, the rate of water flow over the cliff face appeared visually to be similar at both bird inhabited and non-bird inhabited (control) areas. Below the cliff, the topography is characterized by a freely draining steep slope (45°) approximately 70 m in length that appears to consist of well-colonized mixed glacial debris (limestone, red sandstone, etc.; Harland, 1997). Subsequently, this grades into a gently sloping (10°), poorly drained mire area approximately 500 m in length, which is dominated by mosses; abundant surface water channels flow through the mire towards the sea (Fig.6.2).

The plant species under the bird cliff (0-70 m slope section) included *Puccinellia angustata* (R. Br.) Rand & Redf. ssp. Angustata, *Polygonum vivipara* L., *Cerastium arcticum* Lange coll., *Oxyria digyna* (L.) Hill, *Luzula confusa* Lindeb., *Draba* species, *Cochlearia groenlandica* L. coll., *Ranunculus hyperboreus* Rottb. ssp. *arnellii* Scheutz, *Saxifraga cernua* L., *Saxifraga hieracifolia* Waldst. & Kit. ex Willd. *ssp. hieracifolia*, *Saxifraga hirculus* L. ssp. *compacta* Hedberg, *Saxifraga oppositifolia* L., mosses and lichens. Those plant species predominantly absent from the bird cliff transects but present in the control (non-enriched) transects included: *Cassiope tetragona* L.D.Don. ssp. *tetragona* L., *Dryas octopetala* L., *Silene acaulis* (L.) Jacq., *Carex bigelowii* and *Salix polaris* Wahlenb. The species *Dryas octopetala* is associated with nitrogen fixation as are some lichen species.

Fig. 7.1. Seabirds nesting at the study site. The flow of guano down the cliff face is evident from the white streaks.

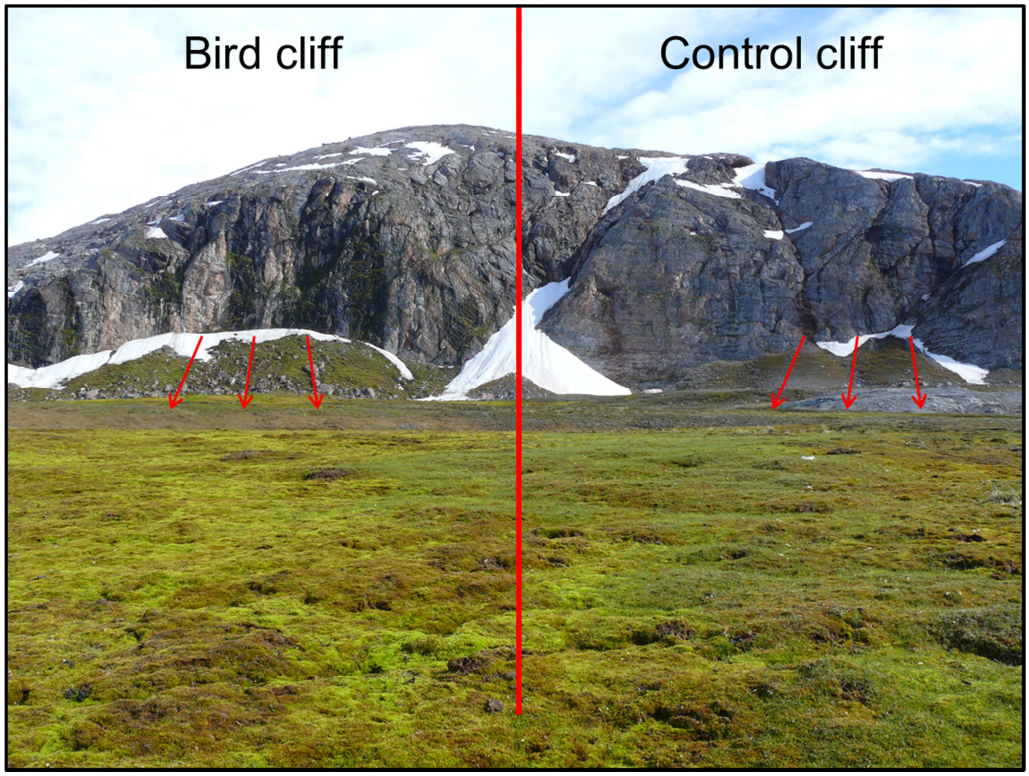

Fig. 7.2. Photograph of the study showing the bird and non-bird cliff sites. The three sampling transects at the site are shown with red arrows.

7.2.2. Site sampling

Six transects were taken at 90° to the cliff face along the steeply sloping area. These included three transects emanating from the bird inhabited area and three from the control (non-bird enriched) transects. Along each transect, samples were taken at increasing distances from the cliff face (0.5, 2, 10, 20, 40 and 60 m), each sample was characterized by a different vegetation community structure. At each location, 10–15 soil samples (0 to 10 cm depth) were taken randomly and stored in gas-permeable plastic bags at 4°C to await analysis. Soil temperature in the 0–10 cm soil layer was also recorded. At each location, 3 randomly positioned quadrats (20 × 20 cm) were placed over the vegetation, the above-ground plant material was harvested, and its biomass determined after drying (70°C, 24 h).

Replicate samples (n = 5) of the water flowing over the cliffs and entering the soil at 0-0.5 m (inflow) and the drainage water emanating from the base of the vegetated slope (70 m; outflow) were also sampled.

7.2.3. Soil analysis

Within 12 h of sample collection, soil solution was recovered from each transect sampling point using the centrifugal-drainage procedure (14,000 *g*, 5 min) of Giesler and Lundström (1993). The soil solutions were further centrifuged for 5 min at 18,000 *g* to remove any suspended particles and the supernatant was stored in polypropylene vials at -21°C prior to analysis.

Dissolved organic C (DOC) and total dissolved nitrogen (TDN) in solution were determined with a Shimadzu TOC-TNV analyser (Shimadzu Corp., Kyoto, Japan). Concentrations of NH_4^+ and NO_3^- in soil solution were determined colorimetrically by microassay (Downes, 1978; Mulvaney, 1996). Dissolved organic nitrogen (DON) was

then calculated as the difference between TDN and dissolved inorganic nitrogen. Total free amino acids (TFAA) were determined by fluorescence using the *o*-phthalaldehyde - β-mercaptoethanol procedure of Jones et al. (2002). Total C and N of soils were determined using a CHN-2000 analyser (Leco Corp., St Joseph, MI, USA). Soil pH (1:1 v/v) was determined in distilled water as described in Smith and Doran (1996), while moisture content was determined by drying at 80°C. Basal soil respiration was determined in the laboratory at 10°C using an automated multichannel soil respirometer (Nordstrom Innovations Ltd., Umea, Sweden) and the steady-state CO_2 production rates from 24 to 72 h were recorded.

Net N mineralization was determined by incubating soil collected from the field shortly after snowmelt at 10°C for 30 d. Briefly, soil from each transect point (100 g) was stored in gas permeable plastic bags and after 0 and 90 d the soil was extracted with 0.5 M K_2SO_4 (1:5 soil:K_2SO_4 w/v; 30 min), centrifuged (18,000 *g*, 5 min) and the supernatant recovered and frozen (-21°C) to await NH_4^+ and NO_3^- analysis as described above. Ammonification was calculated as the total amount of soluble N

7.2.4. ^{14}C mineralization of low and high molecular weight organic matter

Field-moist soil (5 g) was collected from along the two transects and placed into individual 50 cm^3 polypropylene containers. Different ^{14}C-labelled substrates were then added to the soil and their mineralization to $^{14}CO_2$ monitored over time. The four ^{14}C-labelled substrates included: (1) plant shoot litter; (2) a mixture of low MW compounds (simulated root exudates); (3) a mixture of 15 amino acids; and (4) the peptide, trialanine.

The high MW C source consisted of air-dried ^{14}C-labelled ground *Lolium perenne* L. green shoot material (12.3 kBq g^{-1}) labelled as described in Hill et al. (2007)

and characterized and used as described in Glanville et al. (2012). Within the plant material, the soluble ^{14}C component constituted 32.9 ± 1.5 % of the total ^{14}C, while 26.7 ± 1.0 % of the high MW ^{14}C remaining was soluble in 36 mM HCl, 43.8 ± 0.7 % was NaOH (1 M) soluble and 29.5 ± 3.4 % remained insoluble. Briefly, 100 mg of this ^{14}C-labelled plant material was added directly to 5 g of soil (46 mg C g^{-1}). The low MW C source consisted of a mixture of ^{14}C-labelled substrates that were representative of common root exudate compounds: malic acid (1 mM), citric acid (2 mM), glucose (10 mM), fructose (2 mM), sucrose (1 mM), and an amino acid mixture (3 mM) comprising 15 equimolar ^{14}C-labelled L-amino acids (alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tyrosine, valine). The exudates were dried down onto pure quartz sand as described in Glanville et al. (2012). An amount of ^{14}C-labelled sand (100 mg; 2.19 kBq) was added to each 5 g soil sample to give a substrate addition rate of 0.01 mg C g^{-1}. The ^{14}C-labelled amino acids (an equimolar mixture of 15 different L-isomeric amino acids; 666 μM) and peptide (L-trialanine; 10 mM) were added to the soil in liquid form (0.1 ml g^{-1}; 0.09 kBq ml^{-1}) as described in Jones (1999). After the addition of each ^{14}C-substrate to the soil, a $^{14}CO_2$ trap containing 1 ml of NaOH was placed above the soil and the tubes were capped. The tubes were then incubated at 10°C and the NaOH traps changed over time to determine the temporal dynamics of $^{14}CO_2$ production (0-50 d). The time was chosen to reflect the length of a typical growing season. The ^{14}C content of the NaOH traps was determined with Scintisafe 3® scintillation cocktail (Fisher Scientific, Leicestershire, UK) and a Wallac 1404 liquid scintillation counter (Wallac EG&G, Milton Keynes, UK). After 50 d, the soil was extracted with 0.5 M Na_2SO_4 (1:5 w/v;

200 rev min^{-1}; 30 min), centrifuged (16,000 *g*, 10 min) and ^{14}C in the supernatant counted as described above.

The mineralization dynamics of the substrates, peptide, amino acids and root exudates were best described by a triple exponential decay equation:

$$S = [a_1 \times \exp(-k_1 t)] + [a_2 \times \exp(-k_2 t)] \quad \text{(Eqn.1)}$$

While the plant material mineralization was best described by a double exponential decay equation:

$$S = [a_1 \times \exp(-k_1 t)] + [a_2 \times \exp(-k_2 t)] + [a_3 \times \exp(-k_3 t)] \quad \text{(Eqn.2)}$$

Where S is the ^{14}C-label remaining in the soil, k_1 is the exponential coefficient describing the primary mineralization by microbial biomass; k_2 is the exponential coefficient describing the secondary, slower phase of mineralization and k_3 the third and slowest phase.

The parameters a_1, a_2 and a_3 represent the size of the C pools associated with exponential coefficients k_1, k_2 and k_3, and t is time. The half-life ($t_1/_2$) of pool k_1 and k_2 can is calculated as:

$$t_1/_2 = \ln(2)/k_1 \quad \text{(Eqn.3)}$$

(Paul and Clark, 1996). The connectivity and isotope dilution associated with pools k_1, k_2 and k_3 cannot be determined; therefore, reliable calculation of the half time is not possible for pools k_2 and k_3 (Boddy et al., 2007; Farrar et al., 2012).

7.2.5. Statistical analysis

Statistical analyses (paired t-tests, linear regression, ANOVA) were undertaken with Minitab v15 (Minitab Inc., State College, PA) and Excel 2010 (Microsoft UK, Reading, UK). First order kinetics equations were fitted to the ^{14}C mineralization data

with Sigma Plot 12 (Systat Software Inc., Chicago, IL) using a least sum of squares iteration routine.

7.3. Results

7.3.1. ^{14}C-labelled substrate mineralization

The mineralization of the ^{14}C-labelled plant material is shown in (Fig. 7.3) Over the 50 d incubation period, approximately 10% of the plant material was mineralized; however, few significant differences were observed in the mineralization rate both within transects and between the orthinogenic and control treatments. The only exception was the significantly higher rate of mineralization of plant material observed at 2 m in the orthinogenic transect.

The mineralization was approximately 6-fold faster for the low MW root exudate mixture than for the ^{14}C-plant material within the first 7 d (Fig.7.3). Although mineralization was faster in the control transect than in the orthinogenic transect, no clear transect patterns emerged. At the end of the 50 d incubation, a 0.5 M K_2SO_4 extract only recovered 2.1 ± 0.3% of the initial ^{14}C added, indicating that the majority of the ^{14}C had become immobilized in the microbial biomass. In contrast to the results for root exudates, no significant treatment transect effects were seen for the mineralization of the ^{14}C-labelled peptide (Fig. 7.4). The mineralization of the ^{14}C-labelled amino acids was marginally faster in the orthinogenic treatments although, again, no transect pattern was apparent (Fig.7.4).

The decomposition of both simple and complex molecules could be described by either a double or triple exponential decay model and this was not altered by the presence of bird input or distance from the cliff face when maximum N deposition occurs. The triple exponential decay equation model was used and gave the best fit to

the mineralization data for amino acid, peptide and root exudates ($r^2 > 0.998 \pm 0.0006$) in the orthinogenic site and ($r^2 > 0.999 \pm 0.0003$) in the control site while a double exponential decay equation used was fitted to plant material mineralization ($r^2 = 0.997 \pm 0.001$) in the orthinogenic but in control ($r^2 = 0.998 \pm 0.0006$).

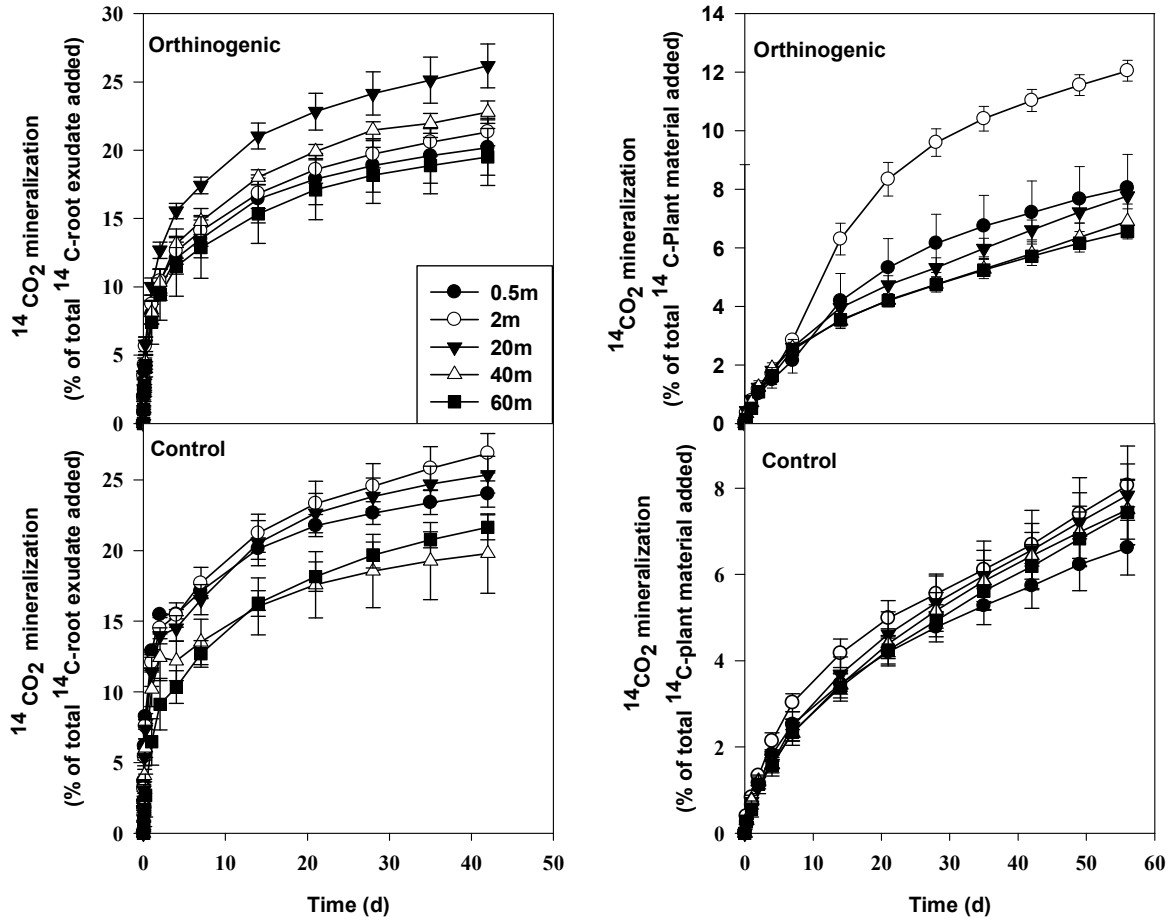

Fig. 7.3. Mineralization kinetics of ^{14}C-labelled root exudates and plant material in soil as a function of increasing distance from cliffs with (orthinogenic) and without (control) bird-derived nutrient inputs. Values represent mean ± SEM (n = 3).

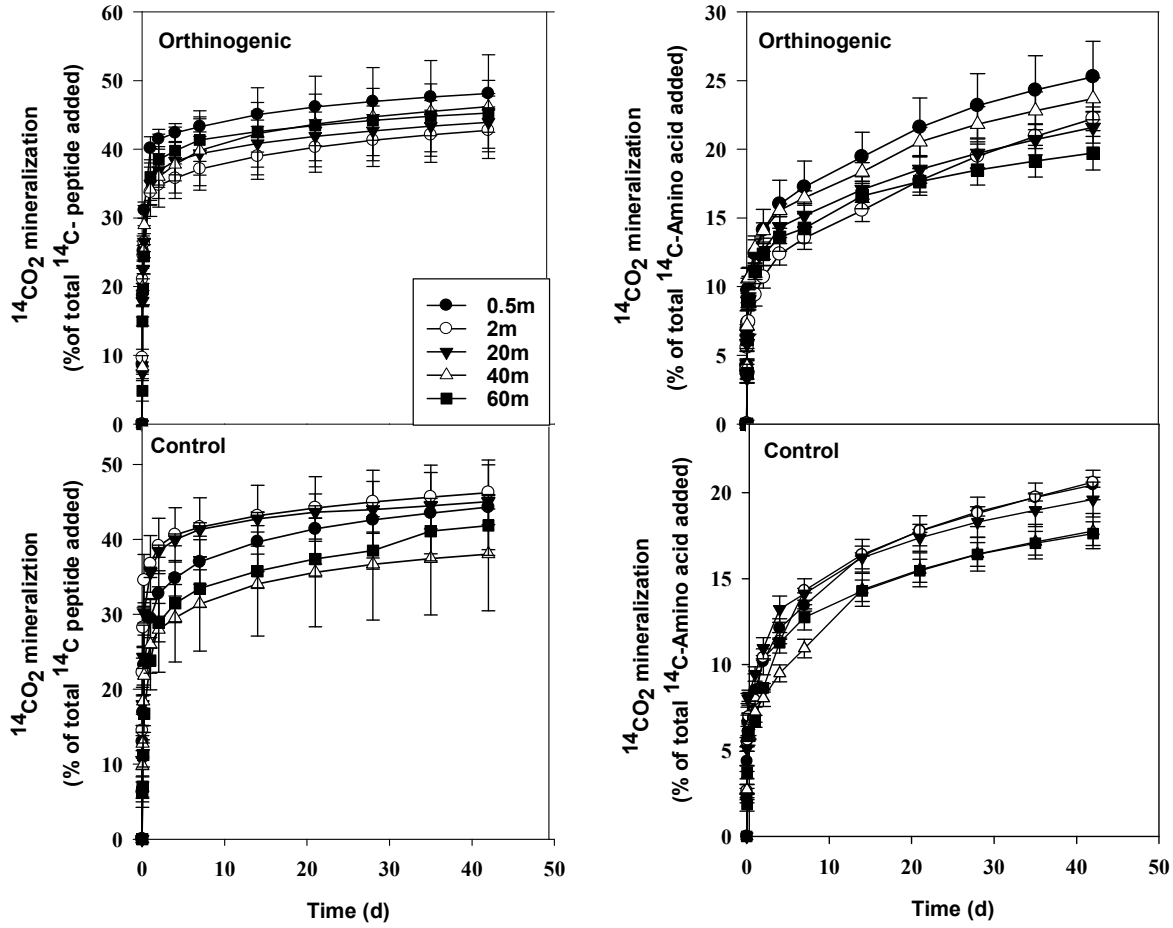

Fig 7. 4. Mineralization kinetics of ^{14}C-labelled Amino acid and peptide in soil as a function of increasing distance from cliffs with (orthinogenic) and without (control) bird-derived nutrient inputs. Values represent mean ± SEM (n = 3).

The fast component (k_1) of the triple exponential decay equation was used to calculate the substrate half- life ($t_{1/2}$).Comparison of the half-life between the orthinogenic treatment and control for each substrate separately, showed no significant difference for amino acids, the peptide and root exudates (P > 0.05) (Table 7.1). The plant material showed some significant difference in half-life between the orthinogenic treatment and the control, but only within 2 m of the cliff. If we compare the half-time for substrates with increasing distance away from the cliff face, the results show no

significant difference in the orthinogenic treatment for the amino acid substrate and for the control only 2 m is significantly different from the other distances ($P < 0.05$). The peptide and root exudates showed no significant difference in half-life across the distance in either the orthinogenic treatment or the control. The plant material showed a significant difference in half-life along the transect in the orthinogenic treatment ($P < 0.05$); it is significantly slower within 2 m of the bird cliff than further away ($P = 0.04$), but the control showed no significance difference in half-time across the distance.

When we compared k_2 between bird cliff and control across the distance, a significant difference was found for the following substrates: amino acids, peptide and plant material. For the amino acid substrate, k_2 was significantly different between the orthinogenic treatment and the control ($P < 0.05$) (Table 7.2). There was no significant difference seen for k_2 when compared across distance for amino acids in the orthinogenic treatment ($P > 0.05$), while in the control treatment, k_2 was not significantly different with the exception of 40 m, which is less than 20 m and 60 m ($P < 0.05$). The peptide substrate showed no significant difference in k_2 between the orthinogenic treatment and the control ($P > 0.05$). Further, no significant difference was observed for k_2 comparing across distance in the orthinogenic treatment or the control, except at 40 m, which is significantly less than 0.5 m in the orthinogenic treatment. The root exudates showed no significant difference in k_2 between the orthinogenic treatment and control ($P > 0.05$), or along either transect. The plant material significant difference in k_2 between the orthinogenic treatment and the control ($P < 0.05$).

Comparison of k_1 and k_2 between the different substrates (amino acids, peptide and root exudates) indicated significant differences between them ($P < 0.05$). The plant material could not be validly compared with the other substrates because the substrates

were analysed with a three phase exponential decay equation (6 parameter) whilst the plant material was analysed with a two phase exponential decay equation (4 parameter). No significant difference was noted between amino acids and peptide for k_1, and no significant difference was observed between amino acid and root exudates for k_2.

7.3.2. N mineralization

No significant difference was seen in soil N ammonification between the orthinogenic treatment and the control, but a significant difference did exist along the transect. At 2 m from the cliff face, N ammonification ($P < 0.05$) was significantly different from all other distances, with the exception of 20 m ($P > 0.05$) in the orthinogenic treatment. Distance showed no significant effect in the control treatment. Nitrification was significantly greater in the orthinogenic treatment than in the control at 2 m from the cliffs. In addition, as with ammonification, nitrification was significantly higher at 2 m than at all other distances tested, except 20 m, in the orthinogenic treatment. See net mineralization (Fig. 7.5).

7.3.3. Soil respiration

The total mineralization for the substrates (amino acids, peptide, root exudates and plant material) showed no significant difference between the bird cliff and control transects ($P < 0.05$). Soil respiration was significantly different ($P < 0.05$) between the orthinogenic treatment and the control. Soil respiration was significantly higher for the first 20 m along the orthinogenic transect in comparison to the control transect ($P < 0.01$). After normalization for soil organic C, rates of soil respiration remained significantly higher in the orthinogenic transect (23 ± 3 mg CO_2 kg soil C^{-1} h^{-1}) in comparison to the control transect (16 ± 2 mg CO_2 kg soil C^{-1} h^{-1}; $P < 0.05$) (Fig. 7.6)

Table 7.1. Half-life of the different substrates in the nutrient enriched (bird cliff) or nutrient limited (control) soil environments. The distances refer to different distances from the cliff face. Values represent mean ± SEM (n = 3).

Substrate	0.5 m	2m	20m	40m	60m
Bird Cliff					
Peptide	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.01	0.03 ± 0.00
Amino acid	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.03 ± 0.01	0.03 ± 0.00
Root exudates	0.27 ± 0.06	0.10 ± 0.03	0.16 ± 0.01	0.19 ± 0.08	0.16 ± 0.02
Plant material	17.62 ± 5.10	16.52 ± 1.42	4.46 ± 0.72	3.01± 0.42	4.28 ± 1.26
Control					
Peptide	0.04 ± 0.02	0.02 ± 0.00	0.03 ± 0.00	0.05 ± 0.03	0.07 ± 0.04
Amino acid	0.03 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
Root exudates	0.13 ± 0.01	0.13 ± 0.02	0.12 ± 0.02	0.18 ± 0.02	0.62 ± 0.33
Plant material	3.22 ± 0.53	3.45 ± 0.24	5.02 ± 0.58	2.99 ± 1.01	3.56 ± 0.42

Table 7.2. k_1 and k_2 of the different substrates in the nutrient enriched (bird cliff) or nutrient limited (control) soil environments. *plant material k_2 in Bird cliff and control= ($d^{-1}*10^{-4}$). Values represent mean ± SEM (n = 3).

(k_1)					
Substrate	**0.5 m**	**2m**	**20m**	**40m**	**60m**
Bird Cliff					
Peptide	26.64 ± 3.62	35.11 ± 1.88	29.42 ±3 .27	27.45 ± 6.23	27.58 ± 4.16
Amino acid	31.41± 4.54	37.77 ± 2.50	25.61 ±1.84	28.73 ± 6.93	27.59 ± 3.86
Root exudates	2.78 ± 0.49	10.47 ± 5.27	4.28 ± 0.29	10.48 ± 7.87	4.49 ± 0.60
Plant material	0.04 ± 0.01	0.04 ± 0.00	0.17 ± 0.03	0. 24 ± 0.04	0.19 ± 0.04
Control					
Peptide	21.26 ± 6.42	37.16 ± 4.93	25.87 ± 1.75	22.83 ± 8.61	20.30 ± 7.88
Amino acid	23.27 ± 1.41	35.92 ± 1.53	22.17 ± 0.54	23.35 ± 1.33	25.29 ± 2.73
Root exudates	5.59 ± 0.41	5.91 ± 1.06	6.06 ± 1.00	3.89 ± 0.35	2.58 ± 1.63
Plant Material	0.23 ± 0.04	0.20 ± 0.01	0.14 ± 0.02	0.33 ± 0.15	0.20 ± 0.02
(k_2)					
Bird Cliff					
Peptide	2.08 ± 0.26	1.48 ± 0.16	1.51 ± 0.09	0.91 ± 0.33	1.58 ± 0.31
Amino acid	0.44 ± 0.07	0.50 ± 0.02	0.47 ± 0.08	0.63 ± 0.23	0.34 ± 0.13
Root exudates	0.15 ± 0.02	0.24 ± 0.07	0.23 ± 0.05	0.21 ± 0.08	0.26 ± 0.13
Plant material	0.26 ± 0.2	3E-13 ± 7E-14	8.60 ± 0.6	8.30 ± 0.80	6.6 ± 0.8
Control					
Peptide	0.76 ± 0.32	2.56 ±1.48	1.16 ± 0.08	0.45 ±0 .28	0.76 ± 0.29
Amino acid	0.22 ± 0.04	0.24 ± 0.01	0.25 ± 0.05	0.11 ± 0.03	0.25 ± 0.01
Root exudates	0.25 ± 0.02	0.2 4± 0.03	0.19 ± 0.03	0.20 ± 0.03	0.16 ± 0.04
Plant Material	8 .00 ± 1.00	8.6 0 ± 0.3	9.00 ± 2.00	11.0 ± 1.0	10.0 ± 1.0

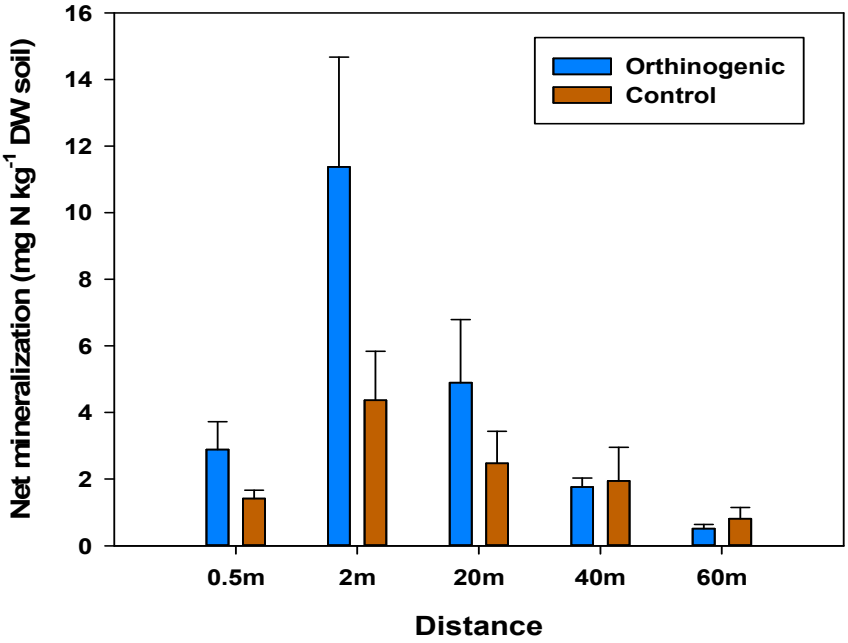

Fig. 7.5. Net mineralization of orthinogenic and control site with increasing distance from the bird cliff. Values represent mean ± SEM (n = 3).

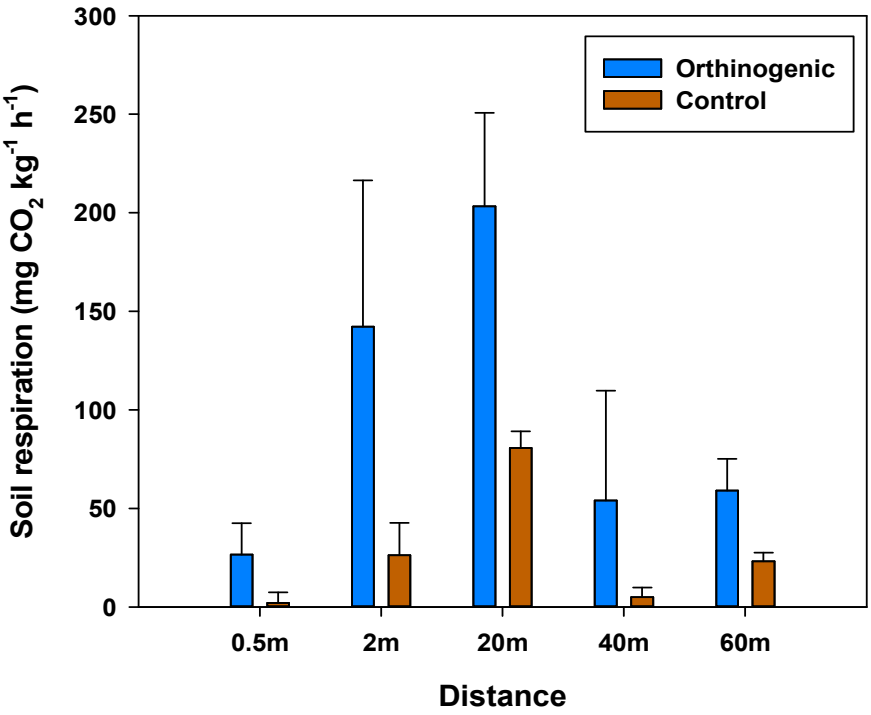

Fig. 7.6. Soil respiration in orthinogenic and control site with increasing distance from the bird cliff. Values represent mean ± SEM (n = 3).

7.4. Discussion

This study compared soil nutrient cycling between a seabird site, which is rich in organic material, and a control nutrient-limited site. Seabirds are migratory, arriving at Kongsfjorden in spring and departing in autumn (May-Aug). The birds are surface or near-surface feeders, consuming zooplankton and pelagic fish. Seabirds typically excrete 10.2% of the mass of their food intake (Brekke and Gabrielsen, 1994; Croll et al., 2005; Stempniewicza et al., 2007). Their excrement contains 21% N by weight (faeces and uric acid together).

Nitrification was significantly greater at the orthinogenic site than at the control 2 m from the cliff. This is similar to results shown by Goulding et al. (1998), where chronic and long-term N fertilization (more than 150 years of 1-150 kg N ha^{-1} yr^{-1}) input in UK grassland has caused high nitrification. The authors suggest that N saturation leads to less efficient cycling of N and a build-up of NO_3^-. However, rates of nitrification reported by Goulding et al. (1998) and Aber (1992) were five times higher than even the highest rate observed in the orthinogenic treatment. This could be due to the higher temperatures in the UK relative to polar regions.

Approximately 10 % of the ^{14}C-labelled plant material was mineralized. However, a few significant differences were seen in mineralization rate, both within transects and between the orthinogenic and control treatments. The only exception was the significantly higher rate of mineralization of plant material observed at 2 m in the orthinogenic transect. Nitrogen mineralization is a sign of C limitation, which might explain the high use of plant material at 2 m in the orthinogenic transect (Schimel et al., 2004). Soil respiration rates were significantly different between the bird cliff and

control transects. Soil respiration was significantly higher for the first 20 m along the orthinogenic transect in comparison to the control transect ($P < 0.01$).

The first phase of mineralization, represented by the parameter k_1 and the half-time, can be attributed to the immediate use of the substrate-C in microbial respiration. The second phase (k_2) represents the turnover of the microbial cell material into which the ^{14}C labelled substrate was incorporated. Half-life time was longer for the plant material than for the amino acids, peptide and root exudates, and k_2 was smaller. Plant material contains high MW complex components that need to be broken down, whereas the low MW compounds are more easily taken up by microbes. This result is supported by Jones et al. (2004), who found a rapid uptake of amino acids from soil solution. In addition, they described how the low concentrations of free amino acids and peptide in the soil solution are due, in part, to the rapid removal by the soil microbial community. In addition, our result is supported by Boddy et al. (2008), who demonstrated that some low MW DOC compounds are rapidly cycled by soil microbes in the High Arctic soils to the same extent as in temperate ecosystems. The results are also supported by Lipson et al. (2001) and Vinolas et al. (2001), who found that amino acids turned over rapidly in summer in Arctic soil, primarily as result of uptake by microorganisms and plant roots.

Mineralization of root exudates was faster in the control soil than in the orthinogenic soil 0.5 m from the cliff. The above-ground biomass is greater in the control soil than in the orthinogenic soil and so the control soil may have a microbial community more suited to using these materials. However, root exudates contain low MW compounds such as amino acids, sugars and organic acids, which are used by a wide variety of microbes (Farrar et al., 2003). The rate of amino acid mineralization was

also higher in the orthinogenic treatment. No difference was observed for the peptide among the sites. The mineralization of plant material was slower within 2 m of the cliff in the orthinogenic transect compared to both the control and further away.

7.5 Conclusion

This study has demonstrated that the mineralization rate was faster for the low MW root exudate mixture than for the plant material (HMW). The k_2 parameter was also smaller for plant material than for the low MW substrates. Mineralization rates and half-lives for all the substrates analysed were similar for both the orthinogenic site and the control, except for the plant material. This had a longer half-life within 2 m of the cliff in the orthinogenic transect than it did anywhere else and yet this 2 m area also showed the maximum total mineralization of plant material. The mineralization of the ^{14}C-labelled amino acids was marginally faster in the orthinogenic treatments, while the mineralization of root exudates was faster at 0.5 m in the control soil than in the orthinogenic soil. When we compared the k_2 values between the bird cliff and control gradients, a significant difference was found for the following substrates: amino acids, peptide and plant material. Amino acids and peptide were significantly higher in the orthinogenic treatment while plant material was lower close to the cliff. Nitrification was also significantly greater at the orthinogenic site than at the control cliff. More work is required to study the effect of N deposition on plant and microbial activity and the factors, such as temperature, which can affect N deposition. This work suggests that an increase in N deposition in the Arctic might increase the degradation of high MW C, which could reduce the potential for C storage in Arctic soils. Increased nitrification could affect the competition for nutrients and have consequences for the plant and microbial communities.

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Chapter 8

General discussion

The aim of this PhD thesis was to challenge the traditional concept of N cycling that views amino acids and inorganic N as the most important forms of N available for plant and microbial use. We have shown that the competition between plants and microbes could occur at a higher level in the N cycle (i.e. for peptides). We have also investigated the N cycle in the subsoil, which is typically neglected in studies of soil N cycling. Organic N is believed to be particularly important in low nutrient environments such as the Arctic. The final part of our research compared N cycling processes in a relatively high N input area with a control N limiting environment. This may give an indication of future conditions, as increased temperatures are predicted to increase soil N availability in the Arctic.

My thesis work focused on the dynamics of organic N forms in the soil, particularly on microbial transformation and the uptake of organic N by plants. There has been a lot of previous research about inorganic N, but relatively little work on organic N and its role in the N cycle. In the first chapter, I examined the mobility of organic N in soil and how the microorganisms affect this process. In the second chapter, I analysed the uptake of organic N by plants and soil microbes and the competition between them for different N sources of ecological relevance. After confirming that plants can compete with microorganisms for LMW organic N sources (amino acid and peptides) directly without extracellular cleavage, the ability of plants to take up intact protein without prior cleavage was tested.

Most previous studies have only focussed on the N cycle in surface soil; however, plant roots are known to extend well into the subsoil. Consequently, I investigated N cycling processes in the soil profile. There are few studies linking root distribution with N cycling. I measured the root distribution down the soil profile of a mature wheat crop and linked this to the forms and turnover of N down the soil profile.

In this chapter, I hope to summarise all the data presented in this thesis and relate the findings to the following 8 objectives:

- To test the the influence of microbial activity and chemical form on the potential for organic and inorganic N to move vertically in soil
- To characterise rates of microbial uptake and mineralization of amino acids and peptides in soils in relation to rates of inorganic N cycling
- To determine the influence of peptide addition on the uptake of inorganic forms of N, and vice versa
- To determine how much N can be taken up as peptides by plants grown in soil
- To determine what forms of N microorganisms prefer
- To investigate microbial N cycling in relationship to root growth in wheat cropping soils
- To quantify the mineralization of N, high MW and low MW carbon forms in polar regions and to determine the effect of long term N deposition on soil microorganisms

8.1. Microbial activity differentially regulates the vertical mobility of nitrogen compounds in soil

The aim of this chapter was to test the influence of microbial activity and the chemical form of N on the potential for organic and inorganic N to move vertically in

soil. Strategies to optimise N management are largely targeted towards inorganic N fertilizers (Jones et al., 2005), whilst the behaviour of dissolved organic N (DON) remains poorly characterised (Gardenas et al., 2011). The results showed that it was mainly high molecular weight (HMW) DON that was leached from agricultural soils, rather than low molecular weight (LMW) DON. Although nitrate was available to microbes, it was not the preferred form of N for the C-limited microbial biomass. It was found that, although electrostatic interactions strongly influenced the movement of N forms in soil, microbial immobilization appeared to be the most important factor limiting the movement of neutrally charged amino acids and peptides.

The uptake of the LMW DON provides microbes with both N and C to fuel respiration and growth (Jones et al., 2004). Low molecular weight DON is also a primary N source for plants (Näsholm et al., 2009), particularly in N-limiting environments; therefore, the next chapter investigated the competition between plants and microbes for this resource. Proteins are a major form of N input into soils, so we examined the fate of its breakdown products, amino acids and peptides, with regards to microbial and plant uptake.

8.2. Nutritional competition between plants and soil microorganisms for inorganic nitrogen as influenced by peptides, and vice versa

More detailed studies are needed about the behaviour of organic N, especially where it comprises a major supply of N in soils (Wanek et al., 2010). It was conventionally thought that primary productivity was limited by the rates of both protein cleavage to amino acids and mineralization to ammonium and nitrate by soil microbes (Chapin et al., 1993). It has been confirmed that most plants and soil microbes have the capacity to take up organic N, and that organic N is important as a nutrient

source for both the plants and microorganisms (Näsholm et al., 2009; Jones and Hodge, 1999). However, work has mostly focussed on the smaller forms of N (i.e. amino acids) rather than peptides. Previous studies have not focussed on the competition between plants and microorganisms for N at this stage in protein breakdown, nor has it been determined if they have a preference for peptides over other organic and inorganic N forms. This chapter contained many experiments in order to achieve the following 4 objectives:

1- To characterise rates of microbial uptake and mineralization of amino acids and peptides in soils in relation to rates of inorganic N cycling;
2- To determine the influence of peptide addition on the uptake of inorganic forms of N, and vice versa.
3- To determine how much N can be taken up as peptides by plants grown in soil;
4- To determine what forms of N microorganisms prefer.

8.2.1. Characterising rates of microbial uptake and mineralization of amino acids and peptides in soils in relation to rates of inorganic nitrogen cycling

Soil is a complex matrix where a range of biological and physical factors interacts to determine a compound's fate. Here, the influence of microbial uptake and soil particle attraction on organic and inorganic N forms was investigated to discover if microbes have a preference for a particular N form and how quickly they are taken up.

In non-sterile soil, amino acids and peptides were all rapidly assimilated by the soil microbes and the rate of uptake of N by soil microbes was significantly greater for the organic N forms. This supports previous research by Farrell et al. (2011b, 2012) and Hill et al. (2011b, 2012). The microbial community readily took up NH_4^+ from the soil, but did not take up significant quantities of NO_3^-, which rapidly leached down the soil.

This is supported by previous work showing that microbial metabolism of NH_4^+ is more energetically favourable than metabolism of NO_3^- (Jones et al., 2004; Hill et al., 2011a). Soils that are more limited by C than N, as studied here, often have a high nitrification potential, and microbes in general uptake NH_4^+ in preference to NO_3^-. This suggests that organic N-based fertilisers might have better N retention in soil and slow down the rate of NO_3^- leaching. Conversely, however, organic N-based fertilisers may increase N_2O emissions, which would be a negative consequence of their use (McLain and Martens, 2005).

In sterile soil, the retention of trialanine, alanine and NO_3^- was low, which supports the results of the first experiment, where microbial immobilization was the key factor in retaining these N forms within the soil profile. However, NH_4^+ was largely held in the soil, suggesting this N form was adsorbed to cation exchange sites. One major caveat to the experiments here is that they were performed on repacked soil columns and therefore there were no macropores present. These are known to be routes of preferential water flow in soil and can accelerate the movement of N down the soil profile (Larsson and Jarvis, 1999). This does imply that some DON may be transported long distances in soil, although it should be noted that these biopores also have large and very active microbial communities relative to the soil within the inner aggregates (Savin et al., 2004). This community would also reduce the vertical migration of DON relative to NO_3^-.

8.2.2. Determining the influence of peptide addition on the uptake of inorganic forms of N and vice versa in sterile plants

Plants are known to have a strong preference for different inorganic N forms; however, the uptake of organic N forms other than amino acids is poorly understood

(Andrews et al., 2013; Nacry et al., 2013). Due to the complexity of N forms that can co-exist in soil solution, it is necessary to study the effect of organic N on the uptake of inorganic N, and vice versa. This follows on from work in cell cultures that has shown that amino acids can repress nitrate uptake (Padgett and Leonard, 1993, 1996). Peptide uptake provided the focus for the work, as this had not been previously studied. The uptake of NO_3^- was significantly slower than the uptake of other N forms; however, nitrate uptake is known to be highly inducible at both the nitrate reductase and transporter level (Kaiser and Huber, 1994). Similar results were found by Hill et al. (2011a). The availability of alternative N forms (NO_3^-, NH_4^+ and alanine) has no significant effect on peptide uptake. This suggests that plants do not have a preference for other N forms over peptides (i.e. that the transporter is probably constitutively expressed and not down-regulated at the transport level). Interestingly, the availability of peptide on NO_3^- uptake also had no significant effect in our short term competition studies. One caveat is that over long time periods there may have been significant changes in gene expression, rather than post-translational modification of the transporters (i.e. phosphorylation). It would be interesting to investigate the longer term adaptation to the different N forms. Generally, when grown under sterile conditions, plants appear to prefer trialanine over alanine and NH_4^+. How the plant utilised and partitioned this N after uptake into the cell changed when different N forms were supplied. More N from alanine and trialanine than NO_3^- went to the roots compared to the shoot, and the proportion of trialanine that went to the root decreased with addition of other N forms. This suggests that the N derived from peptides may be processed differently within the cell in comparison to inorganic N. It would therefore be interesting to undertake a metabolomic and transcriptomic analysis, by ultra-high-

resolution Fourier transform-ion cyclotron MS and DNA macroarray (Aharoni et al. 2002; Hirai et al 2003), of roots and shoots supplied with the different N forms to show any changes to biochemical processes or gene expression. This would help elucidate the different N assimilation and transport pathways within the plant.

8.2.3. The uptake of nitrogen by plants grown in soil

After confirming that peptides constitute important N sources for plants and microorganisms, this experiment helped to find out how much N can be taken up by plants in the form of peptides when plants are in competition with soil microbes. Nitrogen uptake was much higher for plants grown in sterile conditions than for those grown in soil. Microbes have a more uniform distribution in the soil than roots so as a group they have greater spatial access to N forms than plants, giving them a competitive advantage (Kuzyakov and Xingliang, 2013). Fungi as well as bacteria have been shown to uptake peptides. Research into peptide utilization and transport in fungi has generally been limited to the Ascomycete *Neurospora crassa* and the yeasts *Saccharomyces cerevisia*e and *Candida albicans* (Becker and Naider, 1980; Mathews and Payne, 1980). In *S. cerevisiae*, research on growth of amino acid auxotrophs suggested that a variety of di-tripeptides share a common system that is distinct from amino acid permeases (Becker and Naider, 1980; Marder et al., 1977). Research on cell growth suggested that certain strains can utilize tetra-and pentapeptides, but that other strain cannot (Becker and Naider, 1980) with the uptake of peptides larger than tripeptides undetectable in direct transport assays. Peptide uptake has been found in ectomycorrhizal fungi (Benjidia et al., 2003; Chalot et al., 2006).

Sorption to soil particles can reduce N availability to plants, but the results of the previous experiments show that this is likely only important for ammonium. In soil,

NO_3^- was the main form of N taken up by plants, partly due to its mobility and less competition from soil microbes (Abaas et al., 2012).

An increase in peptide uptake occurred when other N forms were added. This could have been caused by higher nutrient concentrations stimulating plant growth. A greater root density can improve a plant's ability to compete with microbes for nutrients. More growth would cause transpiration, increasing a plant's water and nutrient uptake.

Higher peptide concentrations decreased NH_4^+ uptake, but did not affect the other N forms. This was also found in sterile plants, but only at the highest peptide concentration. At higher peptide concentrations, microbes could start producing $^{14}NH_4^+$ as a waste product, diluting the $^{15}NH_4^+$ pool. This would make it appear as though the plant was taking up less NH_4^+ and could explain this discrepancy.

The results in this chapter suggest that, despite plants having the ability, and possibly a preference to take up peptides, when in competition with microbes, nitrate may be the main source of N. However, these experiments suggest that larger plants (i.e. greater root length density) could compete more effectively for peptides. Laboratory experiments do not truly reflect a plant's growth in the field, where plants reach maturity and roots extend to depth within the soil.

8.3. Evaluation of soil nitrogen turnover in relationship to root growth in a wheat cropping system

Most studies on N cycle processes focus on the topsoil (plough layer), as this is where most of the organic matter and nutrient cycling takes place. It is also where root length density is greatest. However, plant roots can extend deep into the soil and potentially acquire some N from this zone. Nitrogen may accumulate in this zone due to

small amounts of N mineralization and from N leached from surface horizons. In some cases, shallow groundwater may also contain high concentrations of N, which can be recovered by plant roots (Jacinthe et al., 1999). In order to utilise the potential of a crop's deep rooting ability, more knowledge on N dynamics and water availability in subsoil is required. This chapter compared the distribution of roots of a mature wheat crop in the soil profile with the turnover and availability of different N forms in the soil profile.

Whilst there is microbial activity in the subsoil, it was much less than in the overlying soil layers. Nitrogen concentrations also decreased with depth, and the N appeared to be in less microbially available forms. Greater root length occurred in the surface soils, corresponding with higher microbial activity, N concentrations and turnover rates. It is likely that more N is taken up by surface roots than by those at depth. The surface soil is also likely the area where N demand is greatest. Greater root length would allow for greater competition with microbes. Microbes appeared to be C limited at depth, likely due, at least in part, to reduced root exudates and root/mycorrhizal turnover. However, roots appeared to remove water from all depths equally, rather than being proportional to root length density, which contradicts the findings of Hinsinger et al. (2009) and Hodge et al. (2009). Thus, nutrient uptake from deeper soils could still be important in some contexts; however, integrated over the growing season, its contribution to total plant uptake is probably quite low. So far, this work has only explored organic uptake in N-rich, C-limited soils. In low N soils, organic N is liable to be more important for plant uptake. The next chapter investigates N turnover in the arctic.

8.4. Long-term nutrient enrichment drives soil and vegetation community development in Arctic habitats

A rapid increase in temperature is predicted for the Arctic, as are changes to precipitation and greater atmospheric N deposition (Førland et al., 2011; Geng et al., 2010). These changes are expected to have a large impact on ecosystem functioning in high latitude ecosystems (White et al., 2000; Myers-Smith et al., 2011). One expected impact is a change in soil nutrient composition and availability. This is predicted to affect plant community behaviour, which will have implications for grazer biodiversity, water quality, greenhouse gas emissions and soil C storage (Natali et al., 2012; Post and Pedersen, 2008). However, many past experiments have subjected arctic soils to sudden, large inputs of N which do not accurately represent the gradual change expected. This chapter aimed to determine the effect of long term N deposition on the cycling of C and N in soil.

Studying areas of high N concentrations in the Arctic could provide useful insights into possible future conditions in this region. At the study site, seabird colonies create areas of high terrestrial nutrient (including N) deposition. This study compared a nutrient gradient transect at a site adjacent to a seabird cliff and a control transect at an adjacent uninhabited cliff. The statistical replication in this study is not ideal, as it would be preferable to have a series of independent replicated transects; however, logistically this was not possible. This chapter investigated N mineralization as well as the turnover of high and low molecular weight organic compounds along these two transects.

As expected, based on the different vegetation communities at the two sites, significant differences in soil C and N cycling were observed between the transects. Nitrification and HMW carbon mineralization was greater close to the cliff at the

orthinogenic site than at the control site, although the turnover of HMW carbon was slower. Soil respiration was higher for the first 20 m along the orthinogenic transect than for the control transect. Differences to LMW turnover were inconsistent, with root exudates having a shorter half-life in the control transect, amino acids having the shorter half-life in the orthinogenic transect and peptides showing no difference between sites. The orthinogenic site had less above-ground biomass, suggesting that nutrient availability was limiting primary productivity. This suggests that increased N deposition in the Arctic could lead to a greater turnover of HMW organic material potential, reducing carbon storage in arctic soils. This could have serious implications for positive climate change feedback. However, how this effect would interact with potential changes in plant cover, precipitation and other anthropogenically induced impacts is unclear. Nutrient enrichment will increase primary productivity, so the balance between C storage in plants and soil remains critical. Changes in greenhouse gas emissions (e.g. CH_4, N_2O, and CO2) also need to be included in any evaluation of C storage potential within these ecosystems.

8.5. Conclusions

This thesis investigated key aspects of dissolved organic nitrogen cycling in soil. The research investigated and advanced the knowledge of vertical N migration in the soil; the importance of organic N to plants and soil microbes, plant and microbial competition for organic and inorganic N, subsoil N and C cycling; and the effect of increased organic N inputs in an N-limited ecosystem. The key findings of the thesis follow below.

(1) This study has shown that HMW DON was the predominant form of DON in soil leachate, and that NO_3^- was the most mobile form of N.

(2) It was confirmed that plants and soil microbes show a preference for organic N over inorganic N, with plants preferring peptides over amino acids such as alanine. However, it was also found that the crop plants studied here were poor at competing for peptides in the soil when microorganisms were present because the microorganisms are ubiquitous and can access their preferential N source first. Increases in the concentration of inorganic N in the root bathing medium increased the capacity of roots to take up peptide, whilst, conversely, increases in external peptide concentration decreased the plant's capacity to take up ammonium, but not NO_3^- or alanine.

(3) It was shown that the carbon limitation at depth in an agricultural Eutric Cambisol soil profile reduces microbial activity and, as a result, rates of C and N cycling. The root length of wheat decreases with soil depth, which is partly responsible for this carbon limitation (reduced C inputs). This finding suggests that roots are adapted to exploiting the greater nutrient availability in surface soils and that engineering plants for nutrient retrieval from subsoil may be challenging. However, the evidence suggests that deeper roots can access greater water reserves within the soil profile.

(4) Long term deposition of organic N in the Arctic appears to change plant composition and increase the decomposition rate of HMW organic carbon. This is important, considering that N availability in the Arctic is predicted to increase with climate change and that the Arctic is currently a major store of soil carbon.

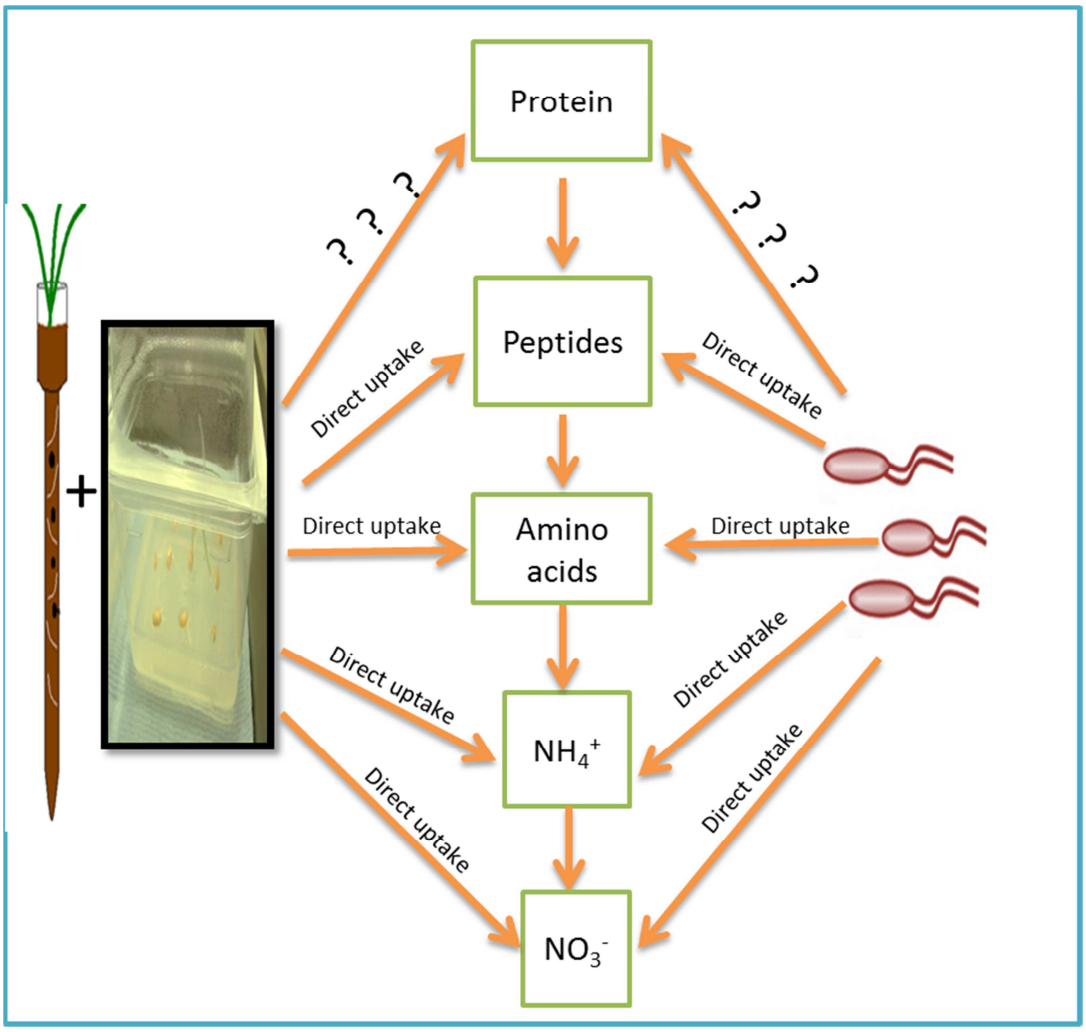

Fig.8.1. Conclusion graphs showing the ability of direct uptake for microbes and plant to for organic and inorganic N grow in soil

8.6. Suggestions for future work

This study has revealed a range of research areas that are worthy of further investigation, as detailed below.

1. The leaching study only examined one soil type. Consequently, it would be worthwhile to study a wider range of soil types and a wider range of organic N forms. Specifically, soils with different textures (and thus different sorption properties) should be studied. The role of N movement down preferential flow pathways (e.g. macropores) would also be useful, as these are known to be highly effective for rapid transport of DOC from topsoil to subsoil. It would also be useful to study a wider range of DON substrates and specifically those compounds of higher molecular weight (e.g. proteins and longer chain peptides). Many peptides are also known to be poorly soluble, and a study of the movement of these in soil would also be informative, as many are known to have bioactive properties (i.e. antimicrobials). The study undertaken here was a laboratory study; therefore, it would be worthwhile to undertake the same study in the field, and to validate the experimental results with a mathematical model. Coupling the flow of N with C and other nutrients (e.g. P) in these mathematical models would also be worthwhile.
2. Dissolved organic nitrogen is a complex mixture of many compounds, so splitting this into HMW and LMW fractions, as we did, is a gross oversimplification. Better ways of fractionating and characterising the DON are therefore required. The bioavailability of these different compound groups then needs evaluating to determine their ecological significance.

3. Here it was shown that plants could readily take up and assimilate tetraalanine from outside the root. This was used as a model peptide; however, it is acknowledged that many thousands of peptides may exist in soil solution. These will have a wide diversity in charge, size, solubility and shape. This may have a major impact on their bioavailability and, therefore, a wider range of peptides needs to be studied in the context of both plant and microbial uptake.
4. Plants also exude amino acids and peptides from their roots. The balance between root exudation and root uptake therefore needs to be determined (i.e. under what conditions doe's net influx occur?). This will probably require the use of mathematical rhizosphere models in order to simulate the mass flow and diffusion of the different N solutes in soil.
5. The nutrient gradient study in the arctic soils only examined one potential effect of climate change and thus could not investigate interactions with other predicted effects. For example, it would be useful to look at the impact of climate warming (reduced snow cover), elevated CO_2, increased O_2, changes in water content and N deposition on rates of nutrient cycling and plant productivity. This could then be linked more explicitly to rates of carbon sequestration in soil and to impacts on the wider food chain (herbivores) and water quality.
6. The results on the soil profile suggested that deeper rooting plants may be of limited importance in capturing nutrients from depth. Further work could look at a wider range of crop plants, as they are known to differ markedly in their rooting strategies. Instead of recapturing nutrients from depth, it may be more

advantageous to develop slow-release fertilisers, which greatly reduce inadvertent nutrient loss to subsoil.

7. In addition to all these suggestion it is necessary to study the ability of uptake the protein by microbes and plants in sterile condition and the competition between them on this N sources.

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Appendix 1

Long-term nutrient enrichment drives soil and vegetation community development in Arctic habitats

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1. Introduction

Net primary production in the terrestrial Arctic is typically limited by short growing seasons, cold temperatures, frequent and strong winds and low nutrient supply (Bobbink et al., 2010). Consequently, the projected rapid increase in temperature,

changes in precipitation pattern and enhanced atmospheric nitrogen (N) deposition (Førland et al., 2011; Geng et al., 2010) are predicted to have profound effects on polar ecosystem functioning (White et al., 2000; Myers-Smith et al., 2011; McManus et al., 2012). One of the primary impacts of these anthropogenically mediated changes are the projected nutrient induced shifts in plant community composition which will impact directly on a range of ecosystem services including water quality, soil carbon storage and food provisioning (grazer biodiversity) (Natali et al., 2012; Post and Pedersen, 2008). Understanding and predicting the long-term resilience and potential feedbacks in response to environmental change therefore remains a central goal in polar ecosystem science.

Short-term (ca. 5-10 y) studies in Arctic tundra have frequently reported that nutrient enrichment (e.g. N and P) causes relatively rapid changes in above and below-ground diversity (Gordon et al., 2001; Madan et al., 2005; Campbell et al., 2010). These studies, however, have often simulated enrichment by pulse additions of high levels of inorganic nutrients which can lead to bias in environments where spatial and temporal niche separation for nutrient and water acquisition occurs (Clarkson et al., 2009; Glanville et al., 2012). While these studies have proved extremely useful in demonstrating the importance of plant-soil-grazer feedbacks, a number of studies have also concluded that these may also not accurately reflect long-term responses to nutrient addition (Dormann and Woodin, 2002; Ellis and Rochefort, 2004).

An alternative strategy to artificial nutrient manipulation is to investigate ecosystem responses at sites which have experienced natural enrichment over much longer time periods (ca. 1000-10,000 y) and which are assumed to be at quasi steady state (Holloway et al., 1998; Osada et al., 2010). Unlike most small-scale experimental

plot additions (<10 m^2), the natural long-term addition of nutrients typically occurs in a spatially heterogeneous pattern often leading to the formation of large scale (>1000 m^2) nutrient enrichment gradients. Further, differential grazing across these expansive gradients over long time periods can induce genuine feedbacks in vegetation and soil ecology. The disadvantage of using these sites, however, is that their full chronology can only be partially resolved from either biomarkers (e.g. pollen records) or geomarkers (e.g. ^{14}C dating, $\delta^{15}N$, $\delta^{13}C$) and that rarely are the nutrients deposited singly (Rozema et al., 2009).

Large scale variation in nutrient enrichment in tundra environments typically occurs due to differences in underlying parent material, hydrology, anthropogenic pollution and homoeothermic animal abundance (Löffler and Pape, 2008; Naito and Cairns, 2011). Of these, seabirds are responsible for the creation of some of the largest tundra nutrient gradients (Stempniewicz, 2005). This arises due to their continual foraging for food at sea with subsequent roosting and breeding on land leading to transfer of nutrients from a highly productive marine habitat to an intrinsically nutrient poor terrestrial habitat. This is exemplified by estimates of annual guano deposition of up to 60 t km^{-2} y^{-1} by Little Auks (*Alle alle*) which commonly exist in dense land colonies (>1000 pairs $site^{-1}$) often located kilometers from the coast (Stempniewicz et al., 2006). Faecal deposition tends to be concentrated close to the colony but also occurs during bird flight leading to a progressive decline in nutrients from the nesting site towards the coast.

Changes in vegetation and herbivore populations in response to orthinogenic nutrient enrichment are well documented (Zmudczynska et al., 2008; Jakubas et al., 2008). In contrast, the impacts of seabirds on soil development, microbial community

functioning and nutrient cycling remain fragmentary (Solheim et al., 1996). The aim of this study was therefore to investigate the potential feedbacks between soil development, microbial growth, biomass and activity, plant community structure and herbivore grazing along two contrasting nutrient enrichment gradients in the high Arctic.

2. Materials and methods

2. 1. Site characteristics

The experimental site was located at Scoresbygrotta on Blomstrandhalvøya Island located in Kongsfjorden close to Ny Ålesund, Svalbard (78°59'10"N, 11°59'12"E). The site has a mean annual air temperature of -6.5°C (minimum -24.8°C, max. 12.1°C) and an annual precipitation of 197 mm with approximately 65% falling as snow. The site consists of a cliff face approximately 600 m in length running parallel to the coast (500 m from the shoreline) beneath which significant vegetation and soil development has occurred in response to the presence of seabirds. The cliff is composed of limestone, 25 m in height and is characterized by a middle section approximately 50 m in length that is heavily inhabited by seabirds (black-legged kittiwake, *Rissa tridactyla; Black guillemot, Cepphus grille; Glaucous gull, Larus hyperboreus*). In contrast, areas where very few seabirds are present flank this central area. Water movement down the cliff face was evident across all areas due to snowmelt and runoff from land directly above the cliff. At the three sampling times, the rate of water flow over the cliff face appeared to be visually similar at both bird inhabited and non-bird inhabited (control) areas. Below the cliff, the topography is characterized by a freely draining steep slope (45°) approximately 70 m in length that appears to consist of well-

colonized mixed glacial debris (limestone. red sandstone etc; Harland, 1997). Subsequently, this grades into a gently sloping (10°) poorly drained mire area approximately 500 m in length which is dominated by mosses and through which abundant surface water channels flow towards the sea.

The plant species under the bird cliff (0-70 m slope section) included *Puccinellia angustata* (R. Br.) Rand & Redf. ssp. Angustata, *Polygonum vivipara* L., *Cerastium arcticum* Lange coll., *Oxyria digyna* (L.) Hill, *Luzula confusa* Lindeb., *Draba* species, *Cochlearia groenlandica* L. coll., *Ranunculus hyperboreus* Rottb. ssp. *arnellii* Scheutz, *Saxifraga cernua* L., *Saxifraga hieracifolia* Waldst. & Kit. ex Willd. *ssp. hieracifolia*, *Saxifraga hirculus* L. ssp. *compacta* Hedberg, *Saxifraga oppositifolia* L., mosses and lichens. Those plant species predominantly absent from the bird cliff transects but present in the control (non-enriched) transects included: *Cassiope tetragona* L.D.Don. ssp. *tetragona* L., *Dryas octopetala* L., *Silene acaulis* (L.) Jacq., *Carex bigelowii* and *Salix polaris* Wahlenb..

2.2. Site sampling

Six transects were taken at 90° to the cliff face along the steeply sloping area. These included three transects emanating from the bird inhabited area and three control (non-bird enriched) transects. Along each transect, samples were taken at increasing distances from the cliff face (0.5, 2, 10, 20, 40 and 60 m) each of which were characterized by a different vegetation community structure. At each location, 10-15 soil samples (0 to 10 cm depth) were taken randomly and stored in gas-permeable plastic bags at 4°C to await analysis. Soil temperature in the 0-10 cm soil layer was also recorded. At each location, 3 randomly positioned quadrats (20 × 20 cm) were placed

over the vegetation and the above-ground plant material harvested and its biomass determined after drying (70°C, 24 h).

Replicate samples (n = 5) of the water flowing over the cliffs and entering the soil at 0-0.5 m (inflow) and the drainage water emanating from the base of the vegetated slope (70 m; outflow) were also sampled.

2.3. Soil analysis

Within 12 h of sample collection, soil solution was recovered from each transect sampling point using the centrifugal-drainage procedure (14,000 *g*, 5 min) of Giesler and Lundström (1993). The soil solutions recovered were further centrifuged for 5 min at 18,000 *g* to remove any suspended particles and the supernatant stored in polypropylene vials at -21°C prior to analysis.

Dissolved organic C (DOC) and total dissolved nitrogen (TDN) in solution were determined with a Shimadzu TOC-TNV analyzer (Shimadzu Corp., Kyoto, Japan). Concentrations of NH_4^+ and NO_3^- in soil solution were determined colorimetrically by microassay (Downes, 1978; Mulvaney, 1996). Dissolved organic nitrogen (DON) was then calculated as the difference between TDN and dissolved inorganic nitrogen. Total free amino acids (TFAA) were determined by fluorescence using the *o*-phthalaldehyde-β-mercaptoethanol procedure of Jones et al. (2002). Total C and N of soils were determined using a CHN-2000 analyzer (Leco Corp., St Joseph, MI, USA). Soil pH (1:1 v/v) was determined in distilled water as described in Smith and Doran (1996) while moisture content was determined by drying at 80°C. Basal soil respiration was determined in the laboratory at 10°C using an automated multichannel soil respirometer (Nordstrom Innovations Ltd., Umea, Sweden) and the steady state CO_2 production rates from 24 to 72 h recorded.

Nitrogen mineralization was determined by incubating soil collected from the field shortly after snowmelt at 10°C for up to 12 weeks (equivalent to the period when the soils are unfrozen). Briefly, soil was from each transect point was stored in gas permeable plastic bags and after 0, 7, 14, 21, 28, 56 and 72 days the soil was extracted with 0.5 M Na_2SO_4 (1:5 soil:Na_2SO_4 w/v; 30 min), centrifuged (18,000*g*, 5 min) and the supernatant recovered and frozen (-21°C) to await N analysis as described above.

2.4. Isotopic analysis

Natural abundance stable isotopic analysis of the soil and vegetation was undertaken as described by Bol et al. (20XX). For comparison, the isotopic signatures of the feathers of the kittiwakes and those of the dominant vegetation grazers at the site were also made. These included feathers and faeces from the barnacle goose (*Branta leucopsis*), tissue from the pink-footed goose (*Anser brachyrhynchus*) and fur, bone marrow and faeces from the Svalbard reindeer (*Rangifer tarandus*). As the grazers range freely within the Blomstrandhalvøya region (i.e. not spatially restricted to the transects) only one set of replicate samples was collected from the general area of the cliff site.

2.5. ^{14}C mineralization of low and high molecular weight organic matter

Field-moist soil (5 g) was collected from along the two transects and placed into individual 50 cm^3 polypropylene containers. Different ^{14}C-labelled substrates were then added to the soil and their mineralization to $^{14}CO_2$ monitored over time. The four ^{14}C-labelled substrates included: (1) plant leaves, (2) a mixture of low MW compounds

(simulated root exudates), (3) a mixture of 15 amino acids, and (4) the peptide, trialanine.

The high MW C source consisted of air-dried ^{14}C-labelled ground *Lolium perenne* L. green shoot material (12.3 kBq g^{-1}) labeled as described in Hill et al. (2007) and characterized and used as described in Glanville et al. (2012). Within the plant material, the soluble ^{14}C component constituted 32.9 ± 1.5 % of the total ^{14}C, while of the high MW ^{14}C remaining, 26.7 ± 1.0 % was soluble in 36 mM HCl, 43.8 ± 0.7 % was NaOH (1 M) soluble and 29.5 ± 3.4 % remained insoluble. Briefly, 100 mg of this ^{14}C-labelled plant material was added directly to 5 g of soil (46 mg C g^{-1}). The low MW C source consisted of a mixture of ^{14}C labeled substrates which represent common root exudate compounds and included: malic acid (1 mM), citric acid (2 mM), glucose (10 mM), fructose (2 mM), sucrose (1 mM), amino acid mixture (3 mM) comprising 15 equimolar ^{14}C labelled L-amino acids (alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tyrosine, valine). The exudates were dried down onto pure quartz sand as described in Glanville et al. (2012). The ^{14}C-labelled sand (100 mg; 2.19 kBq) was added to each 5 g soil sample to give a substrate addition rate of 0.01 mg C g^{-1}. The ^{14}C-labelled amino acids (an equimolar mixture of 15 different L-isomeric amino acids; 666 μM) and peptide (L-trialanine; 10 mM) were added to the soil in liquid form (0.1 ml g^{-1}; 0.09 kBq ml^{-1}) as described in Jones (1999). After the addition of each ^{14}C-substrate to the soil, a $^{14}CO_2$ trap containing 1 ml of NaOH was placed above the soil and the tubes capped. The tubes were then incubated at 10°C and the NaOH traps changed over time to determine the temporal dynamics of $^{14}CO_2$ production (0-90 d). The time was chosen to reflect the length of a typical growing season. The ^{14}C content of the NaOH traps was

determined with Scintisafe 3® scintillation cocktail (Fisher Scientific, Leicestershire, UK) and a Wallac 1404 liquid scintillation counter (Wallac EGandG, Milton Keynes, UK). After 90 d, the soil was extracted with 0.5 M Na_2SO_4 (1:5 w/v; 200 rev min^{-1}; 30 min), centrifuged (16,000 *g*, 10 min) and ^{14}C in the supernatant counted as described above.

A double first-order kinetic equation was fitted to the ^{14}C mineralization for the low MW substrates where

$$f = (a_1 \times \exp^{-k1t}) + (a_2 \times \exp^{-k2t}) \quad \text{(Eqn. 1)}$$

where a_1 describes the amount of ^{14}C allocated to the first mineralizable pool (a_1) and k_1 is the corresponding rate constant (Saggar et al., 1999; Boddy et al., 2008). The proportion of C partitioned to the second slower pool (a_2) is described by the rate constant k_2. The half-life of the first mineralization pool a_1 can be calculated using the following equation:

$$t_{½} = \ln(2)/k_1 \quad \text{(Eqn. 2)}$$

Calculating the half-life for the slower second phase a_2 is subject to uncertainty as the connectivity between these pools a_1 and a_2 is unknown (Saggar et al., 1999; Boddy et al., 2007).

2.6. Microbial community analysis

To assess the impact of nutrient inputs on ectomycorrhizal abundance, individual plants (n = 10) of *Polygonum vivipara* were selected from underneath the cliff face (0-1 m) and from the lower slope in both the control and orthinogenic transect. The roots were then rinsed with tap water and the ectomycorrhizal morphotypes categorized following the schema of XXX. Briefly, three fields of view (1.77 cm^2)

from each root system were studied at x15 magnification and the number of tips corresponding to each morphotype enumerated.

Fungal and bacterial growth estimates were obtained using the acetate-into-ergosterol-incorporation method to determine fungal growth, and leucine incorporation technique to determine bacterial growth. Briefly, bacterial growth was estimated using the leucine (Kirchman et al., 1985) with a homogenization / centrifugation step (Bååth, 1990) to adapt it for use in soil (Rousk and Bååth, 2011). Radiolabelled leucine ([^{3}H]leucine, 37 MBq ml^{-1}, 5.74 TBq mmol^{-1}, Perkin Elmer, UK) combined with non-radioactive leucine for a final concentration of 275 nM at a final concentration of 130 nM, were added to the bacterial suspension. After a 2 h incubation step at 22°C, growth was terminated with thichloroacetic acid, and samples were washed (Bååth et al., 2001) after which incorporated radioactivity was determined using liquid scintillation. Fungal growth was assessed by measuring acetate incorporation into ergosterol (Newell and Fallon, 1991; Rousk and Bååth, 2011), using [1-^{14}C] acetate (sodium salt, 7.4 MBq ml^{-1}, 2.04 GBq mmol^{-1}, Perkin Elmer, UK) combined with unlabeled sodium acetate to create a final concentration of 220 μM in a soil slurry, and an incubation time of 4 h at 22°C, after which growth was terminated by addition of formalin. Ergosterol was extracted, separated and quantified using HPLC with a UV detector (Rousk et al., 2009), and the ergosterol eluent was collected to determine its radioactivity using liquid scintillation. Ergosterol was also used to estimate fungal biomass-C assuming a fungal ergosterol content of 5 mg g^{-1} and a fungal C content of 45% (Joergensen et al., 2001; Ruzicka et al., 2001).

2.7. Statistical analysis

Statistical analyses (paired t-tests, linear regression, ANOVA) were undertaken with Minitab v15 (Minitab Inc., State College, PA) and Excel 2010 (Microsoft UK, Reading, UK). First order kinetics equations were fitted to the ^{14}C mineralization data with SigmaPlot 12 (Systat Software Inc., Chicago, IL) using a least sum of squares iteration routine.

3. Results

3.1. Plant characteristics

The influence of orthinogenic inputs on plant biomass and tissue N concentration is shown in Figure 1. Overall, there were few significant differences in above-ground plant biomass between the two treatments with the marked exception of the area directly under the cliff face in the orthinogenic transect where plant growth was very spatially heterogeneous. In contrast, the foliar N concentrations were significantly higher in the orthinogenic transect than the control transect ($P < 0.05$). Foliar N declined progressively with distance from the cliff face in the orthinogenic transect ($P < 0.001$), however, no such significant trend was observed in the control transect ($P > 0.05$). The C-to-N ratio of the foliage was on average 12 ± 2 in the orthinogenic transect and 33 ± 3 in the control transect ($P < 0.001$; On-line Supplementary Information Figure S1).

3.2. Major soil properties

The temperature of the soil declined away from the cliff face in both transects and was similar in both treatments although it tended to be slightly lower (1.0 ± 0.3 °C) in the orthinogenic transect. In contrast, soil pH was significantly higher in the control transect by on average 1.0 ± 0.1 pH units ($P < 0.01$). Soil moisture tended to be higher

in the orthinogenic soils especially close to the cliff face ($P < 0.05$). Soil respiration was significantly higher at nearly all points along the orthinogenic transect in comparison to the control transect ($P < 0.01$). After normalization for soil organic C, rates of soil respiration remained significantly higher in the orthinogenic transect (23 ± 3 mg CO_2 kg soil C^{-1} h^{-1}) in comparison to the control transect (16 ± 2 mg CO_2 kg soil C^{-1} h^{-1}; $P < 0.05$). If the lower temperature in the orthinogenic transect is accounted for using a Q_{10} factor of 2, then the difference is marginally greater (25 ± 3 mg CO_2 kg soil C^{-1} h^{-1}).

3.3. Plant available N

The concentration of different forms of soluble N in the orthinogenic and control soil transects are shown in Figure 4. Overall, the presence of birds caused a significant increase in total soluble N (TSN) with most N being present as NO_3^- close to the cliff face with concentration of NO_3^- subsequently declining with increasing distance. NO_3^- in the soil solution taken at 40 m were not significantly different to the control transect. The concentration of NH_4^+ was approximately 10-fold lower than that of NO_3^- with the exception of the final transect point at 40 m where NO_3^- was low and NH_4^+ dominated. At a distance of 10 m onwards, however, DON dominated the soluble N pool (83 ± 4% of the total) although only a small fraction of this was present as amino acids (2.8 ± 0.4% of the total DON). In the control transect the pattern was generally similar to the orthinogenic transect except that the concentrations were typically much lower.

Soil solution DOC concentrations were statistically similar ($P > 0.05$) at all points on the orthinogenic transect (mean ± SEM; 82 ± 7 mg C l^{-1}) and the control transect (30 ± 4 mg C l^{-1}) although the concentrations were significantly lower in the latter ($P < 0.01$; On-line Supplementary Information Figure S3). As for the total soil C-

to-N ratio, the DOC-to-DON ratio was also significantly lower ($P < 0.05$) in the orthinogenic transect (8 ± 2) relative to the control transect (14 ± 1). The streams leaving the orthinogenic transects were also enriched in soluble N.

3.4. Natural abundance stable isotope ratios

The $\delta^{15}N$ and $\delta^{13}C$ ratios of the plant and soils collected along the orthinogenic and control transects are shown in Figure 5. Overall, there were large differences in the $\delta^{15}N$ ($P < 0.001$) signatures but not the $\delta^{13}C$ signatures ($P > 0.05$) between the orthinogenic and control treatments. There were, however, no differences in the soil or vegetation $\delta^{15}N$ at individual points along the transects within an individual treatment ($P > 0.05$). The $\delta^{15}N$ signature of the soil was significantly more negative than that of the vegetation in the orthinogenic treatments ($P < 0.05$), however, a significant contrasting trend was seen in the control treatment ($P < 0.001$).

The $\delta^{13}C$ signatures the vegetation samples were significantly more negative than the soils in both the orthinogenic and control treatments ($P < 0.01$). Significant differences were also seen along the orthinogenic transect ($P < 0.05$) but not in the control transect.

The $\delta^{13}C$ and $\delta^{15}N$ signatures of the birds and their primary food source (polar cod and capelin) were extremely similar (Fig. 5). While the $\delta^{13}C$ signals of these were very different from any of the vegetation and soil samples, their $\delta^{15}N$ signals remained extremely similar to the vegetation and soils collected from the orthinogenic transects.

3.5. ^{14}C-labelled substrate mineralization

The mineralization of the ^{14}C-labelled plant material is shown in Figure 6A-C. Over the 90 d incubation period approximately 10% of the plant material was mineralized, however, there were few significant differences in mineralization rate both within transects and between the orthinogenic and control treatments. The only exception was the significantly higher rate of mineralization of plant material observed at 2 m in the orthinogenic transect.

The mineralization of the low MW root exudate mixture was approximately 6-fold faster than that of the ^{14}C-plant material within the first 7 d (Fig. 6C-D). Although mineralization was faster in the control transect in comparison to the orthinogenic transect, there were no clear transect patterns. At the end of the 90 d incubation, a 0.5 M K_2SO_4 extract only recovered 2.1 ± 0.3% of the initial ^{14}C added indicating that the majority of the ^{14}C had become immobilized in the microbial biomass. In contrast, to the root exudates, there was no significant treatment r transect effect on the mineralization of the 14C-labelled peptide (On-line Supplementary Information S4). The mineralization of the 14C-labelled amino acids was marginally faster in the orthinogenic treatments although again no transect pattern was apparent (On-line Supplementary Information S4).

3.6. Abundance of N cycling genes in the microbial community, fungal and bacterial growth and fungal biomass

The abundance of key genes involved in soil N cycling are presented in Figure 7. Within the ammonia oxidizing archaea, the copy number of the *amoA* gene was more abundant at all locations along the control non-nutrient enriched transect in comparison to the orthinogenic transect (transect average; control XX ± X gene copies g^{-1};

orthinogenic XX ± X gene copies g^{-1}). In contrast, the abundance of the *amoA* gene within the bacterial community showed the opposite trend with highest levels of *amoA* seen close to the cliff face in the orthinogenic transect and very little presence in the control transect. The abundance of the nitrous oxide reductase (*nosZ)* gene in bacteria showed an almost identical trend to that of bacterial *amoA* with maximal activity directly at the cliff face and subsequently declining with increasing distance away from the cliff face. The same level of abundance of *nosZ* were seen at all locations along the control transect.

Bacterial growth measured as leucine incorporation into extracted bacteria tended to be generally higher in bird-inhabited areas, with markedly higher rates 0.5 and 2 m from the cliff face (both $P < 0.01$; Fig. 9A). Fungal growth measured with acetate incorporation into ergosterol were at similar levels in both bird-inhabited and bird-free areas, and showed a similar, if less pronounced, stimulation to bacterial growth close to the bird cliff-face ($P < 0.05$; Fig. 9B), after which rates converged further from the cliff. Fungal biomass, as estimated from the ergosterol concentration, did not show pronounced dynamics in the bird-free area, except at 0.5 m from the cliff face, where rates were markedly lower than at the same distance from the bird-inhabited cliff ($P < 0.01$; Fig. 9C). A further 2-10 m from the cliff face, the fungal biomass was indistinguishable between bird-inhabited and bird-free areas, while they subsequently diverged in the bird-inhabited area, reaching levels more than two-fold that of the bird-free area at 20 m and beyond ($P < 0.05$).

In both treatments, the *Polygonum vivipara* plants were heavily colonized by ectomycorrhizas all along the transect with three dominant morphotypes comprising 95

± 2% of the total colonized tips. Overall, there were no major significant differences between the two treatments (Fig. 8).

4. Discussion

Seabirds are migratory leaving kongsfjorden in winter – arrival in spring and departure in autumn (May-Aug). Surface or near-surface feeders. Feed on zooplankton and pelagic fish. Seabirds excrete 10.2% mass of the food intake (Brekke and Gabrielsen (1994) of which excrement contains 21% N faeces and uric acid together

Context for chronic and long-term N fertilization input – compare with park grass experiment, UK. More than 150 years of 1-150 kg N ha-1yr-1.
E.g. FEMS Microbiology ecology 76 (2011) 89–99.

Carcasses, shed feathers and eggs
An explanation might be that this species feeds on polar cod and Themisto spp., which transfer the Calanus signature. They are pelagic species preying on capelin and polar cod.

Acknowledgements

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Figure legends

Fig. 1. Above-ground biomass and foliar N content of tundra vegetation as a function of increasing distance from cliffs with (orthinogenic) and without (control) bird-derived nutrient inputs. Values represent mean ± SEM (n = 3). *, **, *** and *NS* represent $P < 0.05$, $P < 0.01$, $P < 0.001$ and $P > 0.05$ respectively. The legend is the same for all panels.

Fig. 2. Soil organic C and N content as a function of increasing distance from cliffs with (orthinogenic) and without (control) bird-derived nutrient inputs. Values represent mean ± SEM (n = 3). *, **, *** and *NS* represent $P < 0.05$, $P < 0.01$, $P < 0.001$ and $P > 0.05$ respectively. The legend is the same for all panels.

Fig. 3. Soil characteristics as a function of increasing distance from cliffs with (orthinogenic) and without (control) bird-derived nutrient inputs. Values represent mean ± SEM (n = 3). *, **, *** represent $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively. The legend is the same for all panels.

Fig. 4. Soil solution concentration of different forms of soluble organic and inorganic N as a function of increasing distance from cliffs with (orthinogenic) and without (control) bird-derived nutrient inputs. Values represent mean ± SEM (n = 3). *, **, *** represent $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively. The legend is the same for all panels. Note the different y-axis scales between the upper and lower panels.

Fig. 5. $\delta^{13}C$ and $\delta^{15}N$ isotopic values for tundra soil and vegetation collected from transects with (orthinogenic) and without (control) bird-derived nutrient inputs. The isotopic values of Kittiwake muscle from birds at the same location is also

shown (data from Wold et al., 2011). Values represent mean ± SEM (n = 3). The legend is the same for both panels.

Fig. 6. Mineralization kinetics of ^{14}C-labelled plant material and root exudates in soil as a function of increasing distance from cliffs with (orthinogenic) and without (control) bird-derived nutrient inputs. Values represent mean ± SEM (n = 3). * represents significant differences between the control and orthinogenic treatment (paired t-test) at the $P < 0.05$ level.

Fig. 7. Quantification of amoA in ammonia-oxidizing *archaea* (*AOA*) and bacteria (AOB) and nitrous oxide reductase (nosZ) in bacteria as a function of increasing distance from cliffs with (orthinogenic) and without (control) bird-derived nutrient inputs. Values represent mean ± SEM (n = 3).

Fig. 8. Quantification of the three main ectomycorhizal morphotypes found on *Polygonum vivipara* L. at sites with (orthinogenic) and without (control) bird-derived nutrient inputs.

Fig. 9. Bacterial growth (panel A), fungal growth (panel B) and fungal biomass (panel C) along distance from cliffs with (orthinogenic) and without (control) bird-derived nutrient inputs. Values represent mean ± SEM (n = 3). * and ** represent $P < 0.05$ and $P < 0.01$ respectively. The legend is the same for all panels.

Figure 1

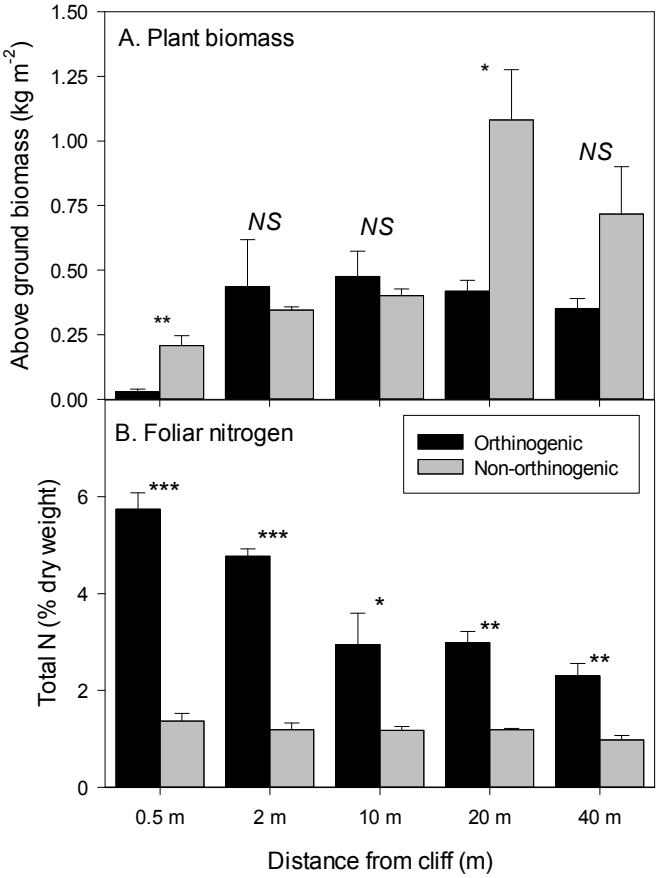

A. Plant biomass
Above ground biomass (kg m-2)
1.50
1.25
1.00
0.75
0.50
0.25
0.00
**
NS
NS
*
NS
B. Foliar nitrogen
Orthinogenic
Non-orthinogenic
Total N (% dry weight)
6
4
2
0
***
***
*
**
**
0.5 m
2 m
10 m
20 m
40 m
Distance from cliff (m)

Figure 2

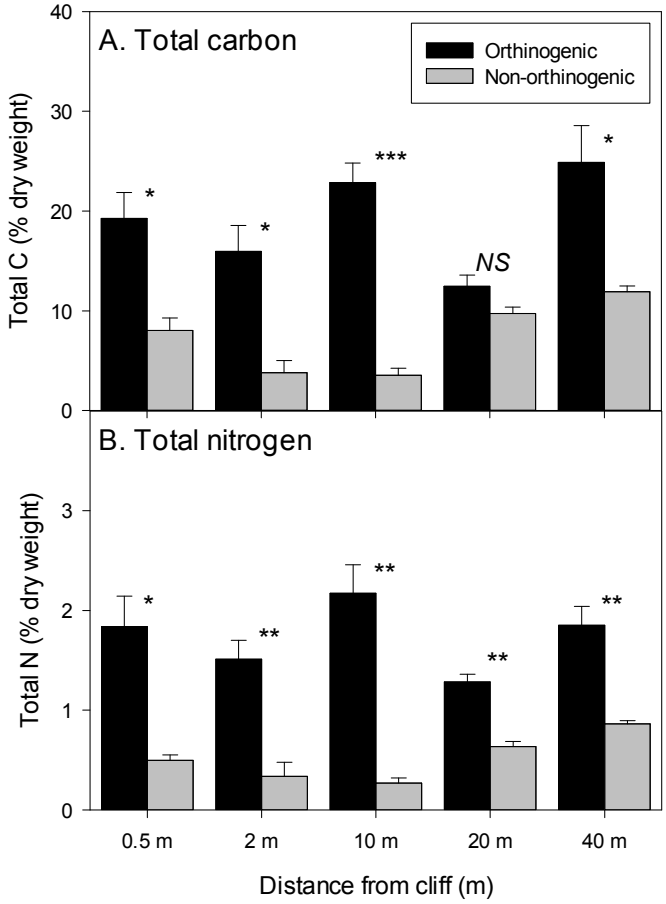

A. Total carbon
Orthinogenic
Non-orthinogenic
Total C (% dry weight)
40
30
20
10
0
*
*
***
NS
*
B. Total nitrogen
Total N (% dry weight)
3
2
1
0
*
**
**
**
**
0.5 m
2 m
10 m
20 m
40 m
Distance from cliff (m)

Figure 3

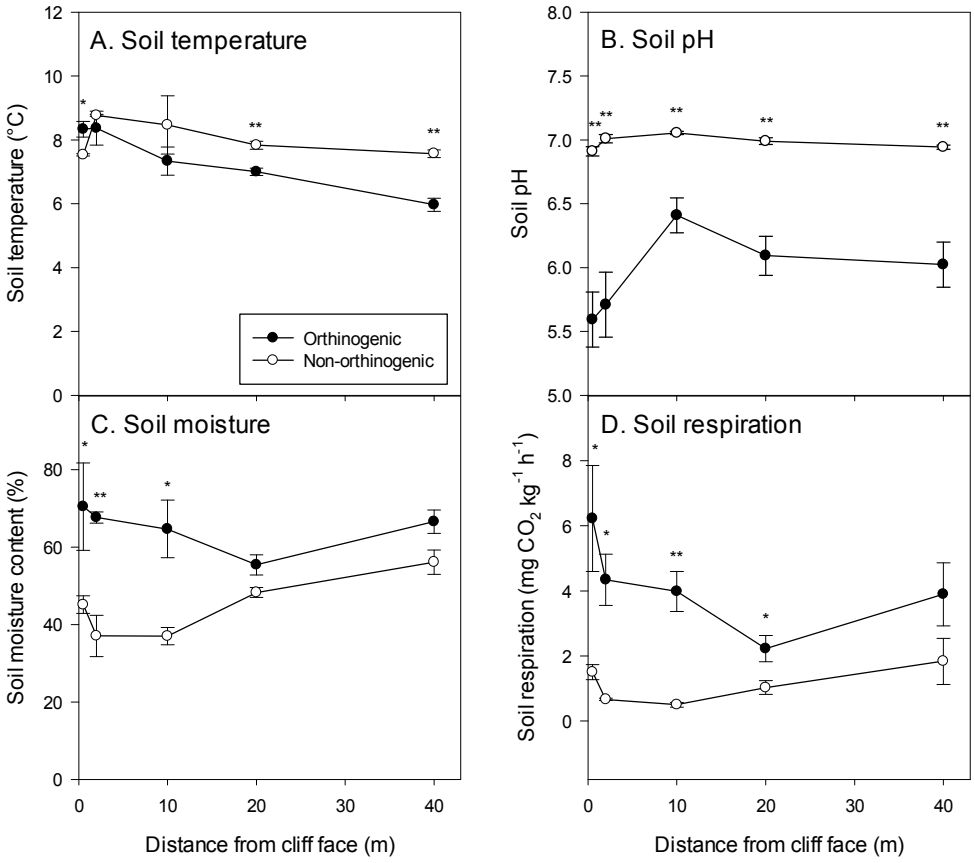

A. Soil temperature
Soil temperature (°C)
Orthinogenic
Non-orthinogenic
B. Soil pH
Soil pH
C. Soil moisture
Soil moisture content (%)
D. Soil respiration
Soil respiration (mg CO2 kg-1 h-1)
Distance from cliff face (m)
Distance from cliff face (m)

Figure 4

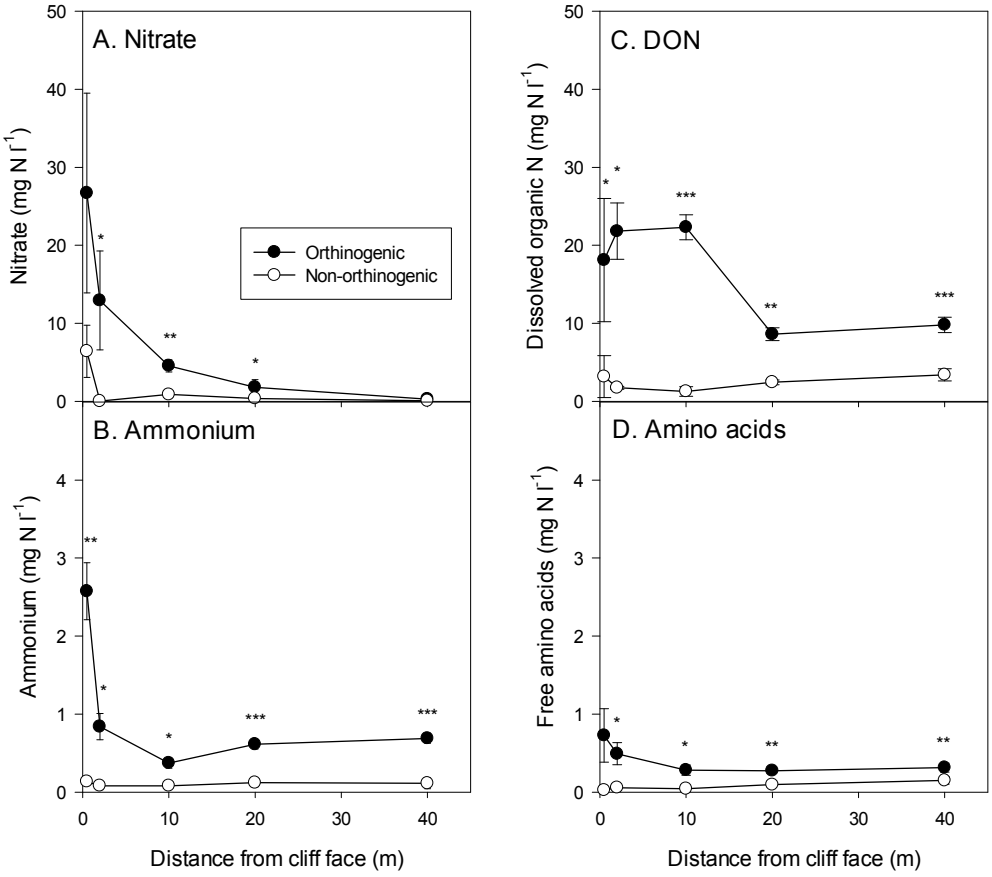

A. Nitrate
B. Ammonium
C. DON
D. Amino acids
Nitrate (mg N l-1)
Ammonium (mg N l-1)
Dissolved organic N (mg N l-1)
Free amino acids (mg N l-1)
Orthinogenic
Non-orthinogenic
Distance from cliff face (m)
Distance from cliff face (m)

Figure 5

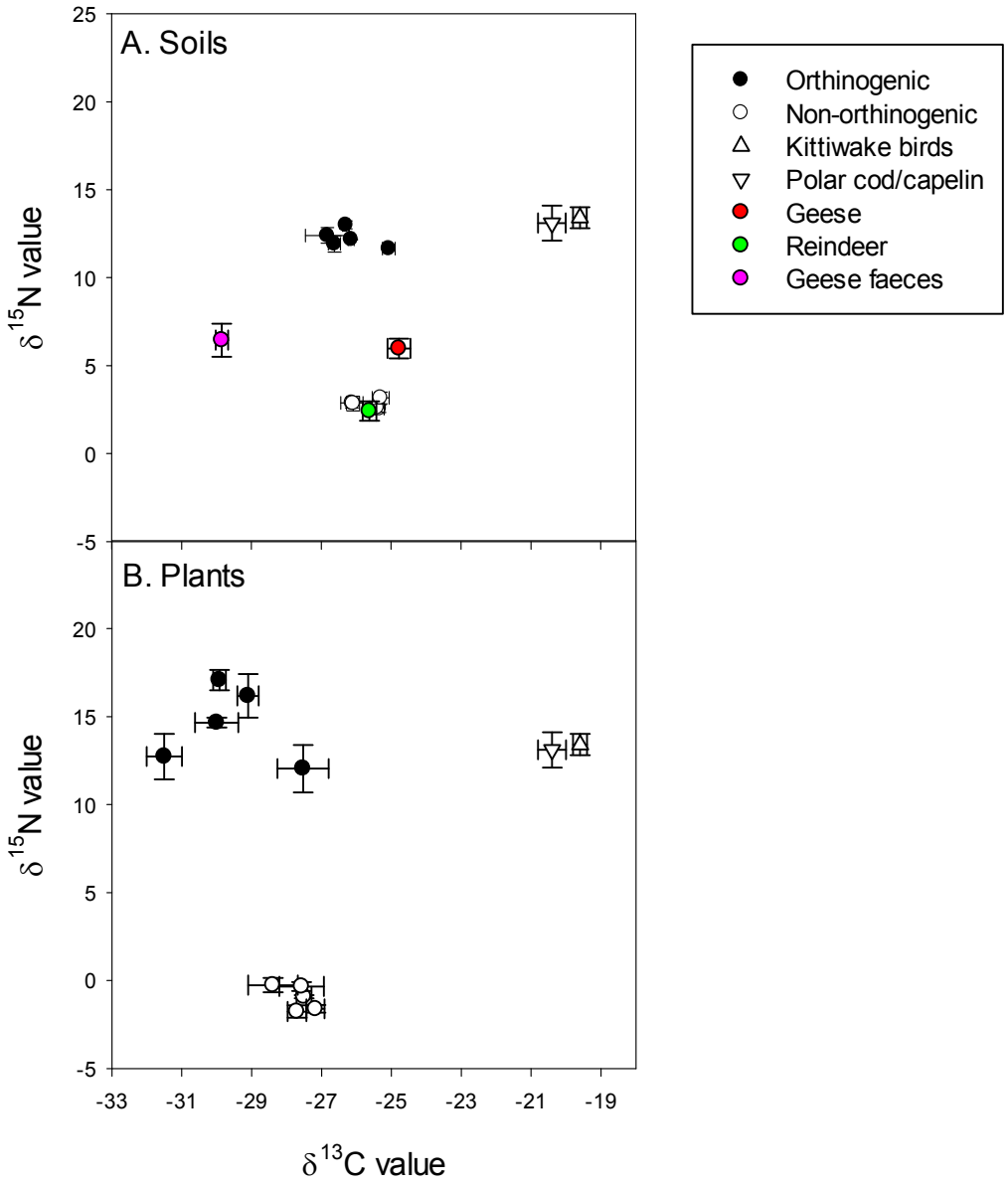

A. Soils
B. Plants
δ15N value
δ13C value
25
20
15
10
5
0
-5
-33
-31
-29
-27
-25
-23
-21
-19
Orthinogenic
Non-orthinogenic
Kittiwake birds
Polar cod/capelin
Geese
Reindeer
Geese faeces

Figure 6

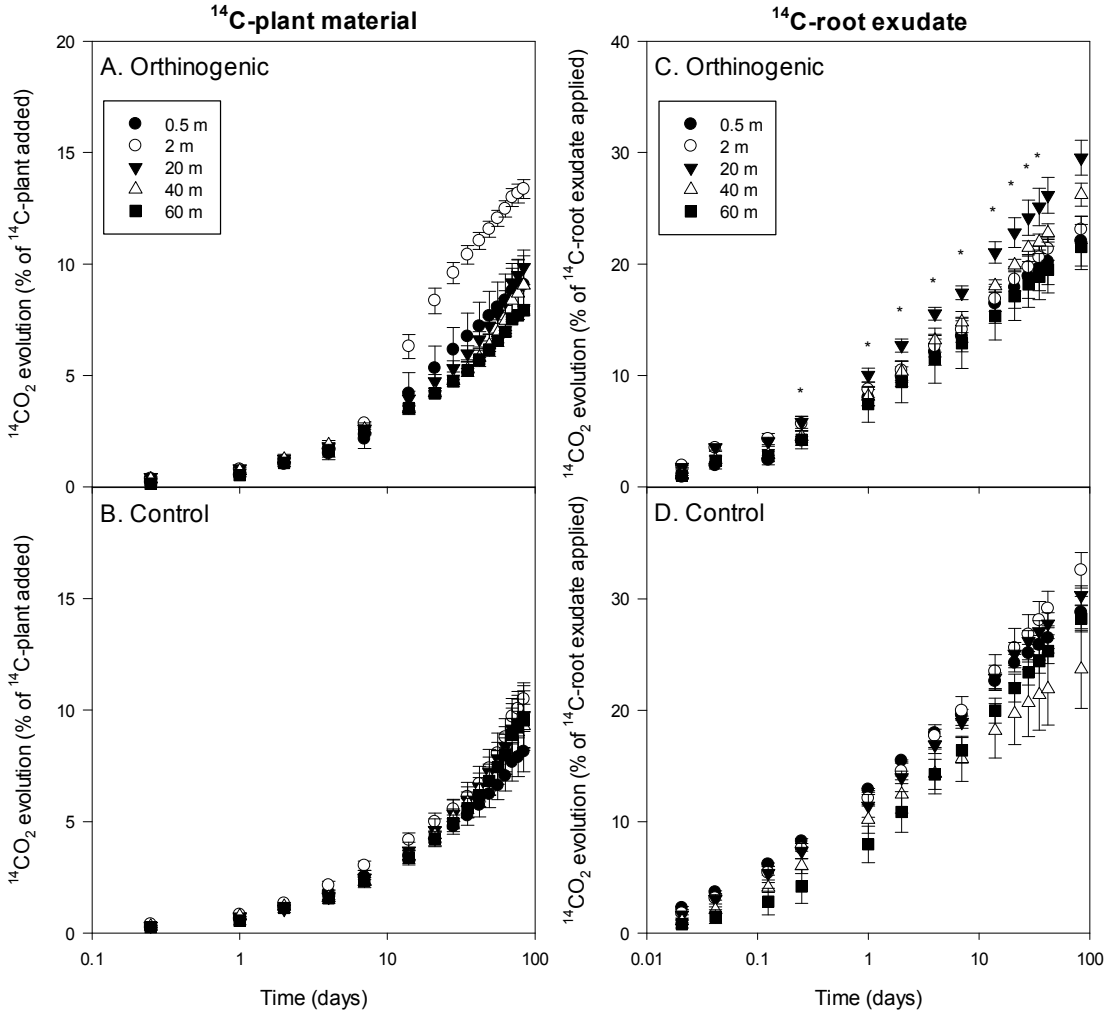
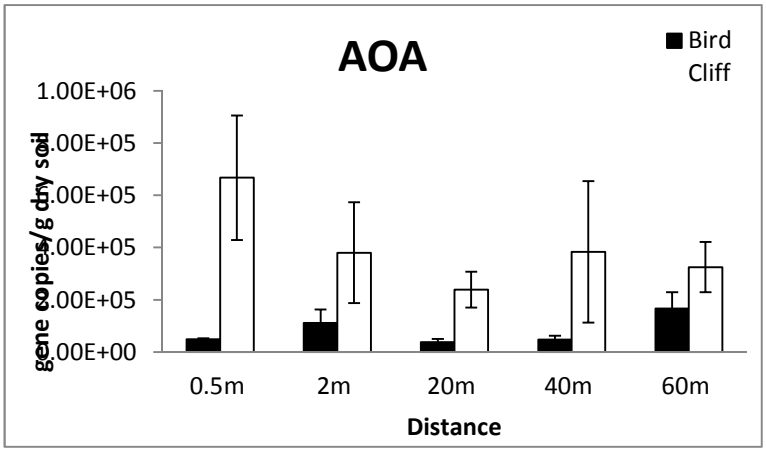
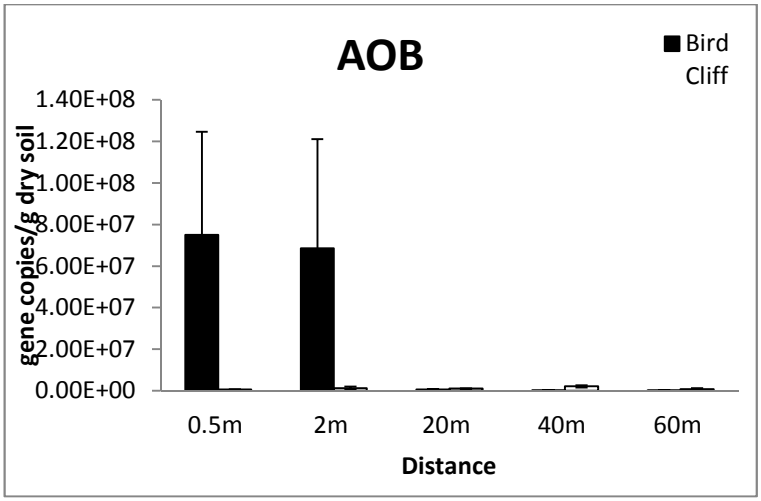

14C-plant material
14C-root exudate
A. Orthinogenic
B. Control
C. Orthinogenic
D. Control
0.5 m
2 m
20 m
40 m
60 m
14CO2 evolution (% of 14C-plant added)
14CO2 evolution (% of 14C-root exudate applied)
Time (days)

Figure 7


AOA
Bird
Cliff
1.00E+06
gene copies/g dry soil
0.5m
2m
20m
40m
60m
Distance


AOB
Bird
Cliff
1.40E+08
1.20E+08
1.00E+08
8.00E+07
6.00E+07
4.00E+07
2.00E+07
0.00E+00
gene copies/g dry soil
0.5m
2m
20m
40m
60m
Distance

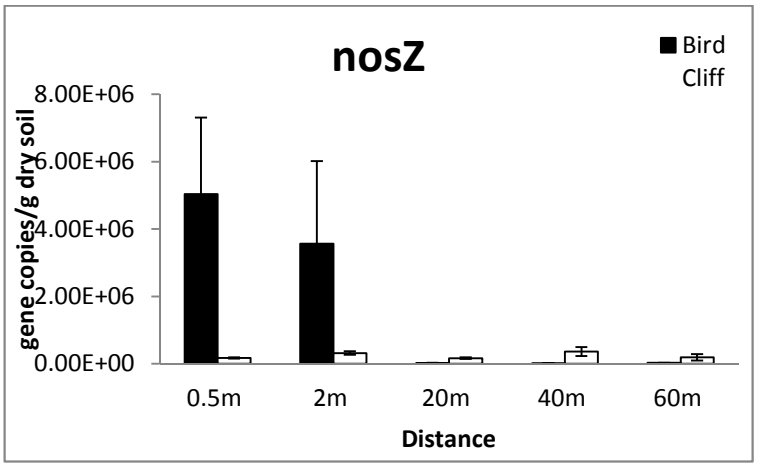

nosZ
Bird
Cliff
8.00E+06
6.00E+06
4.00E+06
2.00E+06
0.00E+00
gene copies/g dry soil
0.5m
2m
20m
40m
60m
Distance

Figure 8

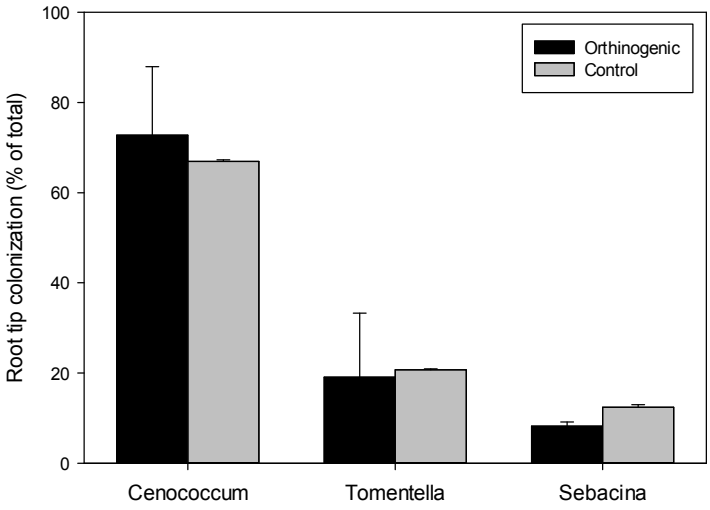
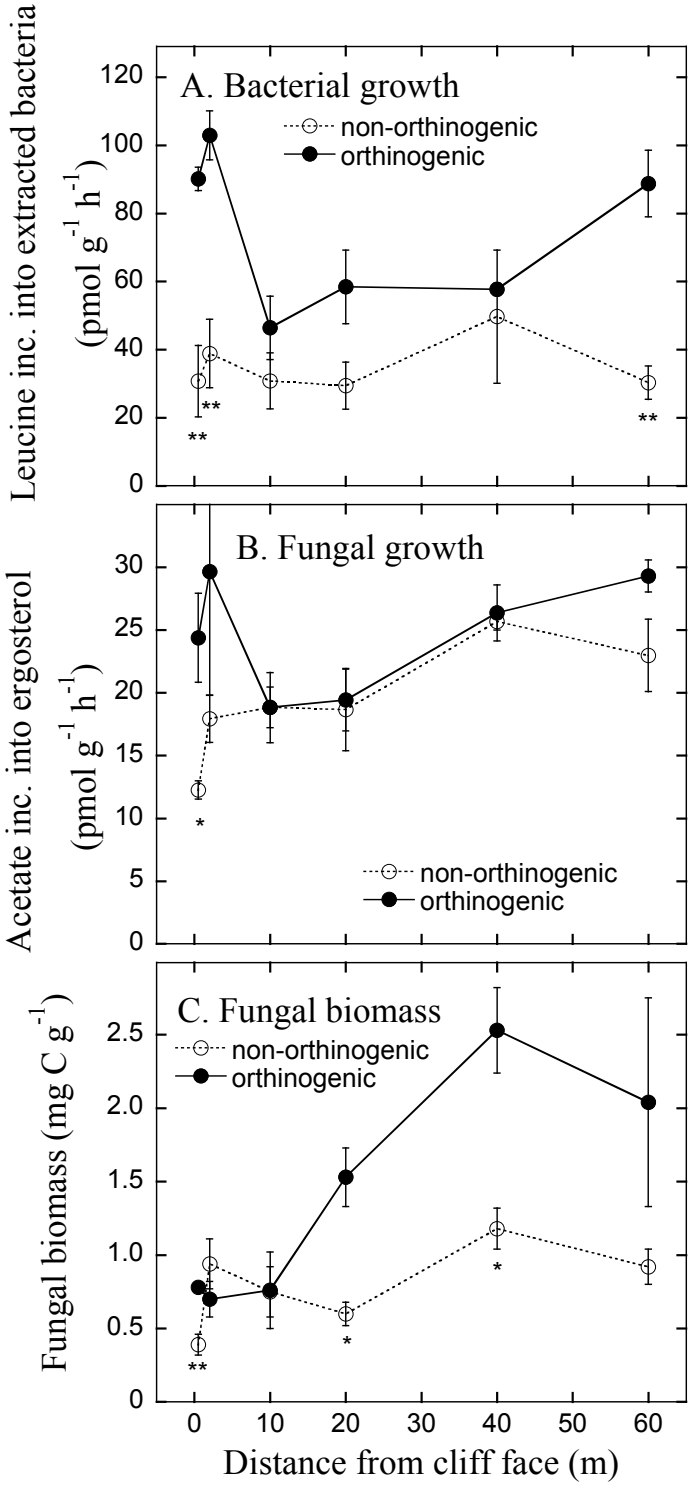

Orthinogenic
Control
Root tip colonization (% of total)
100
80
60
40
20
0
Cenococcum
Tomentella
Sebacina

Figure 9


A. Bacterial growth
non-orthinogenic
orthinogenic
Leucine inc. into extracted bacteria (pmol g-1 h-1)
B. Fungal growth
Acetate inc. into ergosterol (pmol g-1 h-1)
C. Fungal biomass
Fungal biomass (mg C g-1)
Distance from cliff face (m)

Supplementary Figure 1

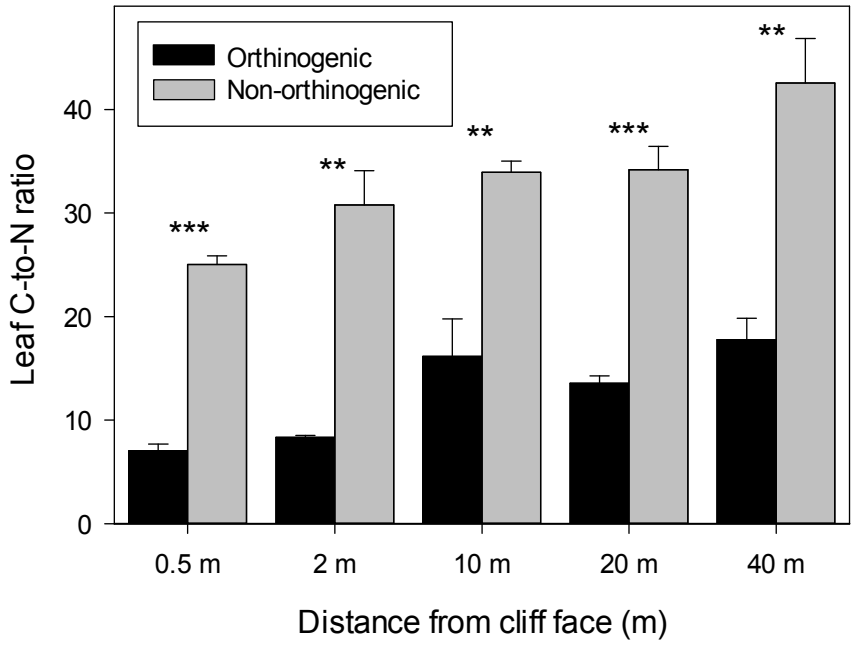


Fig. S1. Above-ground C-to-N ratio of tundra vegetation as a function of increasing distance from cliffs with and without orthinogenic nutrient inputs. Values represent mean ± SEM (n = 3). *, ** and *** represent significant differences at the $P < 0.05$, $P < 0.01$ and $P < 0.001$ level respectively.

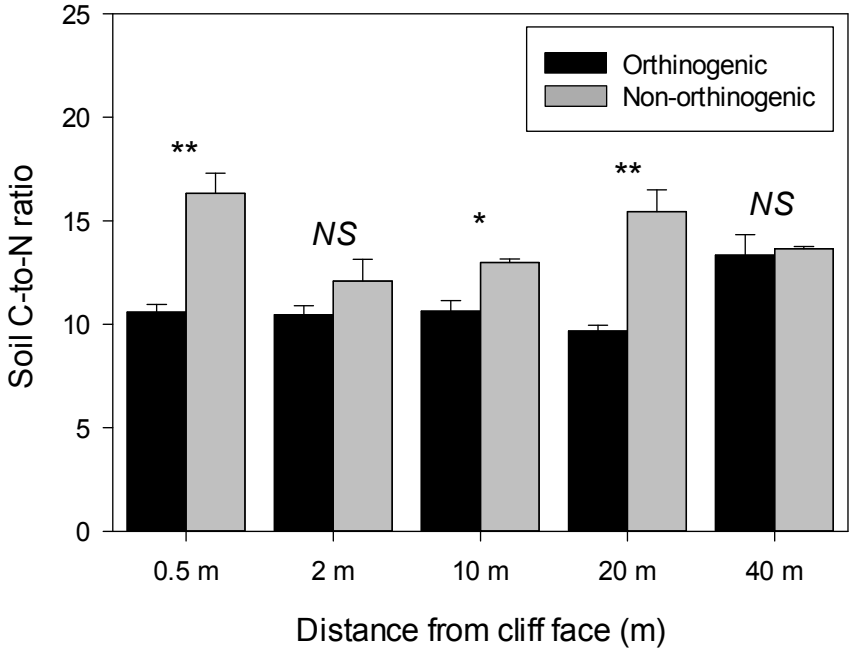


Fig. S2. Soil C-to-N ratio as a function of increasing distance from cliffs with and without orthinogenic nutrient inputs. Values represent mean ± SEM (n = 3). *, ** and *NS* represent significant differences at the $P < 0.05$, $P < 0.01$ and $P > 0.05$ respectively.

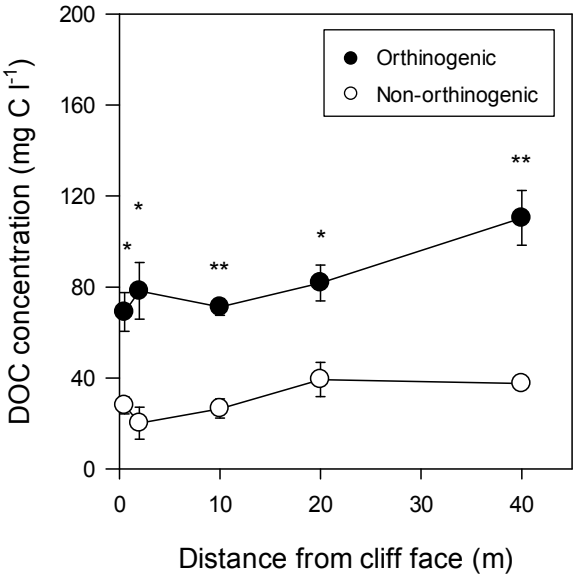


Fig. S3. Soil solution dissolved organic carbon (DOC) concentration as a function of increasing distance from cliffs with and without orthinogenic nutrient inputs. Values represent mean ± SEM (n = 3). * and ** represent statistical differences at the $P < 0.05$ and $P < 0.01$ level respectively.

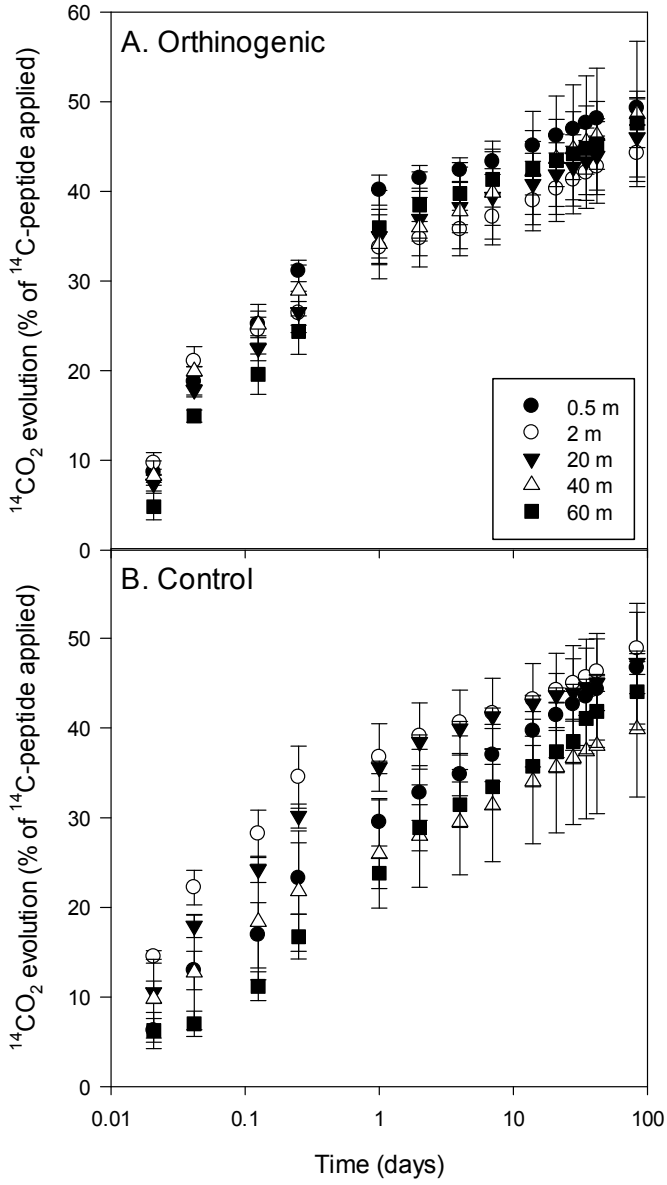


Fig. S4. Mineralization kinetics of ^{14}C-labelled peptides in soil as a function of increasing distance from cliffs with and without orthinogenic nutrient inputs. Values represent mean ± SEM (n = 3).

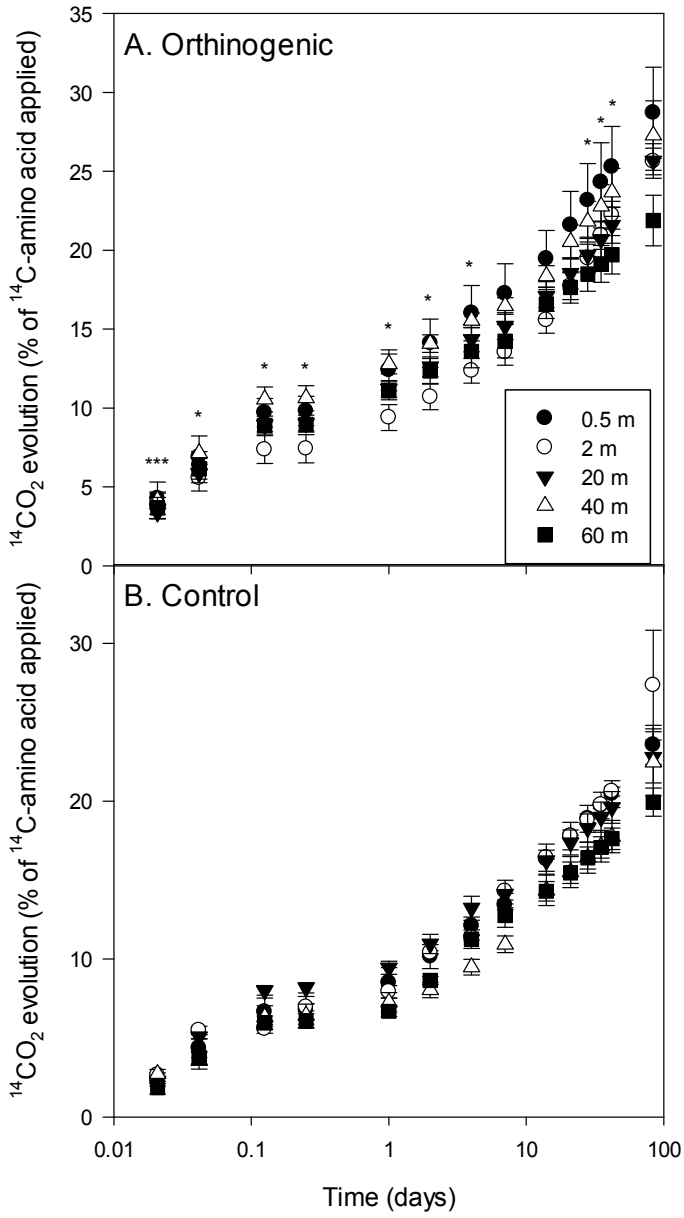


Fig. S5. Mineralization kinetics of ^{14}C-labelled amino acids in soil as a function of increasing distance from cliffs with and without orthinogenic nutrient inputs. Values represent mean ± SEM (n = 3). * and *** represent significant differences between the control and orthinogenic treatment (paired t-test) at the $P < 0.05$ and $P < 0.001$ level respectively. At the end of the 90 d incubation, 1.1 ± 0.1% of the ^{14}C could be recovered from the soil by a 0.5 M K_2SO_4 extract when averaged across all treatments (n = 10).