MASTERS BY RESEARCH

Hotspots and Hot Moments of Amino Acid Nitrogen: Real-time Insights Using Continuous Microdialysis Sampling

Hill, Elliot

Award date:
2019

Awarding institution:
Bangor University

MASTERS BY RESEARCH

Hotspots and Hot Moments of Amino Acid Nitrogen: Real-time Insights Using Continuous Microdialysis Sampling

Hill, Elliot

Award date:
2019

Hotspots and Hot Moments of Amino Acid Nitrogen: Real-time Insights Using Continuous Microdialysis Sampling

Elliot James Hill

School of Environment, Natural Resources and Geography

Bangor University

Bangor, United Kingdom

31st March 2019

A dissertation submitted for the degree of Masters of Science by Research

Bangor University

Supervisors:

Dr. Paul W. Hill

School of Environment, Natural Resources and Geography
Bangor University
Bangor, United Kingdom

Professor David L. Jones

School of Environment, Natural Resources and Geography
Bangor University
Bangor, United Kingdom

Dr. Eric Paterson

The James Hutton Institute,
Craigiebuckler, Aberdeen
United Kingdom

Professor Kevin Newsham
British Antarctic Survey
Cambridge, United Kingdom

Abstract

Nitrogen (N) is a principal component of biological activity and quantitatively the most important nutrient involved in plant growth, which in turn may present a significant limiting factor for plant productivity in both natural and anthropogenic ecosystems. Other researchers have identified three key determinants of plant N nutrition. Namely, these are; (1) availability: specifically the access which the plant has to the N form within the soil, e.g. pool size and nutrient fluxes close to plant roots (2) uptake: does the plant possess the capacity to acquire the target N compounds from the soil, and (3) metabolism: does the plant possess the capacity to assimilate the target N compound into its own biomass? These works focus predominately on number one, availability.

Protein hotspots in soil, such as those associated with decaying soil fauna or plant litter, may produce ephemeral patches of disproportionately high nitrogen forms. These hotspots may occur at the macro- and microscale in close proximity to plant roots, however, the likely concentration of soluble products produced in these hotspots remains poorly understood. To address this, we buried two contrasting biomass residues in soil, namely earthworm (*Lumbricus terrestris*) and clover (*Trifolium repens*). Their transformation to amino acids, NH_4^+ and NO_3^- were monitored continually over 6 days using microdialysis. Microdialysis is a novel, membrane based sampling technique with origins in medical science, which has seen limited use in environmental sampling, and to our knowledge, has never been used to quantify fluxes of soil organic matter breakdown products in continuous real time before. For the purposes of our experiment, microdialysis acted biomimetically as a plant root, as it allows bidirectional flow of perfusate across the probe membrane (simulating root exudates and uptake).

Through the use of microdialysis we are able to show that soil nutrient hotspots may provide nearby roots with concentrations of amino acids and NH_4^+ several orders of magnitude higher than found in the bulk soil solution; a highly significant step towards understanding the mechanistic processes which likely occur in soil nutrient hotspots close to plant roots.

Acknowledgements

Many thanks to my supervisors at Bangor, Dr. Paul W. Hill and Professor David L. Jones, without whom this work wouldn't have been possible. Thanks also to my secondary supervisors Dr. Eric Paterson and Professor Kevin Newsham for guidance. Thanks to the lab technicians at Environment Centre Wales and the STARS group.

Contents

List of Figures

List of Tables

Chapter 1 – Literature Review

1.1 Introduction

Nitrogen (N) is a principal component of biological activity and quantitatively the most important nutrient involved in plant growth, which in turn may present a significant limiting factor for plant productivity in both natural and anthropogenic ecosystems (Rentsch *et al.*, 2007; Vitousek *et al.*, 1997, Gruber & Galloway, 2008; Tartowski & Howarth, 2013; Jones *et al.*, 2013). An essential element for biological processes, N is required in DNA synthesis, enzyme production and function for a diverse range of organisms; consequently controlling net primary productivity in the majority of terrestrial ecosystems (Chapman, *et al.*, 2006; Lambers *et al.*, 2008; Tartowski & Howarth, 2013). Additionally, in plants, N is a constituent element of proteins, purines, and pyrimidines, alongside many enzymes and co-enzymes, and is therefore concomitant with sustained growth.

Owing to this, N deficiency causes chlorosis, resulting from inhibition of chlorophyll synthesis, and greatly reduces the productivity of the affected plant; in addition to overall reduction of leaf area, and a reduction of storage compounds such as oils and proteins (Epstein, 1971; Tegeder & Rentsch, 2010). The majority of N enters the soil as organic matter, which is frequently the most dominant portion of the soil N pool, with estimates of up to 95% of total soil N being comprised of organic forms (Schnitzer & Khan, 1989; Jones & Willett, 2006); with peptides and amino acids released by microorganisms via the breakdown of plant and animal residues (Schnitzer & Khan, 1989; Schulten & Schnitzer, 1998).

Previous understanding of plant N nutrition was dominated by the view that microbial mineralization of organic N (oN) to inorganic N (iN) acted as the rate-limiting step in plant N availability, which subsequently formed a 'bottleneck' within the terrestrial N cycle (Schimel & Bennett, 2004; Weigelt *et al.*, 2004). This was supported by the conventional view that plants which lacked an N_2 fixing symbiont acquired N solely in the form of ammonium (NH_4^+) and nitrate (NO_3^-) directly from the soil (Kielland, 1994; Jones *et al.*, 2013).

However, a significant body of evidence has now identified a 'short-circuit' in the N cycle, whereby plants, from a diverse range of agricultural & non-agricultural species, in addition to high– & low-productivity ecosystems, have been shown to bypass the need for microbial mineralisation and uptake oN; particularly the resultant short peptides and amino acid monomers produced early in protein decomposition, from the dissolved organic N (DON)

pool directly (Chapin *et al.*, 1993; Martens & Frankenberger, 1994; Kielland, 1994; Atkin, 1996; Neff *et al.*, 2003; Schimel & Bennett, 2004; Näsholm *et al.*, 2009). Despite current studies which explicate plants ability to bypass oN mineralization, the exact mechanisms and controlling factors of oN uptake by plants; alongside the quantitative importance and assimilation of oN, remains to be fully elucidated.

Furthermore, competition for oN between microbes and plant roots at the root:soil interface has been shown to be particularly fierce, more so than for inorganic N forms, with a rapid turnover of and response to amino acids by the microbial community (Kaye & Hart, 1997; Owen & Jones, 2001; Bardgett *et al.*, 2003; Dunn *et al.*, 2006; Jones & Murphy, 2007; Farrell *et al.*, 2014). Reviews by Lipson & Näsholm (2001), Näsholm *et al.*, (2009), and Warren (2014), highlight a need for the accurate quantification of the soil oN pool, identification of species which possess an intrinsic capacity to utilise the oN fraction of the soil N pool, and for a greater understanding of the mechanistic processes by which oN is acquired at the root:soil interface; to better inform ecosystem models.

Within the review by Näsholm *et al.*, (2009), the authors identify three important criteria which an N source must meet to contribute to plant N nutrition. Namely, these are; (1) availability: specifically the access which the plant has to the N form within the soil, e.g. pool size and nutrient fluxes close to plant roots (2) uptake: does the plant possess the capacity to acquire the target N compounds from the soil, and (3) metabolism: does the plant possess the capacity to assimilate the target N compound into its own biomass? It is further stated that the importance of a target N compound for plant nutrition is therefore a function of both plant and soil interactions simultaneously. It is proposed herein that a fourth aspect of N nutrition is considered, (4) competition: whereby the microbial community and their interactions at the root:soil interface, specifically for acquisition of the rapidly transformed oN pool, is viewed as a separate criterion, and examined under field relevant conditions, as noted in the above review.

These four thematic areas shall form the basis for this review. What remains to be fully established is the hypothesis that certain plants display a ‘preference’ for oN forms. Whilst this term had been used widely to describe oN uptake, its accuracy is unestablished, as plants may act opportunistically for oN resources, rather than show a strong preference. It is therefore congruent that these interactions are fully understood to allow greater resolution within ecosystem modelling and to better inform our knowledge of the N cycle. In lieu of this, the impetus of these works is to elucidate if oN uptake in plants is a ubiquitous and intrinsic ability

within many different genera, the mechanisms of uptake, and the influence of competition at the root surface; in addition to the effect that symbiotic associations exert on plant oN uptake.

1.1.1 Agricultural nitrogen reliance

Whilst the focus of this review is by no means solely restricted to the understanding of agricultural systems, it is important nonetheless to familiarise oneself with mankind's reliance on N cycling, the availability of N to our crops, and the issues associated with the perturbation of the N cycle; understanding that this subsequently forms at least part of the impetus for our continued research in this area. A complete understanding of the mechanisms by which plants acquire N in all available forms is crucial for our continued production of ecosystem models in natural ecosystems, yet it also has great bearing on our agricultural systems.

It has been suggested that an ever burgeoning human population is linked to increased agricultural growth; prompting humanity to find continuous methods of enhancing yields. Consequently, the manipulation of N for anthropogenic use has a history stretching far into antiquity, with one of the earliest European acknowledgements of the need for improved soil fertility being postulated by the Roman scholar Columella, who proposed that soil fertility could be improved by ploughing legume crops in to soils between yearly rotations. Earlier still, in the Shang dynasty of China, three millennia ago, there exists records of green manure application using leguminous crops in rotation with rice (Evans, 1998). These records, alongside other historical sources, indicate humanities ongoing efforts to augment their N supply, and thus increase food security. Similarly, mankind historically enhanced its access to N resources by utilising naturally N-rich reserves, such as potassium nitrate and animal manures; alongside the manipulation of biological N fixation (BNF) by legume cultivation as described above (Galloway *et al*., 2014). Nitrogen used in this manner already represented a fixed source of N and therefore required no further processing, besides extraction and incorporation into the soil.

These methods of anthropogenic N enhancement were un-intensive and contributed little to the global N cycle, and until the end of the 19th century, most reactive N was produced naturally (Shibata *et al.*, 2016). Despite this, certain scholars at this time recognised that natural sources of N were finite, and steps to increase fertiliser supply would have to be taken to avoid a Malthusian disaster in the face of a growing population. A seminal address on this issue was given in 1898 by Sir William Crookes, a renowned chemist and physicist, who called for the widespread adoption of 'nitrate of soda', produced through electrical fixation, to augment the

diminishing supplies of Chilean nitrate and manure, and subsequently off-set drastic wheat shortages in Europe and America (Crookes, 1899). At the time this address was met with scepticism. In credit to Crookes, it is now widely accepted that without fertiliser use, 'organic' agriculture in the absence of N fertiliser could support an upper estimate of just 4 billion people (Connor, 2008).

Although improving technology and farming practices allowed for steady increases in productivity, BNF and natural reactive N (N_r) remained ubiquitous and widely used until the 1940's, when the widespread utilisation of industrial N fixing techniques (most notably the Haber-Bosch process), the contribution of fossil fuel powered industry, and a rising post-war population became the catalysts for a dramatic shift in anthropogenic N production (Erisman *et al.*, 2008). This shift significantly changed mankind's relationship with both N and the wider environment. It is reported that as of 2010, around 75% of all terrestrial reactive N was produced through anthropogenic means, both intentionally and as a by-product of other industrial processes, with an estimated fixation of 210 Tg N yr^{-1} from anthropogenic sources, as a portion of the total annual global fixation of 413 Tg N yr^{-1} from natural (biotic and abiotic) and anthropogenic sources combined. More recent estimates attribute that ~ 100 Tg of N_r yr^{-1} is produced by the Haber-Bosch process alone (Vitousek *et al.*, 1997; Fowler *et al.*, 2013; Ladha *et al.*, 2016). Compare this to the N_r production of our ancestors 2000 years ago, a somewhat insignificant 3 Tg N_r yr^{-1} arising almost solely from legume production (Galloway *et al.*, 2013), and it becomes apparent the extent to which humanity has perturbed the natural N cycle over an incredibly short temporal scale.

Nitrogen produced industrially as a result of the Haber-Bosch process is used primarily in agriculture as fertilizer. Of the 172.2 million metric tonnes (Mt) of fertilizer (N, P, & K) consumed in the years 2010 - 2011, 104.5 Mt of this was N (IFA, 2013). Of the aforementioned 100 Tg N_r yr^{-1} produced industrially, 50% of this is applied to the three major cereal crops rice, maize and wheat (16%, 16%, and 18% respectively) (Ladha *et al.*, 2016), giving insight into the extent to which N fertiliser underpins our global food security. Furthermore, the demand for N fertilizer is growing, with a projected growth of 1.4% yr^{-1} until 2018 (FAO, 2015). A growing demand for N shall be compounded further by increasing population growth, which although has seen a slight lapse in recent years, is projected to increase to 9.7 billion by 2050 (UN - DESA, 2015).

What does this mean in the context of this study? As our current systems for producing N fertiliser relies on the burning of fossil fuel, and with the looming challenges of climatic change, it is important that we fully understand the mechanisms of plant N nutrition, including elucidating the role that N forms previously dismissed as of little relevance to plant nutrition may play, and their pool/flux size.

1.2. Nitrogen Biogeochemical Processes

Nitrogen is the fourth most abundant element in the biosphere (0.3% by weight) after carbon, oxygen and hydrogen respectively (Ward, 2012). However, > 99% of the N on Earth is molecular N (N_2), which is present most abundantly in the atmosphere and oceans[*]. Despite its ubiquity, the majority of organisms, save some free living and symbiotic microorganisms (section 2.3.1), are not able to utilise N_2, and therefore there exists a clear distinction between N_2 and other N forms (Ward, 2012; Tartowski & Howarth, 2013). Perhaps most pertinent to these works is the distinction between soil oN and iN forms, their quantity within the soil matrix, and their importance for plant growth.

1.2.1. Inorganic nitrogen

As briefly discussed within the introduction, despite being well established that plants may acquire oN, it was the long held belief that iN forms were the only quantitatively relevant source of N which provided a significant contribution to plant growth. Inorganic N forms include ammonium (NH_4+), nitrate (NO_3^-), nitrite (NO_2^-), nitrous oxide (N_2O), nitric oxide (NO) and molecular N (N_2) (Tisdale *et al.*, 1990). In terms of overall contribution to plant nutrition, NH_4+ and NO_3^- are currently the predominant N compounds on which most commercially important fertilisers are based (Raghavan & Torrey, 1964). Ammonium and NO_3^- arise from the decomposition of soil organic matter, as the end products of mineralization, following ammonification and nitrification.

1.2.2. Organic nitrogen

Organic N assumes manifold forms within the soil. It occurs predominately as proteins, peptides (polymers of amino acids), amino acids and amino sugars from the breakdown of

[*] As reported in Tartowski & Howarth (2013), other authors provide different methods of quantification and ratios. E.g. Sprent & Sprent (1990), report a figure of 78% N in the *atmosphere*, and Postgate, (1998), reports *atmospheric* N at 80%. For the purpose of this review, it is sufficient to recognise that N_2 is the dominant form of N globally.

organic matter in the soil, such as plant, microbe or animal residues (Schulten & Schnitzer, 1998). However, many more forms exist, including purines, pyrimidines, and amines (Tisdale *et al.*, 1990). In addition to its multitude of forms, oN is also referred to using many different terms. Whilst many authors simply refer to constituent compounds such as amino acids, purines, peptides etc. of which 'oN' is formed, others refer more broadly to 'DON', or 'SON' (soluble organic N). The distinction between the two is important, as SON is the fraction of soil N which is extracted using water, potassium chloride, potassium sulfate or other extractants, whereas DON is the fraction of total SON which can be collected *in situ* without the use of extractants (van Kessel *et al.*, 2009); for example using a tension lysimeter or microdialysis probe. Researchers have previously classified DON compounds into high - and low-molecular weight forms (HMW and LMW respectively), in relation to their availability for plant and microbial utilisation.

Generally, those oN forms with a LMW such as free amino acids and peptides, represent a highly bioavailable source of N for direct plant acquisition; therefore the LMW pool has a high turnover rate and its accumulation within the soil is relatively small (Jones, 1999; Jones *et al.*, 2004; Jiang *et al.*, 2016). High molecular weight DON compounds on the other hand are less rapidly consumed and therefore are implicated with leaching and runoff into water courses. The quantitative amount of oN within the soil matrix shall be discussed in detail below (section 3.1.) in context of its availability to plants; though there are widely different estimates of its ubiquity in the soil. This is a crucial area of plant N nutrition which requires further investigation and is the key impetus for these works.

1.2.3. The Nitrogen Cycle

The N cycle is well understood and extensively covered within the literature to a greater degree than can be discussed within the confines of this review (Galloway, 1998; Galloway *et al.*, 2008; Gruber & Galloway, 2008; Fowler *et al.*, 2013; Galloway *et al.*, 2013; Tartowski & Howarth, 2013). Briefly, the N within the terrestrial N cycle is composed of fluxes and pools. Pools are accumulations of various N forms, and fluxes are the movement and transformations of N forms between these pools (fig. 1). Work done by Ward, (2012; *& references therein*) compiled informative data on the major global N pools and fluxes, their magnitude and flow rates, which show that the atmospheric and oceanic N_2 pool is several orders of magnitude higher than that of the terrestrial pool, on which plant productivity relies (Table 1). The steps of the N cycle shall be briefly discussed in the following sections, in relation to their importance for oN nutrition.

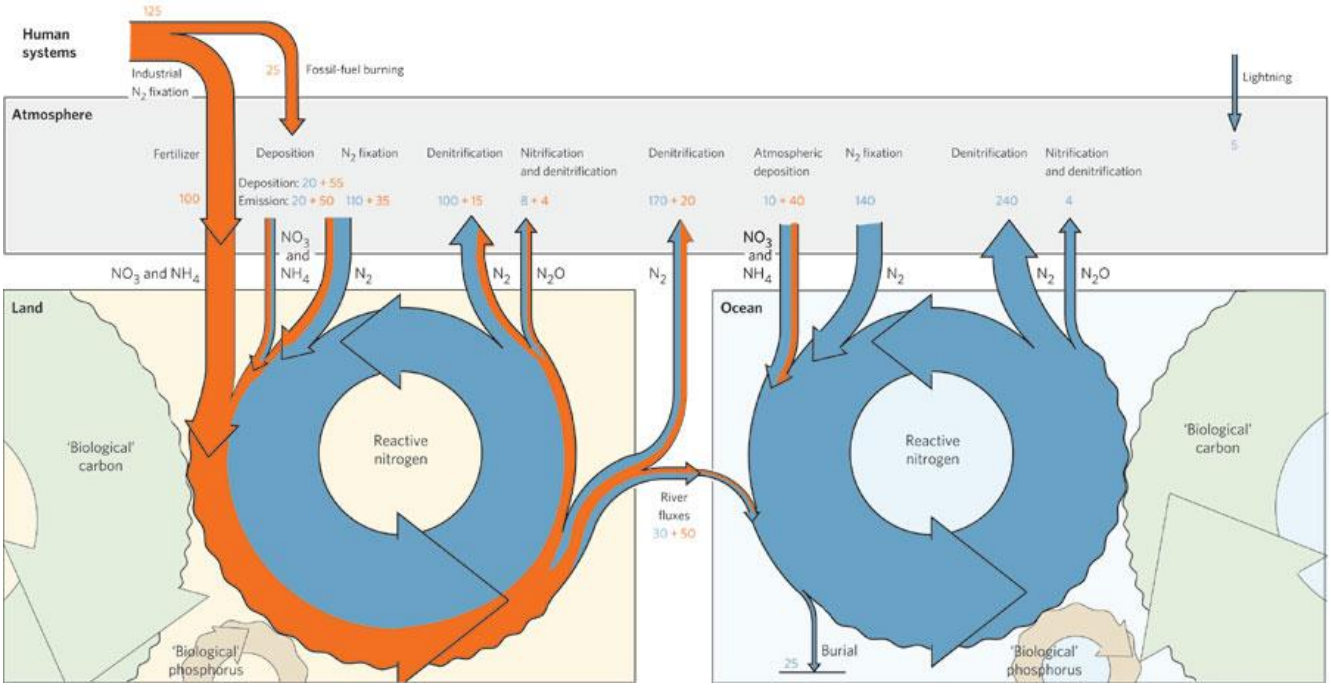

Figure 1.1 *"Depiction of the global nitrogen cycle on land and in the ocean."* Major processes that transform molecular nitrogen into reactive nitrogen, and back, are shown. Also shown is the tight coupling between the nitrogen cycles on land and in the ocean with those of carbon and phosphorus. Blue fluxes denote 'natural' (unperturbed) fluxes; orange fluxes denote anthropogenic perturbation. The numbers (in Tg N per year) are values for the 1990s. Few of these flux estimates are known to better than ±20%, and many have uncertainties of ±50% and larger. *Adapted from:* Gruber & Galloway, (2008)

Reservoir		Tg
Atmosphere	N_2	3.7×10^9
	N_2O	1.4×10^3
Biosphere	Marine	3.0×10^3
		$5\,0 \times 10^2$
	Terrestrial	5.4×10^3
		2.9×10^4
		7.7×10^3
Ocean	N_2	1.46×10^6
	N_2O	0.34
	NO_3^-	6.0×10^5
	PON	9.0×10^4
	DON	8.1×10^5
Geological	Continental crust	1.3×10^9
	Crustal rocks	6.4×10^8
	Oceanic crust	8.9×10^5
	Coastal sediments	3.2×10^4
	Deep ocean sediments	2.0×10^9
	Soil	1.4×10^5
		2.2×10^4

Flux		Tg yr^{-1}
Inputs		
Fixation	Natural terrestrial	107
	Natural oceanic	121
		110
		130–58
	Leguminous crops	31.5
	Chemical fertilizer	100
	Fossil fuel combustion	24.5
Lightning		21
Volcanoes		5
Losses		0.04
Denitrification	Natural terrestrial (land and rivers)	115
		154
	Natural oceanic	123
		193
		285
		400
		2030
Industrial combustion		7
Biomass burning		41.6
Burial (ocean sedimentation)		25

Table 1.1 *Left:* Major nitrogen reservoirs (pools) on Earth. DON = dissolved organic nitrogen; PON = particulate organic nitrogen. *Right:* Major fluxes in the global N cycle. Adapted from: Ward, (2012 & *references therein*)

1.2.4. Fixation

Atmospheric N_2 requires 'fixing', that is, breaking the N≡N triple bond and thus reducing N_2 to ammonia (NH_3) or nitrate (NO_3^-), before it becomes widely biologically available; therefore the rate at which this reaction occurs in nature is potentially limiting for plant growth (Postgate, 1998; Dixon & Kahn, 2004; de Bruijn, 2015). Fixation occurs in three main ways, abiotically through lightning in the atmosphere, biological N_2 fixation, or anthropogenic N fixation. Of these, lightning is the least significant contributor fixing just 5 Tg N yr^{-1} as NO_x; although lightning fixation maintains an important role in the formation of ozone, and delivery of N_r across the troposphere (Fowler *et al.*, 2013). Fixation of N is a highly energy intensive process, as the triple bond in N_2 is one of the most stable found in nature, with a bond dissociation energy of 945 kJ (Lee *et al.*, 2014). Indeed, the Haber-Bosch process, despite utilising modern methods and optimum catalysts, requires temperatures up to ~ 500 °C and pressures reaching 200 bar (Kandemir *et al.*, 2013; Vojvodic *et al.*, 2014). The catalytic reduction of dinitrogen to ammonia via the Haber process follows the reaction: $N_2 + 3H_2 \rightarrow 2NH_3$ (Postgate, 1998; table 2 [1]). However, biological N fixation, by organisms referred to as 'diazotrophs', achieves conversion of N_2 to NH_3 in the soil, catalysed by the enzyme nitrogenase, utilising energy produced in the hydrolysis of ATP, at ambient temperatures and pressures (Postgate, 1998). Nevertheless, the reaction is metabolically expensive and requires the hydrolysis of 16 units of ATP per N_2 fixed (Raymond, 2003).

1.2.5. Mineralization: aminization → ammonification → nitrification

As mentioned within the introduction, mineralization of oN to iN was considered the rate limiting step for plant acquisition of N. Whilst a 'short-circuit' has been identified, the process of oN to iN mineralization remains an integral part of the terrestrial N cycle. Mineralization is comprised of three 'steps', performed by numerous heterotrophic and autotrophic microorganisms, in which the decomposition of the initial substrate undergoes aminization → ammonification → nitrification. Of principal interest within the subject of amino acid acquisition is the fate of amino acids, short peptides and amines released during aminization, the initial step of organic matter (particularly protein) decomposition by heterotrophs, and plants capacity to compete for these oN forms released early-on in protein hydrolysis before ammonification (table 2 [2]) occurs (Tisdale *et al.*, 1990; Hodges, 2010). Also competing for this early released oN are the large populations of soil microbes which may be better placed to access spatially diverse pools of oN, as shall be discussed later in this review.

Subsequently, microbes that acquire these early released amino acids and peptides further decompose these substrates utilising deaminases into ammoniacal compounds, termed ammonification (Ladd & Jackson, 1982; Postgate, 1998). The principal resultant compound, NH_4^+, can be directly assimilated by plants, fixed to clay colloids, or released into the atmosphere as elemental N, though in most circumstances it rapidly undergoes microbial nitrification. Microbial nitrification is an integral step of the terrestrial N cycle mediated by two groups of bacteria, *Nitrosomonas* and *Nitrobacter* (nitrobacteria) which convert NH_4^+ to NO_2^- (table 2 [3.1]), and then subsequently to NO_3^- (table 2 [3.2]), respectively. The rates of ammonification and nitrification may be limited by temperature (for example low temperature environments, such as the Antarctic, have comparatively slow mineralisation rates), moisture, pH, microbial populations, and the aeration status of the soil (nitrobacteria are obligate aerobes).

1.2.6. Leaching, immobilization & denitrification

A major issue facing agricultural systems is the loss of applied N before it is utilised by crops. One of the major pathways of applied N loss is the leaching of NO_3^- (Dee & Cameron, 2002). Nitrate added to crops is mobile within the soil solution phase, and subsequently under heavy precipitation or irrigation regimes, is subject to leaching from the upper soil horizons. Therefore over fertilization, especially prior to periods of heavy rain, may result in a plethora of plant health issues (Albornoz, 2016) and environmental issues associated with leaching, for example eutrophication of watercourses (Smith & Schindler, 2009).

Availability of oN is subject to the dynamics and availability of other soil nutrient cycles; and N may become immobilised by the microbial community (Hodge et al., 2000; Jackson et al., 2008). If the C:N ratio of a decomposing substrate is greater than that of the decomposers themselves, for example wheat straw which has a high cellulose/lignin content, but a comparatively poor N content, then the associated microorganisms and fungi shall conserve oN resources to facilitate their own biological processes, and may even take up N from the inorganic pool to meet their requirements; thus immobilizing part of that N pool and reducing its availability to plants (Hodge et al., 2000; Kardol et al., 2015).

Essentially, immobilization is the inverse of mineralization and creates N limiting environments. Similarly, NH_4^+ can become strongly fixed to clay minerals. Denitrification (table 2 [4]) occurs under anaerobic conditions, whereby bacterial groups such as *Bacillus*, *Pseudomonas*, and *Paracoccus*, in addition to certain species of *Chromobacterium*,

Corynebacterium, *Hyphomicrobium* and *Serratia*, remove nitrate in conjunction with an organic carbon energy source and subsequently it is released back into the atmosphere in its primary state, N_2 (Tisdale *et al.*, 1990; Bernhard, 2010). Therefore, in addition to loss of NO_3^-, denitrifying bacteria may take up and utilise amino acids and peptides to facilitate their carbon requirements, thereby reducing availability of two important N pools.

Table 1.2 Major Reactions Involved Within the Nitrogen Cycle.

Nitrogen Cycle Step	Reaction	Reference
1. Fixation	$N_2 + 8\ H^+ + 8\ e^- \longrightarrow 2\ NH_3 + H_2$	(Bernhard, 2010)
2. Ammonification	$NH_2 + HOH \longrightarrow NH_3 + R - OH + \textit{energy}$ $\longrightarrow NH_4^+ + OH^-$	(Tisdale *et al.*, 1990)
3. Nitrification	**1)** $NH_3 + O_2 + 2\ e^- \longrightarrow NH_2OH + H_2O$ **2)** $NH_2OH + H_2O \longrightarrow NO_2^- + 5\ H^+ + 4\ e^-$	(Bernhard, 2010)
4. Denitrification	$NO_3^- \longrightarrow NO_2^- \longrightarrow NO + N_2O \longrightarrow N_2$	(Tisdale *et al.*, 1990); (Bernhard, 2010)

1.3. Organic Nitrogen Availability

1.3.1. Proteins, peptides and amino acid pools

In order to assess the importance of oN for plant nutrition, quantification of the oN pool and its availability to plants must be established. A recent review by Warren, (2014) which assessed the quantity of existing studies for each major class of nitrogenous compounds within the soil solution; subsequently reported that whilst our current knowledge of the amino acid fraction of the soil solution is 'good', our knowledge of the oligopeptide, polypeptide and protein fraction of the soil solution is comparatively poor. This is of course further compounded by the highly heterogeneous nature of the soil matrix (Miller & Cramer, 2005; Richardson, *et al.*, 2009) even within one specific ecosystem and the aforementioned rapid microbial turnover times of oN; much less considering the entire global variations of soil types, climate, vegetation, parent material and microbial communities and the interactions of these factors. Establishing oN availability and spatial arrangement within the soil matrix is the first step to understanding the role of oN for plant nutrition.

Early workers give widely different estimates of oN pool size in relation to total soil N, ranging from very early estimates of 37% (Kojima, 1947), 45 - 50% (Bremner, 1949), 90% (Bremner, 1967; Stevenson, 1982); and more recently up to 95% (Schnitzer & Khan, 1989);

possibly reflecting the increasing effectiveness of extraction methods through time; though clearly there exists differences between ecosystems. Of the total soil oN fraction it has been suggested, using NMR spectroscopy, that the majority of this pool occurs as free peptide structures (Knicker, 2000). This supports earlier research which suggested that the majority of the oN pool was comprised of amino acids held in peptidic form and to a lesser degree free amino acids (Stevenson, 1982; Jones, 1999).

It has been shown that plants ability to uptake amino acids from the soil is proportional to the size of the amino acid pool within the soil, with low concentrations resulting in plants poor ability to compete with microbes for these resources (Jones *et al.,* 2005). It is expected that in the vicinity of N hotspots, such as decaying soil fauna residues, the level of soil organic N will be significantly increased.

1.3.2. Climatic influences and geographic variability

Though it has been suggested that the innate capacity of soils to retain their intrinsic functions, such as mineralization, is independent of global position due to the similar worldwide composition of plant and animal residues (Jones *et al.*, 2009), pools of oN and N cycling processes may nevertheless be directly influenced by their geographic location in conjunction with local and global climatic conditions and changes. Early attempts at quantifying N pools under different climatic zones was undertaken by Sowden *et al.,* (1977), who concluded that the amino acid and amino sugar pool represented a larger portion of the total N pool in warmer climatic areas than that of colder regions. The data also show that in all ecosystems tested, the amino acid N pool is larger than that of ammonium (Table 3). Potentially, understanding of oN soil dynamics under different climates is particularly relevant considering current trends of global warming. For example, larger pools of oN in warmer ecosystems coincides with experiments on Antarctic hair grass (*Deschampsia antarctica*), where it was elucidated that

Table 1.3 - *"Nitrogen distribution in soils from widely differing climatic zones (means and standard deviations)" - Adapted from*: Sowden *et al.,* (1977).

		(%) of total soil soluble N					
Climatic Zone	**Total Soil N Range (%)**	**Total N**	**Amino acid**	**Amino sugar**	**Ammonium**	**Unidentified N**	**Non-hydrolysable N**
Arctic	0.02 - 0.16	86.1 ± 6.6	33.1 ± 9.3	4.5 ± 1.7	32.0 ± 8	16.5	13.9 ± 6.6
Cool temperate	0.02 - 1.06	86.5 ± 6.4	35.9 ± 11.5	5.3 ± 2.1	27.5 ± 12.9	17.8	13.5 ± 6.4
Subtropical	0.03 - 0.30	84.2 ± 4.9	41.7 ± 6.8	7.4 ± 2.1	18.0 ± 4.0	17.1	15.8 ± 4.9
Tropical	0.24 - 1.61	88.9 ± 3.8	40.7 ± 8.0	6.7 ± 1.2	24 ± 4.5	17.6	11.1 ± 3.8

D. antarctica could rapidly acquire and compete for amino acid N (Hill *et al*., 2011). Subsequently the authors hypothesised that this ability had facilitated this species rapid proliferation under warming maritime Antarctic conditions, whereby higher temperatures mediated increased breakdown of moss-derived peat, rich in oN; within an otherwise N limited ecosystem whereby mineralization via bacteria is slow due to extreme low temperatures.

Other authors have given evidence to support this, for example Kielland, (1994), demonstrated that in Arctic tundra, where N mineralization is also low, there was order of magnitude higher concentrations of free amino acids within the soil compared to iN forms; subsequently hypothesising that amino acid in these polar ecosystems mediates plant-microbial interactions. This contradicts data from Sowden *et al.* above (table 3), which appear to show that there is little difference between the Arctic amino acid and ammonium pools. This could perhaps be due to the rapid turnover of amino acids and subsequent mineralization within samples that was overlooked.

1.3.3. Seasonal variability

As would be expected oN, being comprised of biological material as described in section 2.2, is heavily influenced by seasonal and diurnal changes (Miller & Cramer, 2005). Several authors have observed seasonal changes in DON pools. Experiments made across a montane → lowland grassland productivity gradient in Wales, UK, to quantify seasonal DON concentration, showed that, in addition to the DON pool constituting the dominant fraction of total N in the higher altitude/lower productivity sites, there was a marked increase in DON in spring in all sites tested (Farrell *et al.,* 2011). The authors hypothesised that this increase coincides with warming soils, an increase in microbial activity and increased plant growth.

Other studies have observed change in the total amino acid/amino sugar fraction of the soil, in their relation to mean annual temperature (MAT). Interestingly, whilst no significant relationship was found between the two variables, there was a clear convex parabolic relationship when plotted as a scatter plot, showing a marked increase in the concentration of soil amino acids between 10 and 15 °C MAT; however the concentration of amino acids fell when temperatures increased (Amelung *et al.*, 2006). These findings have large implications when considering current climatic changes and the potential effects of climate change on soil organic N cycling.

1.4. Organic Nitrogen Acquisition, an Intrinsic Capacity?

Despite our heavy reliance on N fertilisers, our understanding of plant N acquisition and preference for N forms remains incomplete. It is conceivably beneficial for plants to exploit the 'short-circuit' of the N cycle and bypass the mineralization of oN to iN forms, thus reducing the effects this rate limiting step has on N acquisition; and likewise to facilitate continued growth in iN limited environments. However, it has yet to be fully elucidated if plants from a diverse range of species, origins and habitats display an 'intrinsic' capacity to acquire oN. Providing proof that plants do indeed have an intrinsic capacity to preferentially acquire oN, and showing that this is a ubiquitous trait across many genera, would have a multitude of benefits that could be exploited for ecosystem modelling and agricultural practices. As of yet however, actual preference for oN forms has not been adequately demonstrated. As the following section shows, there are manifold studies which show plants possess the capacity to acquire a range of oN forms, yet establishing if this is a preferential, rather than an opportunistic capacity, is integral to our understanding of the N cycle.

1.4.1. Early research

Many studies assume that a paradigm shift in our understanding of N nutrition has occurred in the last twenty years or so, yet evaluating oN uptake in plants is by no means a recent development. This misconception may overlook older and often important early research. The ability of plants grown in sterile media to acquire oN was first demonstrated in solution culture studies in the late 19th century, though several researchers had investigated the importance of oN prior to this. Early investigators in the mid-19th century examined the uptake of urea in the absence of other N forms, concluding that urea contributed to plant growth and was therefore an effective N source; however these early studies were not performed in sterile soils and subsequently did not account for the mineralization of urea prior to plant uptake (Brigham, 1917; McKee, 1962; *& references therein*). Nonetheless, it was now a matter of profound interest to establish if plants acquired oN in intact forms. Comprehensive reviews by Hutchinson and Miller, (1912); Brigham (1917) and Nightingale, (1937) [†], alongside others at this time, provide insight into the significant amount of studies undertaken at this time; indeed Brigham identifies eighteen

[†] Nightingale's review provides reference to many early studies performed by German workers on the role of oN nutrition in higher plants. As many of these papers are now unobtainable, the reader is directed instead to pp. 148 - 152 of said review, which summarises these studies in more detail than can be reported herein.

Table 1.4. Organic nitrogen uptake studies pre-1950 which report significant oN uptake under reportedly sterile conditions.

Author(s)	**Plant(s) *spp.***	**oN forms supplied**
Baessler, (1884)‡	*Z. mays*	Asparagine
Dachnowski & Gormley, (1914)	*Scheuchzeria palustri, Eriophorum vaginatum, Vaccinium oxycoccos, Coleus spp.,*	Glycine
Ghosh & Burris, (1950)	*Trifolium pratense, Solanum lycopersicum*	Alanine asparagine, histidine, phenylalanine, glutamic acid
Johnson, (1866)*	*Z. mays*	Uric acid
Klein & Tauböck, (1932)*	*Phaseolus, Z. mays*	Arginine
Lefevre, (1906)†	Unreported - Multiple plant *spp.*	Glycine, alanine, leucine, tyrosine
Lutz, (1898)	*Cucurbita maxima, Zea mays, Cucumis prophetarum, Helianthus annuus, Ipomoea purpurea, Cnicus benedictus, Cucumis melo*	Amines (x6)
Molliard, (1910)	*Raphanus raphanistrum*	Glycine, asparagine, leucine
Nakamura, (1897)	*Hordeum vulgare, Allium cepa*	Asparagine
Schreiner & Skinner, (1912)	*T. aestivum*	Arginine, histidine, creatine, creatinine
Schulze, (1901)†	*Lupinus spp., Vicia spp., Ricinus communis*	Leucine, tyrosine
Suzuki, (1897)†	*Lupinus arboreus, Solanum tuberosum, Triticum aestivum, Haledia hispida*	Urea
Tanaka, (1931)*	*Sisyrinchium bermudiana, Brassica chinensis, Plantago major*	Urea
Thompson, (1899)	*H. vulgare, Avena sativa*	Urea, uric acid
Virtanen & Linkola, (1946)	*Pisum, Trifolium*	Glutamic acid, aspartic acid
White, (1937)	*S. lycopersicum*	Glutamic acid, lysine, histidine, phenyl-alanine,

		leucine, isoleucine, valine, serine, proline
White, (1939)	*S. lycopersicum*	Glycine
White, (1943)	*S. lycopersicum*	Glycine, pyridoxine, thiamine, nicotinic acid
Wolf, (1868)*	*Secale cereale*	Tyrosine
Yamaguchi, (1930)	*Z. mays*	Urea

**In:* McKee, (1962) †*In:* Brigham (1917) ‡*In:* Hutchinson and Miller, (1912)

separate experiments which demonstrate the uptake of one or more oN forms in a number of plant species (table 4). Of particular significance among these early works is the hypothesis of Schreiner, (1912) which assertively states, after observing enhanced plant growth when supplied with oN forms (including the amino acids arginine & histidine), that the degradation products of proteins are acquired intact by plants, directly from the soil. Schreiner continues to hypothesise that N acquired in this way would confer significant energy benefits to the plant during the synthesis of proteins over N acquired via nitrates owing to the presence of C within the oN; subsequently Schreiner's paper is among the first to indicate that the acquisition of oN forms may be an important and hitherto unrecognised constituent of plant N nutrition. Additional experiments conducted by Schreiner at this time found that the growth of wheat seedlings was enhanced by 11 - 23% when receiving a mixture of oN and iN compounds, as opposed to those which received solely nitrate (Schreiner & Skinner, 1912). In the following decades many studies continued to investigate the intrinsic capacity of plants to acquire oN. Later works by Bollard, (1966), examined over 160 organic nitrogenous compounds supplied as sole N sources, including amino acids, peptides, amines and amides, and compared growth rates to those plants supplied just with iN. Bollard's paper concluded that some forms of oN compounds were equally as sufficient for plant N nutritional requirements as iN forms*. However, despite the magnitude of early interest in oN nutrition of higher plants, it wasn't until decades later, perhaps due to provision of adequate N produced via the Haber-Bosch methods, that the subject was revisited on a large scale. What is evident within these early studies is that although a wide range of plants are tested, many studies at this time do not examine in detail the relationship between species, plant physiology, and the presence of symbionts. Owing to the difficulties involved in creating pure sterile cultures at this time, in addition to examining

the roots of the plants for mycorrhizal symbionts that may have colonised early on in plant germination or on the seed surface, some reported results of these early workers may be influenced by unobserved rapid mineralization at the root surface, or enhanced uptake via root colonists.

1.4.2. Recent publications

Although there is scarce recent research on organic N's relevance to plant nutrition, it has since been elucidated that all plant species tested to date, regardless of mycorrhizal symbionts or lack thereof, have some basic capacity to acquire amino acids (*As reviewed in:* Lipson & Näsholm, 2001; Näsholm *et al.,* 2009). Having established the ubiquity of oN in the soil (section 3) using modern methods, many researchers have expressed a need to revisit the question of oN nutrition and define the environmental and ecological factors which drive plants to acquire oN. There is currently a large body of recent research which shows plants capacity to acquire oN resources (table 5). Some particularly significant studies shall be discussed. An agriculturally important crop, *Zea mays*, was assessed by Jones & Darrah, (1994), whereby metabolic inhibitors were employed to ascertain if glycine influx and efflux across the root surface was the result of passive leakage, or mediated by an active transport mechanism under different temperatures. Using sterile cultures to exclude the possibility of microbial mineralization of oN compounds prior to uptake, the authors were able to conclude that plants placed in a 120 μM bathing solution of glycine (in the presence of an inhibitor) exhibited influx of glycine via an active transport mechanism, and efflux via passive diffusion.

Furthermore, to measure the spatial characteristics of glycine and aspartic acid influx, the authors measured uptake at all zones along the root profile, as shown by loss of analytes in the agar solution, concluding that uptake was spatially homogenous along the root profile. The implications of this finding are important within the rhizosphere, in regard to the aforementioned high degree of spatial variation of oN compounds within the soil matrix, alongside competitive considerations discussed in detail below, whereby plants with active oN transporters at all depths of the rooting profile may compete more effectively for oN. Further experiments by Streeter et al. (2000), compared grass species which dominated high and low productivity ecosystems, namely improved (Dominated by: *Lolium-Cynosurus spp.*) and unimproved (Dominated by: *Festuca-Agrostis-Gallium spp.*) grasslands. It was hypothesised

* Bollard's paper tests the organisms *Neurospora crassa, Chlorella vulgaris* and *Spirodela oligorrhiza,* of which, though taxonomically unrecognised at the time, only *Spirodela* is a higher plant, the other two being fungi and green algae, respectively. Therefore only results relevant to *Spirodela* are reported herein.

that the unimproved grassland *spp.*, which dominate low fertility sites, would preferentially uptake supplied oN, to a greater degree than those grasses from the high fertility sites. Using dual labelled ^{13}C - ^{15}N glycine and ^{15}N labelled NH_4^+, total uptake was measured between grass *spp.* from the contrasting fertility sites. Interestingly it was found that all grasses from both fertility sites took up significant amounts of glycine, and it was concluded that as ^{13}C and ^{15}N enrichment was observed in shoot biomass of all *spp.*, that intact uptake of glycine occurred. Furthermore, no preference was observed for NH_4^+ derived N over that of glycine N in any *spp.* tested under N limiting microcosm conditions. The authors concluded that whilst their findings supported direct amino acid uptake in plants from both high- and low-fertility ecosystems, it was not known to what extent their results were influenced by microbial mineralisation prior to uptake.

Table 1.5. Organic nitrogen uptake studies 1990 onwards. *Key*: **(S - MC)** = Soil. Microcosm / **(Sand/S/V- MC)** = Sand. Soil. Vermiculite / **(Sol.)** = Solution culture / **(S - *IS*)** = Soil, *in situ* / **(SW - MC**) = Sea Water. Microcosm / **(×)** = Non sterile *or* No sig. uptake / **(✓)** = Sterile *or* Uptake in some *spp*. / **(✓✓)** = Uptake in all *spp.* tested.

Author(s)	**Plant *spp.***	**Biome**	**oN Form(s)**	**Conc. N**	**Growth Media**	**Sterile**	**oN Uptake**	**Further Information**
Uscola *et al.*, (2017)	*Q. ilex, P. halepensis*	Mediterra-nean	^{13}C -^{15}N glycine	200 ml - 1 mM	S - MC	×	✓✓	Performed on fine tree roots. Both *spp.* took up intact glycine. However, the author's state that the low levels of dual tracer found within the roots could be due to rapid metabolism of compounds *in planta*, yet the paper does not offer the other consideration that perhaps the majority of the glycine was taken up following microbial mineralisation.
Hill, Marsden & Jones, (2013)	*T. aestivum*	Agri	^{13}C -^{15}N ala/ ^{13}C -^{15}N tetra-ala/ ^{15}N labelled	4 ml - 1 mM ala, 4 ml - 250 µM tetra-ala, 4 ml - 1 mM	Sol.	✓	✓✓	This study was carried out to assess the importance of intact microbe uptake by the commercially important *spp. T. aestivum.* Uptake of intact

			intact microbes					microbes occurred but was not considered quantitatively important for plant N nutrition.
Hill *et al.*, (2011)	*T. aestivum*	Agri	^{14}C Alanine - differing chiralities - L-alanine, D-alanine, L-trialanine, D-trialanine	4.5 ml - 10 mM	Sol.	✓	✓×	This study examines the fate of oN compounds of different chirality and their acquisition in sterile, excised roots. It is concluded that D-peptide oN is unavailable for utilisation by plants and observed uptake is likely passive. However other chiralities of alanine are taken up and utilised in significant quantities. Concludes that peptidic N may be important for plant nutrition. Importantly, this study was performed with field relevant concentrations.
Vonk *et al.*, (2008)	*T. hemprichii, H. uninervis, C. rotundata*	Marine	^{15}N Urea, 'algal amino acids'	60 mL seawater solution - 10, 1, 2 & 5 µM	SW - MC	×	✓✓	Performed on marine *spp.* which have a considerably different physiology to terrestrial *spp*. Nevertheless, sig. uptake of urea and amino

								acids was demonstrated by roots at field relevant concentrations (amino acids were took up in far larger concentrations). Reported that the importance of amino acid nutrition was sig. > uptake of NO_{3-} , amino acids showed uptake rates comparible to NH_4+.
Nordin, Schmidt & Shaver, (2004)	*E. vaginatum, C. tetragona, B. nana, R. chamaemorus*	Arctic tundra	^{13}C -^{15}N aspartic acid, ^{13}C -^{15}N glycine	210 mL - 4.7 mM	S - *IS*	×	×	Minor (< 1%) of added N was recovered in plant biomass. Instead, 49% was recovered in microbial biomass.
Weigelt *et al.*, (2003)	*H. lanatus, A. capillaris, L. perenne*	Temp. grassland	^{13}C -^{15}N gly	4.8 µg N/g soil^{-1}	S - MC	×	✓✓	> oN uptake occurred in low fertility soil
Miller & Bowman, (2003)	*A. rossii, A. scopulorum, C. leptosepala, F. brachyphylla, D. cespitosa, K. myosuroides, C. rupestris, C. scopulorum, L. spicata*	Alpine	^{13}C -^{15}N glycine	200 mL - 1 mM	Sand/S/V-MC	×	✓✓	Similar conditions to Miller and Bowman, (2002). All *spp.* tested showed significant ^{13}C enrichment of below ground tissue, however only 5/9 *spp.* (*Acomastylis, Caltha, Luzula, C. rupestris,* & *Festuca*) showed significant uptake of

								intact gly. Remarks that measured ^{13}C enrichments were low.
Miller and Bowman, (2002)	*A. rossii, A. scopulorum, F. brachyphylla, C. rupestris, K. myosuroides, L. spicata, C. purpurascens*	Alpine	^{13}C -^{15}N gly	200 mL - 1 mM	Sand/S/V-MC	×	✓✓	Only *Festuca* exhibited > gly uptake than NH_4+ & NO_3-
McKane *et al.*, (2002)	*C. bigelowii, E. vaginatum, V. vitis-idaea, L. palustre, B. nana*	Arctic tundra	^{15}N glycine	0.862 L - 1 mM	S - *IS*	×	✓✓	Only *Vaccinium* and *Eriophorum* relied on oN as a main N source. *Ledum, Eriophorum, Betula* & *Carex* all preferrentially took up glycine over NH_4+ in solution culture.
Nordin *et al.*, (2001)	*P. sylvestris, V. myrtillus, V. vitis-idaea, M. bifolium, O. acetosella, P. abies, R. idaeus, A. septentrionale*	Boreal Forest	^{13}C -^{15}N glycine	200 ml - 3.2 mM	S - *IS*	×	✓✓	Species from high prod. sites (*Picea, Rubus, Oxalis,* and *Maianthemum*) took up NH_4+ > NO_3- > gly. The low prod. sites showed > affinity for gly uptake, though still took up > volume of supplied NH_4+. Concludes that oN is an important N resource in N limited systems.

Nasholm *et al.*, (2000)	*P. pratense, T. hybridum, T. pratense, R. acris*	Agri	$^{13}C_2$ - ^{15}N gly	100 mL - 1 mM	S - MC	×	✓✓	Conducted using agriculturally important plant *spp.*, it was concluded that as enrichment of ^{13}C -^{15}N occurred that glycine was taken up intact.
Streeter, Bol & Bardgett, (2000)	*Festuca, Agrostis, Galium Lolium, Cynosurus*	Grassland - improved/ unimpro-ved	^{13}C -^{15}N glycine, ^{15}N - NH_4^+	7 ml - 4.76 & 42.86 mM	S - MC	×	✓✓	Examined grasses from high- and low-fertility ecosystems, compared labelled gly uptake to NH_4^+ uptake. Concluded that there was no sig. diff. in uptake between grasses from different sites, and furthermore, *spp*. showed no sig. diff. in preference for uptake of gly or NH_4^+. Concludes that this indicates an intrinsic capacity of all grass *spp*. tested to acquire oN.
Nasholm *et al.*, (1997)	*P. sylvestris, P. abies, V. myrtillus, D. flexuosa*	Boreal Forest	^{13}C -^{15}N gly	250 mL - 1.2 mM	S - *IS*	×	✓✓	Rates of glycine uptake were similar to those of ^{15}N - ammonium.

Chapin *et al.*, (1993)	*E. vaginatum, Hordeum vulgare*	Arctic tundra	Gly, glu, asp, ala, asn (Unlabelled) ^{14}C - gly	Once germinated plants were grown in 1.8 litre solution cultures with amino acid concentrations of 1 mM	Sol.	×	✓✓	Both *spp.* acquired amino acids from the solution, though not in significant quantities. *Eriophorum* accumulated > biomass with oN, the inverse was observed for *Hordeum,* reflecting their niches. *Hordeum* oN cultures showed a 35 * > bacterial community; reflecting *Eriophorums* > capacity to compete for oN. *Eriophorum'*s > affinity for intact oN forms was shown with ^{14}C labelling techniques.

1.4.3. Limitations

The above data show, alongside the reviews of several authors, that certain issues require consideration when assessing plants ability to intrinsically acquire oN. Firstly, the relative scarcity of studies which examine acquisition at field relevant concentrations of oN (table 5) gives a potentially unrealistic view of the amount of oN available within the soil solution; as many uptake mechanisms have developed to function at low soil oN concentrations (Näsholm *el al.*, 2009). Certain studies have accounted for this and supplied oN concentrations that closely match that of the field (Hill *et al.*, 2011); these studies have subsequently shown that oN is also taken up under field conditions; however more studies which observe relevant concentrations are needed. In addition, many studies have been carried out in non-sterile or *in situ* conditions, which may not account for the rapid mineralisation of added oN by soil microbes, a process which has been shown several times to be in the order of minutes to hours (Jones, 1999ab).

One method typically employed to avoid such confusion is the use of dual labelling techniques, whereby dual labelled isotopes (e.g. ^{13}C, ^{15}N) are utilised, with the inference that if both isotopes are present within the plant, and a relationship exists between them, then intact uptake of oN compounds has occurred (Nasholm *et al.*, 1997; Jones *et al.*, 2005). Dual labelling is not without its flaws however, and recently it has been suggested that it may have several flaws. For example, Nordin *et al.* (2004), state that the presence of both C and N labels may result from the uptake of degradation products of metabolism prior to uptake from the soil solution. Furthermore, it has been suggested that pulse labelling techniques are unrepresentative of nutrient flux within the soil solution. Novel methods, such as microdialysis, may be employed to circumvent this issue and deliver amino acids within a relevant production period, close to plant roots.

1.5. Transport and Metabolism of Organic Nitrogen Compounds

1.5.1. Amino acid and short peptide transport

When considering transport of oN within plants, and their subsequent partitioning, most studies focus upon amino acids and short peptides, as these constitute the major forms of transported N in plant biomass (Fischer *et al.*, 1995; Fischer *et al.*, 2002). Having established that plant oN uptake occurs via active processes rather than a product of passive transport, identification of amino acid specific carrier proteins (transporters) within plant plasma membranes is essential. Active transport is the net flux of the target compound across the cell membrane, against the potential energy gradient between the intra- and extra-cellular environments (Bush, 1993). Whilst it is widely known that transport of amino acids across the plasmalemma occurs via transporters, the specificity, mechanisms, and quantity of transporters has been widely debated (Weston *et al.*, 1995). Transport of amino acids and amino sugars from the extracellular environment has been demonstrated via active proton-coupled symports,

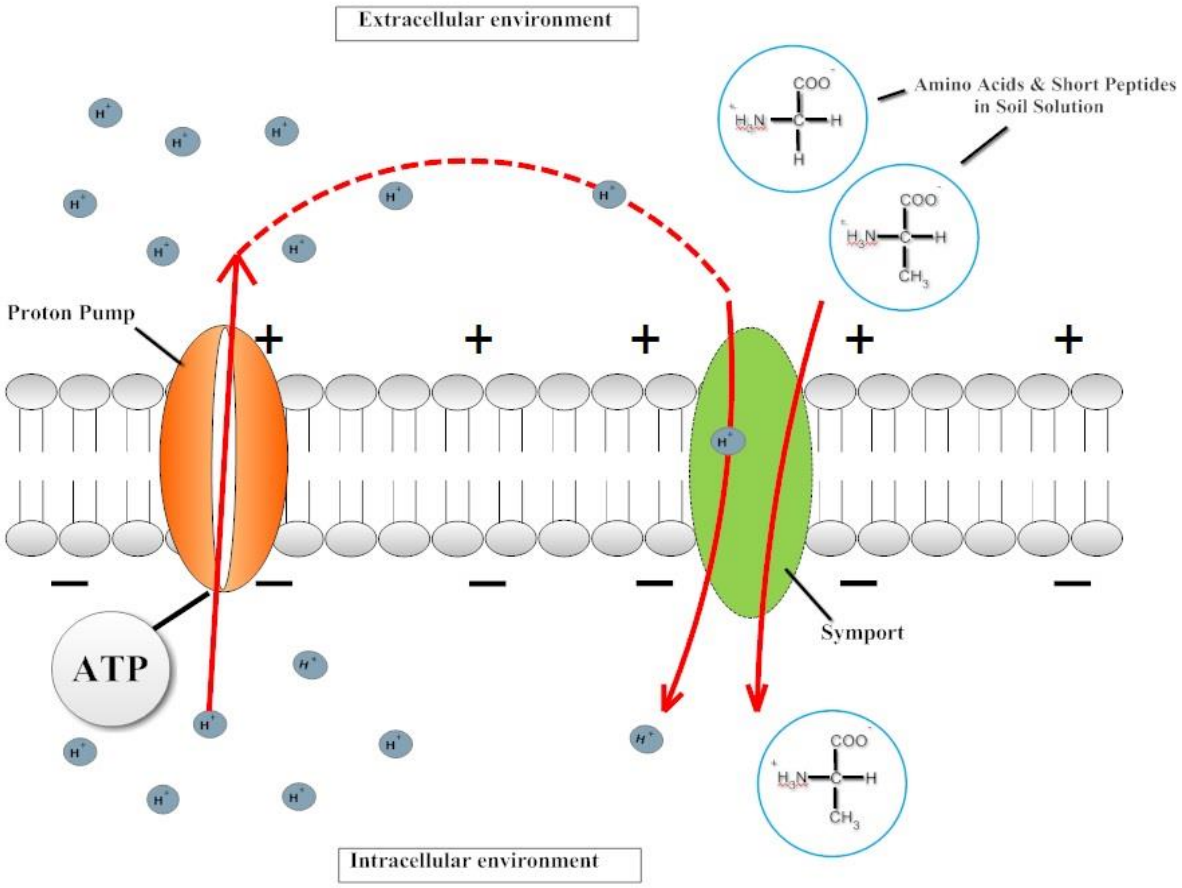

Figure 1.2. The proton coupled symport amino acid transport system. Flux of amino acids occurs from the positively charged extracellular environment into the negatively charged intracellular environment as maintained by the proton pumps transfer of H^+ ions.

whereby a H^+ ion gradient is maintained, inducing a flux of both the target amino acid and the H^+ ion (fig. 2). This gradient is maintained by the transfer of protons across the plasmalemma via an ATPase complex (Reinhold & Kaplan, 1984; Bush, 1993).

Therefore, energy from the plant in the form of ATP is required to perform this process. Reinhold & Kaplan's (1984) paper suggested that a single symport transport system was responsible for the full range of amino acid uptake; Kinraide (1981) showed that at least two transporter systems existed, one general system with an affinity for each amino acid, and another basic transporter system which has a high affinity for cationic (basic) amino acids. Later, two neutral, one acidic and a basic symport system were identified in sugar beets (Li & Bush, 1990). With the advent of molecular and genomic techniques a large number of specific amino acid transporters (AATs) and at least four super families of AATs have been identified within certain model organisms; alongside those expressed through molecular cloning of AAT genes within yeast mutants (Fischer *et al.,* 1998; Ortiz-Lopez *et al.*, 1999). The four most studied families are AAPs, ProTs, LHT-proteins, and AUX1 proteins (Fischer *et al.,* 1998; Rentsch *et al.,* 2007). Within roots in particular, genes revealed to be associated with amino

acid and dipeptide uptake in the model plant *Arabidopsis* are: AAP1, AAP5, LHT1, ProT2, and PTR1 (fig.3) (Tegeder & Rentsch, 2010).

Additionally, it has been elucidated through yeast cloning and gene expression that plants may possess active transport mechanisms for other N containing compounds, for example heterocyclic compounds such as allantoin, uric acid, and xanthine (Desimone *et al.*, 2002). However it has been suggested that there may be a high degree of functional redundancy within genes associated with amino acid and peptide uptake, with many transporters showing no specificity for acidic, basic or neutral amino acids ('general' transporters); in addition to overlapping specificity between transporter genes (Fischer *et al.,* 1998). Of particular importance to the knowledge of acquisition of oN at the root surface, it has been shown that despite expression of multiple transporter genes in root tissue, that only two of these, AAP1 and LHT1, are directly involved in the uptake of amino acids from the soil; additionally a peptide transporter, PepT1, was expressed in *Hakea actities* cluster roots, indicating that plants have developed uptake mechanisms for higher molecular weight compounds; or at least more

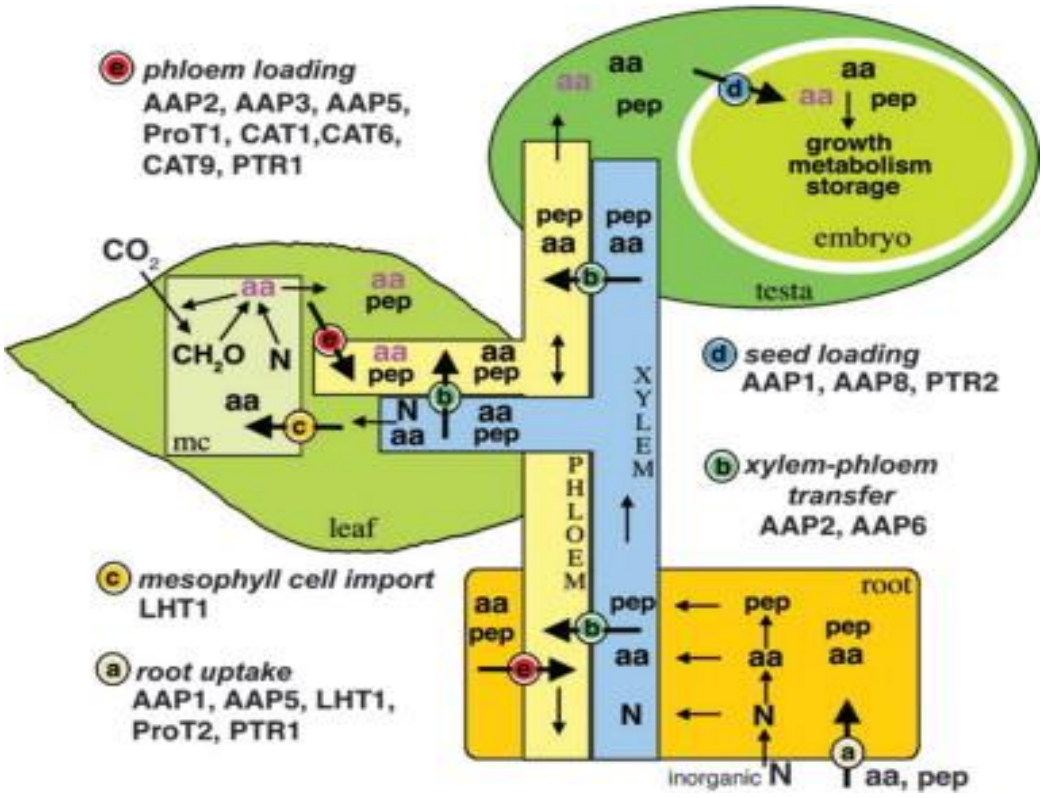

Figure 1.3. "*Model of Amino Acid (aa) and Peptide (pep) Transporters with Demonstrated Function in Arabidopsis."* (A) - Root uptake. (B) - Xylem–phloem transfer. (C) - Import into mesophyll cells (mc). (D) - Seed loading. (E) - Function of transporters in phloem loading has not been shown up to date, but potential candidates have been identified based on expression or localization studies (see text). *Adapted from:* Tegeder & Rentsch, (2010)

complex forms of N rather than free amino acid compounds (Schmidt *et al.*, 2003; Rentsch *et al.*, 2007).

1.5.2. Assimilation, allocation and metabolic benefits

Plants, in terms of nutrition, may be divided in to source organs, such as leaves and roots, and sinks, such as newly developing leaves and reproductive organs (Rentsch *et al.*, 2007). Plants capacity to assimilate and incorporate oN into their biomass has been shown in a range of species and examined at the cellular scale. One such pathway, the glycine decarboxylase complex, as reviewed by Oliver, (1994), is a multi-enzyme process which converts one molecule of glycine to one molecule of serine, CO_2 and NH_3 respectively; which are subsequently incorporated into the plants biomass. The majority of root-acquired and synthesised amino acid is transported to the shoots and other growing biomass to support growth (Tegeder & Rentsch, 2010).

Most importantly in the context of these works, as hypothesised by Schreiner, (1912), is the suggestion that amino acids may confer a significant metabolic benefit to plants due to the presence of both C and N within amino acid compounds. This has been more recently examined, in Arabidopsis, poplar, pine and spruce, where N use efficiency (NUE) was shown to be 20% higher in those plants supplied with oN compared to iN *in vitro*. Furthermore, it is suggested that oN uptake increases overall root:shoot biomass and therefore confers important benefits for the acquisition of more nutrients at the root surface (Franklin *et al.*, 2016). The authors suggest that this significant increase in NUE is due to C content of oN, which significantly lowers C costs when assimilating oN into proteins and subsequent incorporation into the plants biomass. Such metabolic benefits could also increase the plants access to conventional iN, by yielding a greater root area, and therefore more research is needed to assess the quantitative importance of oN over iN in plant metabolism.

1.6. Competition for Organic Nitrogen at the Root:Soil Interface

As organic N presents an important source of C as well as N, it is rapidly consumed by microbes and some fungi to assimilate into their biomass, inducing rapid turnover and resulting in the rapid growth and reproduction of rhizosphere microorganisms. The rhizosphere plays host to a great diversity and quantity of microbial organisms and therefore understanding the dynamics of competition between these groups, and the mechanisms by which they compete, should be one of the foremost aims when attempting to parameterise plant oN nutrition models.

It has long been recognised that microorganisms within the rhizosphere may have deleterious effects on plant growth via limiting access to nutrients (Schippers *et al.*, 1987).

1.6.1. Rhizosphere microbial populations

As briefly mentioned within the introduction, competition for oN between plants and the microbial community at the root:soil interface is fierce. The quantity of soil microbes is significantly greater within the rhizosphere environment, with populations 19 to 32 times greater than average in bulk soil (Kuzyakov, 2002). Termed the 'rhizosphere effect', this increase is predominately due to microbes increased access to carbon rich exudates (rhizodeposits) released via plant roots; which originate from sloughed root cells, mucilage, root hairs, and root turnover (Marschner *et al.*, 2001; Kuzyakov, 2002; Kuzyakov *et al.* 2006; Berg & Smalla, 2009).

Lorenz Hiltner coined the term rhizosphere in 1904 and stated that plant nutrition is directly influenced by the microbial communities living in close proximity to roots, and the diversity of these groups in the context of their relationship with plants (Hartmann *et al.*, 2007; Berg & Smalla, 2009). Previous characterisation of the relationships between plants and soil microorganisms have specified three relationships, (1) pathogenic relationships, (2) positive or associative interactions (symbiosis), whereby neither host nor the infective organism is harmed and one/both parties receives quantitative benefits, or (3) neutral relationships, whereby neither organism exhibits direct benefit from the relationship, nor sustains damaging effects (Singh *et al.*, 2004).

1.6.2. The role of root exudates in the manipulation of rhizosphere microbial communities

It has previously been described that plants release exudates through their roots into the rhizosphere. Root exudate compounds come in a multitude of forms including organic acids, sugars, amino acids, purines, nucleosides, and enzymes (Dakora & Phillips, 2001). Despite the incredibly high numbers of microbes which inhabit the rhizosphere, it has been hypothesised that plants may exert selective pressures upon microbes living in their immediate vicinity and directly influence the rhizosphere microbial community structure through the exudation of specific compounds (Haichar *et al.*, 2008). In doing so, it has been suggested that plants may 'select' those microbes which confer benefits to their growth; and perhaps most importantly within the context of these works, plants may increase their access to N through release of

specific compounds; in addition to potentially mobilising those nutrients with comparatively low solubility, particularly metal oxides and hydroxides (Jones & Darrah, 1994; Jones, 1998; Carvalhais *et al.,* 2011). An example, explicated by Hamilton & Frank, (2001), was observed utilising a ^{13}C tracer, whereby a commonly grazed grass from the Yellowstone ecosystem, *Poa pratensis,* when grazed, triggered increased root exudation of multiple C containing compounds, thus increasing the microbial population within the rhizosphere. Subsequently, positive feed-back effects led to an increased plant N uptake as greater microbial mineralization took place, resulting in increased plant growth rapidly following the grazing event. It was further hypothesised, that in addition to increased mineralization, the greater microbial population following exudation would provide increased nutrients upon their death, with the subsequent addition of oN within the rhizosphere as a result of microbial lysis.

1.6.3. Symbiotic associations

Symbiotic associations between plants and microorganisms may confer benefits for both parties. It has long been established that mycorrhizal associations, by those fungi which form extra- and intracellular associations with plant roots, can have important nutritional benefits for their host plants, effectively vastly extending the colonised area of plant roots in the soil, and exploiting spatially diverse nutrient pools within the soil matrix via fast growing hyphae (John *et al.,* 1983); in addition to 'mining' primary nutrients directly from parent material (with the exception of N) (Landeweert *et al.,* 2001). Mycorrhizal fungi connect plant roots within ecosystems and deliver a range of nutrients to the plants in exchange for carbohydrates (Nehls *et al.,* 2009).

Whilst the enormous scope and body of research into the interactions of mycorrhizal fungi and their hosts remains largely outside the scope of this review, the reader is directed to publications from workers such as Malloch *et al.*, (1980); Allen, (1991), and Heijden *et al.*, (2015) which provide a comprehensive body of knowledge on mycorrhizal interactions. Here it shall instead be discussed in detail the quantitative benefits which mycorrhizal fungi offer their hosts in the acquisition of oN from the soil matrix. Mycorrhizal fungi have been shown to facilitate amino acid acquisition and transfer to their plant hosts in a number of different species (both fungi and plants), across a range of ecosystems (Lipson *et al.,* 1999; Hawkins *et al.,* 2000). Though it has been shown that even plants which do not form mycorrhizal associations have the ability to take up oN, it has recently been shown that plants grown in microcosms, when inoculated with the mycorrhizal obligate biotrophs *Glomus hoi* (fungi which

may exist solely through symbiosis with plants) are able to acquire three times as much added N as untreated controls. Furthermore, the same study showed that the hyphae of *G. hoi* displayed a preference to grow towards nutrient rich organic matter patches, and suggested that this was both a strategy to acquire a greater quantity of nutrients and locate new hosts (Hodge *et al.*, 2017).

By displaying chemotrophic mechanisms such as these, mycorrhizal fungi could potentially provide valuable spatial advantages to the host plant, allowing surfaces associated with nutrient acquisition to be vastly extended outside of the rhizosphere and into the bulk soil, where spot sources of N, such as decaying soil fauna, dead roots, and sloughed off cells, may provide a valuable amino acid and peptide source. Hill *et al.*, (2011), suggested that the ability of *Deschampsia antarctica* to acquire oN in quantities relevant for growth may be mediated via an association with a dark septate endophyte (DSE), endophytic fungi which occupy root cells. A relationship with a fungi could prove beneficial for plants adapted to N limited environments.

1.6.4. Spatial and temporal considerations

It has previously been discussed in detail that oN pools within the soil matrix display high degrees of heterogeneity and subsequently under N limited conditions plant roots proliferate into nutrient-rich patches; or form mycorrhizal relationships to extend root surface area, increasing the root mass area to meet N requirements (Roy *et al.*, 1994; Miller & Cramer, 2005). Several investigators have suggested that microbial or plant success for oN resources is due in part to spatial and temporal considerations of oN pools within the soil, and the rate at which they are accessed and assimilated by each organism (Hodge *et al.*, 2000). The spatial arrangement of microbial communities may result in microbes being 'better placed' to access oN. Furthermore, microbial lifespans and rapid proliferation must be taken into account, whereby microbes can rapidly colonise those niches with new oN deposits.

Despite this, it has been suggested that plants may eventually acquire more oN over long temporal scales. The relative longevity of plant roots in comparison to soil microbes has led many authors to hypothesise that as microbes reach the end of their lifespan and lyse, plant roots are able to take up the resultant oN molecules that are released upon their death. Thus, although it may seem that plants cannot immediately acquire oN forms at a rate comparable to microbes, over longer temporal scales they may effectively compete for these nutrients.

In conclusion, it is apparent that oN is relevant for plant nutrition, and therefore research is needed to elucidate whether plants acquisition of oN resources is a result of preferential uptake for these N forms, a result of a passive mechanism, opportunistically when oN becomes available, or mediated by the microbial community and fungal symbionts.

To investigate these relationships, and to properly explicate whether plant oN acquisition is preferential or opportunistic, novel and conventional methods may be employed simultaneously, such as microdialysis and isotope labelling, to discern the fate of amino acids and peptides over production periods that are relevant to conditions and nutrient flow rates *in situ* and *in planta*. Potentially, establishing if oN uptake occurs truly preferentially rather than as an opportunistic response to oN availability could have profound implications on our current understanding of the terrestrial N cycle. A thorough investigation of plants intrinsic capacity to acquire oN intact, directly from the rhizosphere and in competition with microbes, would suggest that current fertiliser additions in our agricultural systems would serve plant nutrition better if they contained a larger proportion of organically derived N.

1.7. Hypothesis and Objectives

Having established the importance of N to plant nutrition and a whole host of other rhizosphere processes, and identified the need for a greater understanding of the mechanistic processes of N in close proximity to plant roots, this work set out to add to the body of knowledge pertaining to the first stage of Näsholm *et al.'*s, (2009) criteria for N contribution to plant nutrition, namely availability. Specifically, the following experiment used a novel sampling technique, microdialysis, to measure fluxes of amino acid N, ammonium and nitrate, and subsequently estimate their pool size, which could reasonably be found within the vicinity of three decaying organic materials commonly found within the soil 'A' horizon.

It is expected that the results of this experiment will add significantly to the body of knowledge surrounding rhizosphere nutrient acquisition and competition with microbes, which as elucidated within the review above, is one of the most important stages of plant N nutrition. We hypothesise that fluxes and estimated pools of all forms of N will be orders of magnitude higher in close vicinity to decaying soil organic matter than would normally be expected within bulk soil. Through microdialysis sampling, which as a novel soil sampling method we shall give a brief explanation of below, we are able to observe soil processes in a much higher spatial and temporal resolution than using other sampling methods, i.e. lysimeters.

Our hypothesis is as follows, 1) that labile protein transformation products occur in such quantities to be considered relevant to plant nutrition in close spatial proximity to soil macro organic matter additions and 2) that levels of amino acids in the vicinity of decaying soil flora and fauna are orders of magnitude higher than those typically found in bulk soil.

Chapter 2 - Materials and Methods

"Hotspots and hot moments of amino acid N in soil: real-time insights using continuous microdialysis sampling."

The following chapter is a transcript of work which was accepted for publication as a short communication in: *Soil Biology and Biochemistry*, and constitutes the methods, results and discussion of the bulk of the experimental works which have gone into this thesis.

Here, we buried two contrasting biomass residues, earthworm (*Lumbricus terrestris*) and clover (*Trifolium repens*). Earthworm was chosen both for its ubiquity within the top horizons of the soil and therefore its likelihood to expire in close proximity to plant roots, and also its importance within the soil ecosystem. Fresh clover was chosen both because of its common occurrence in the British Isles, and likewise to mimic fresh plant material which may become naturally incorporated within the soil, for example from macro-fauna activities (e.g. *Talpidae spp.*). Conversely, dried clover was chosen to mimic cut and dried plant material incorporated into the soil through activities such as ploughing.

This chapter is the unformatted transcript of the paper published and cited below. **N.B.** Section titles have been added for readability within these works, though the published manuscript does not include defined sections – see Appendix 'B'.

Published as:

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

2.1 Microdialysis

Microdialysis has recently been used in soil research, as a non-invasive sampling technique which allows for the collection of specific molecular weight compounds dissolved within the solution phase in soils. (Inselbacher *et al.*, 2011; Brackin *et al.*, 2015).

Microdialysis allows continuous measurement of free, unbound analyte concentrations in the soil solution phase of analytes smaller than 100 kDa. (Sulyok *et al.*, 2005). Bi-directional flow across the probe membrane maintains soil solution balance throughout the sampling period, and doesn't require the suction of soil solution (i.e. Rhizons), which are by nature invasive sampling methods.

As a sampling method, microdialysis, while limited by its lack of active transporter cells or evapotranspiration as in plant roots, mimics roots in other ways by its ability to be placed in close spatial proximity to soil nutrient hotspots, its minimally invasive nature, and its ability to capture unbound analytes from the soil solution phase.

Furthermore, microdialysis can be pumped continuously, collecting target analytes over a long time scale and providing insights in to specific temporal concentration changes, perfect for detecting fluxes from soil nutrient hotspots.

2.2 Abstract

Protein hotspots in soil, such as those associated with decaying soil fauna or plant litter, may produce ephemeral patches of disproportionately high soil nutrients. These hotspots may occur at the macro- and microscale in close proximity to plant roots, however, the likely concentration of soluble products produced in these hotspots remains poorly understood. To address this, we buried two contrasting biomass residues in soil, namely earthworm (*Lumbricus terrestris*) and clover (*Trifolium repens*). Their transformation to amino acids, NH_4^+ and NO_3^- were monitored continually over 6 days using microdialysis. All treatments showed greater soluble N concentrations compared to the unamended controls. The highest concentrations of both amino acids (12.9 mM after 12 h) and NH_4^+ (45.3 mM after 6 h) were generated in the vicinity of decomposing earthworm. In comparison, dried clover residues yielded 2.7 mM of amino acids at 6 h. After 12 h, amino acid and NH_4^+ concentrations in both earthworm and dried clover treatments showed a steep decline, returning close to background levels (<20 µM). Through the use of microdialysis we are able to show that soil nutrient hotspots may provide nearby roots with concentrations of amino acids and NH_4^+ several orders of magnitude higher than found in the bulk soil solution.

2.3 Introduction

Amino acids (AAs) and oligopeptides are the most abundant and first quantitatively-significant protein breakdown products to be directly available as N sources for plants and soil microbes (Sauheitl et al., 2009; Farrell et al., 2011; Warren, 2013; Moran-Zuloaga et al., 2015). Consequently, transformation of proteins to AAs is a major factor limiting N availability in soil and competition between plant roots and soil microbes for AAs is fierce (Jones and Kielland, 2002; Bardgett et al., 2003; Jan et al., 2009; Hill et al., 2011; Hill and Jones, 2018).

The concentration of AAs in soil appears to be a key determinant in the outcome of competition, with higher concentrations probably favouring plant AA-N capture (Jones et al., 2005). Therefore establishing true concentrations of AAs in soil is crucial to our understanding of plant N acquisition, and N cycling in soil. Typically, total free AA concentrations in contrasting ecosystems have been reported to remain fairly constant at 23 ± 5 µM, with the concentration of individual AAs typically ranging from 0.1-5.0 µM (Jones et al., 2009). These measurements in the bulk soil reflect the balance between slow rates of AA production and, rapid microbial AA consumption. However, biogeochemical 'hotspots', ephemeral patches

which yield disproportionately high nutrient levels relative to the surrounding soil matrix, may supply high quantities of AAs to nearby roots (McClain et al., 2003; Schimel and Bennett, 2004; Jones et al., 2005; Kuzyakov and Blagodatskaya, 2015).

Using plant and soil fauna residues and microdialysis, we measured concentrations of protein breakdown products which may realistically occur close to roots. Microdialysis is a membrane-based sampling technique which offers non-invasive measurement of the soil solution phase, allowing probes to be positioned in close spatial proximity to samples and therefore yielding a high spatial resolution (Miro & Frenzel, 2004; Inselsbacher et al., 2011; Inselsbacher & Näsholm, 2012). We hypothesised that AAs generated from soil hotspots of protein breakdown transiently occur at concentrations greatly exceeding those found in measurements of bulk soil solution.

2.4. Methods

Microdialysis probes were single use, 100 kDa cut-off, 10 mm membrane length, 0.5 mm membrane diameter, 2.6 µL membrane internal volume and inlet internal volume of 1.4 µL (CMA Microdialysis, Torshamnsgatan, Sweden). Probes with a 100 kDa membrane pore size have been shown to recover a great fraction of amino acids, compared to 20 kDa probes used by other researchers (Buckley et al., 2017). Probes were calibrated for relative recovery using amino acid (L-alanine), NH_4Cl and KNO_3 standard solutions of 0.1, 0.25, 0.5, 0.75, 1 and 25 mM (Inselsbacher et al., 2011; Lange, 2012). Probes (n = 8) were positioned in standard solutions at room temperature, and perfused with ultra-pure deionised-water (5 µL min^{-1}). Aliquots of dialysate were collected at 30 min intervals during a 120 min perfusion period.

Total amino acids in dialysate were measured fluormetrically according to Jones et al. (2002) while NH_4^+ and NO_3^- were determined colorimetrically according to Mulvaney (1996) and Miranda et al. (2001), respectively. Relative recovery was found to be 14.5 ± 5.5% of the supplied standard solution, irrespective of N-form, standard solution concentration or time. The relative recovery was later used to estimate actual concentrations of target analytes in the soil; though there may be some difference from standard solution calibration given the heterogeneous nature of soil. Collection vials were weighed before and after perfusion to ensure that there was no net loss or gain of water due to transmembrane flux or leakage.

Table 2.1. Physiochemical properties of the A-horizon of the Eutric Cambisol and Total C & N ratios of protein additions, measured in percentage dry weight (Means ± SEM).

Soil parameters	Values
Soil moisture (%)	31.47 ± 0.22
pH	5.33 ± 0.04
EC (ms^{-1})	45.62 ± 3.25
Total carbon (%)	2.55 ± 0.18
Total N (%)	0.28 ± 0.02
Nitrate (N mg kg^{-1} oven dry soil)	0.872 ± 0.10
Ammonium (N mg kg^{-1} oven dry soil)	3.94 ± 1.02
Clover Carbon (%)	48.6 ± 0.2
Clover N (%)	4.81 ± 0.06
Earthworm Carbon (%)	43.73 ± 3.78
Earthworm N (%)	9.72 ± 1.03

Soil (0-15 cm) was collected from the Ah horizon of an agricultural grassland at Henfaes Agricultural Research Station, N. Wales, UK (53°23´N, 4°01´W, 19 m.a.s.l). The sandy clay loam textured soil is classified as a Eutric Cambisol (FAO), derived from post-glacial alluvial deposits. The main properties of the soil and added protein sources are shown in Table 1.

Soil was sieved to 2 mm, homogenised and added to 1.5 ml microcentrifuge tubes, each tube receiving *ca.*1.5 g of field-moist soil. Three protein sources were added to the soil in a 1:10 w/w protein source to soil ratio as follows: a) fresh necromass of the earthworm, *Lumbricus terrestris* L., b) dried foliage of *T. repens*, and c) fresh foliage of *Trifolium repens* L. (clover), with soil controls receiving no protein addition. All treatments were replicated three times. These protein sources, added by weight, represent different levels of N input, however our hypothesis is predicated on a greater understanding of realistically occurring hotspots in the soil; rather than a direct comparison between treatments. Protein sources were added to the

centre of each tube. Each replicate was sealed using gas-permeable film (Parafilm M®, Bemis Inc., USA) to restrict water loss by evaporation. Probes were positioned to a depth of 10 mm in the centre of the tube, using a syringe introducer and perfused with ultra-pure deionised water, at a continuous rate of 5 μL min^{-1}, using a multi-channel syringe pump (NE-1200 Multi-Phaser, New Era Pump Systems Inc., NY, USA) over a sampling period of 144 h. Samples were taken at hourly intervals for the first 6 h, then every 6 h thereafter (samples at 30 h were contaminated during storage and therefore omitted from analysis). Samples were analysed for amino acids and inorganic N as described above and for pH and electrical conductivity (EC) with standard microelectrodes. Outliers were identified using Grubbs' test (9 of 1008 measurements were removed). Data were analysed using a mixed two-way ANOVA with a Tukey HSD post-hoc test (SPSS v22; IBM, New York, USA), with a $p \leq 0.05$ cut-off for statistical significance.

Chapter 3 – Results and Discussion

3.1. Results

Amino acid concentrations in controls did not exceed a mean concentration of 0.018 mM at any point during the experiment. Initially, levels of AA's were significantly higher in all treatments within the first 4 h following protein addition (Fig. 3.1). We attribute this to immediate loss of soluble components e.g. from damaged cells. All treatments had greater ($p \leq 0.05$) AA and NH_4^+ concentrations than controls during the majority of sampling intervals. Amino acids in dried clover and earthworm treatments showed a sharp rise in concentration after ~4 h, with those in dried clover rising to 2.7 mM after 6 h and to 12.9 mM in the earthworm treatment after 12 h, before sharply decreasing and levelling off after ~72 h. In contrast, fresh clover showed initially low AA concentrations (lowest mean concentration of 0.006 mM at 12 h), with only slightly higher concentrations than the control at some time points until ~54 h, thereafter reaching a peak of 0.099 mM at ~72 h, which was conversely the time at which the other treatments returned to levels comparable with controls. In comparison to the air-dried clover which was dead at the point of addition to the soil, it is likely that the fresh clover leaves underwent slow autolysis and death after excision. During this autolysis, leaf protein and amino acids are catabolised internally for energy production and are less likely to be lost to the soil (Marella et al., 2017). The single highest concentration of AAs was observed in the earthworm treatment at 6 h, with one replicate of 29.3 mM of AAs, though this was identified as an outlier.

Concentrations of NH_4^+ in the control treatment did not exceed 0.21 mM during the entirety of the experiment. In contrast, in the earthworm treatment, concentrations peaked at 45.3 mM at 12 h within the same replicate as the greatest AA concentration. Generally, however, ammonium concentrations in other treatments were low in comparison to the earthworm, reaching peak mean concentrations of 2.67 mM and 1.71 mM for the dried and fresh clover respectively. Likewise, there were significantly ($p \leq 0.05$) greater concentrations than the control observed at every time point for dried clover, and from 24 h onwards for fresh clover (Fig. 3.2). Interestingly, mean nitrate concentrations of all treatments remained low throughout the sampling period (< 1 mM) with the highest concentration of 0.71 mM seen in the earthworm treatment at 6 h. We observed no clear relationship between pH and treatment and/or time (Fig. A.1.), however, EC was significantly higher in the earthworm and dried clover treatments than the control during the majority of sampling times (12-144 h) (Fig. A.2.).

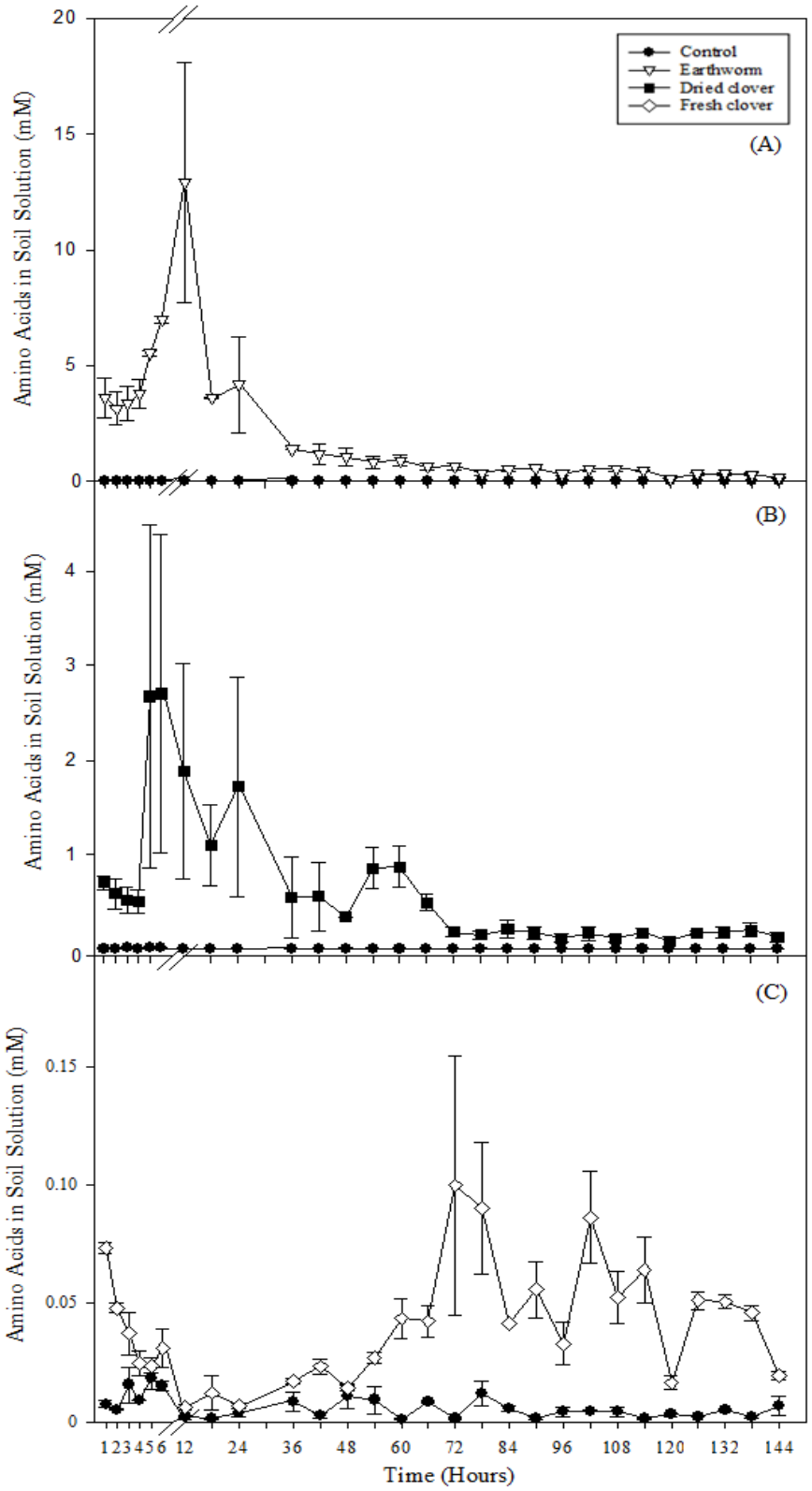

Figure 3.1. Temporal dynamics of soil solution amino acid concentrations in response to the addition of protein-rich hotspots (dead earthworm (A), dried (B) or fresh clover leaves (C)) to soil and measurement by microdialysis. Values represent means + SEM (n=4).

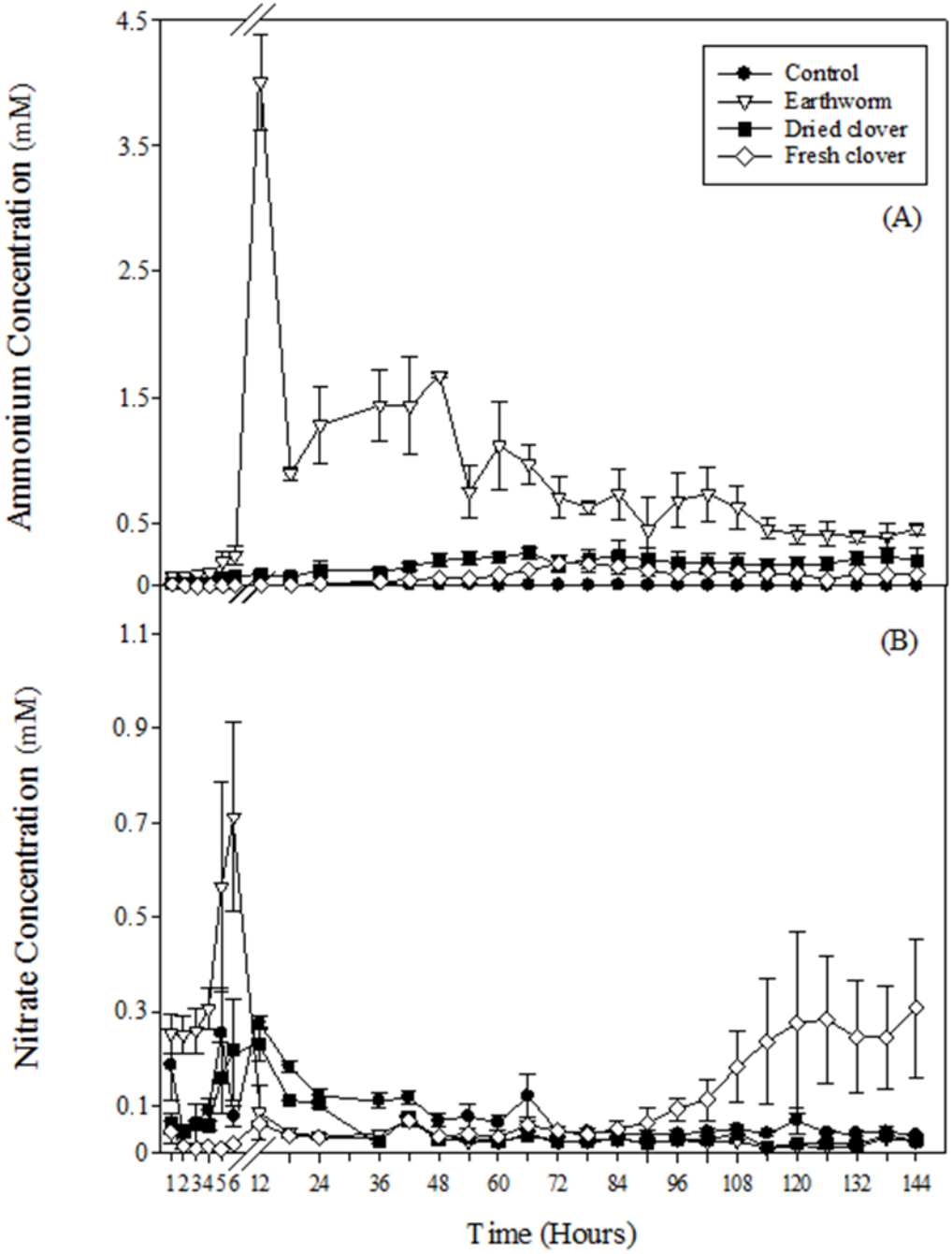

Figure 3.2. Temporal dynamics of soil solution ammonium (A) and nitrate concentrations (B) in response to the addition of protein-rich hotspots (dead earthworm, dried or fresh clover leaves) to soil and measurement by microdialysis. Values represent means + SEM (*n*=4).

3.2. Discussion

The temporal pattern of our results suggests that the rise in levels of free AA's is matched by increased microbial consumption and growth that rapidly lowers the concentration. This suggests that a distinction should be made between concentration hotspots (where breakdown rate is high and exceeds microbial demand leading to accumulation in solution) and flux hotspots (where breakdown rate is high but is matched by microbial demand and where little change in solution concentration occurs). Likewise, microbial demand may have become saturated earlier on in the sampling period due in part to adding N at a higher concentrations (despite being the same weight) in earthworm and dried clover treatments than that of fresh clover. However, hotspots occurring naturally within the soil would reflect this, yielding a range of concentrations from the macroscale, down to very fine microscales.

Results indicate that transformation of proteins to amino acids in ephemeral hotspots of protein addition may provide orders of magnitude greater levels of amino acids than previously measured in bulk soil solution (Jones et al., 2002; Jones et al., 2005; Hill et al., 2011). Further, although our experiment used relatively large fragments, it seems likely that hotspots occur at a much finer scale, for instance in the vicinity of dead microbial cells or damaged root cells in the detritusphere, as hypothesised by other researchers (Marschner et al., 2012; Kuzyakov and Blagodatskaya, 2015). As a consequence of this, it is therefore probable that the form and quantity of N acquired by plants varies considerably on a fine temporal and spatial scale.

Published Manuscript End

3.3. – Images from the Experimental Phase

Figure 3.3. Protein hotspot additions - fresh clover, dried clover and earthworm.

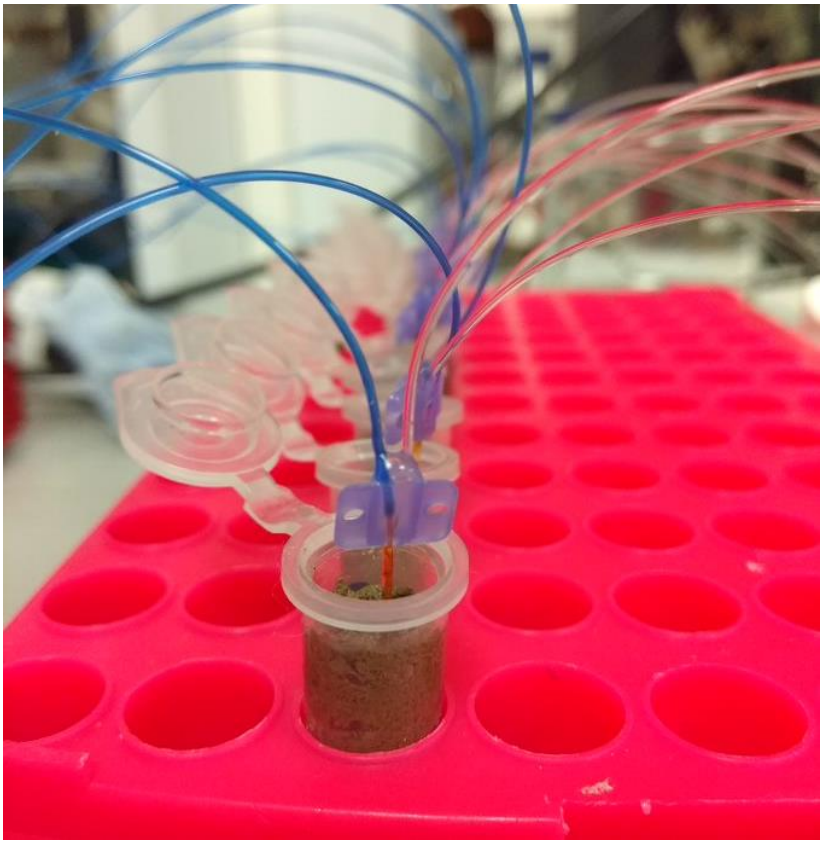

Figure 3.4. Microdialysis positioning in soil samples.

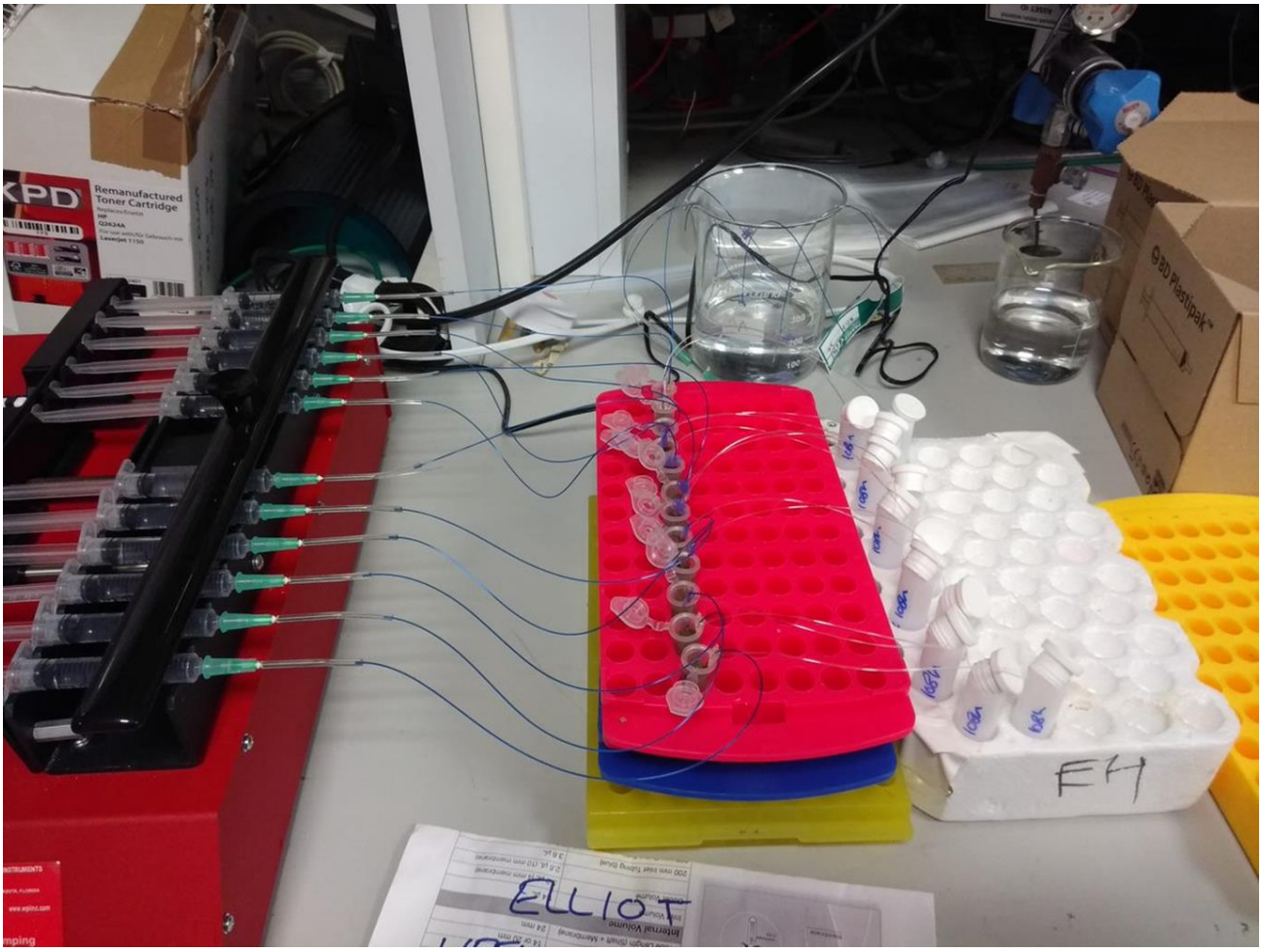

Figure 3.5. Microdialysis and syringe pump set up with dialysate collection vials.

Chapter 4 - Conclusion

Although the preceding publication provides a short discussion of experimental works in keeping with the requirements of a short communication, for the purposes of this thesis, it's necessary to discuss some further considerations from the data collected, especially in the context of their importance to the field of plant N nutrition.

To our knowledge, this study was the first to use microdialysis for the measurement of unbound analytes transformed from a protein addition in soil. We have shown, that in contrast to previous estimates, the levels of N which may be directly available to plants in transient hotspots of soil organic matter are orders of magnitude higher than those found in bulk soil.

Furthermore, as we carried out our experiment under non-sterile conditions congruent with normal soil conditions, we can expect that the levels of recovered N during the course of our study was the fraction which remained unbound or assimilated within the soil solution phase – *i.e.* directly available to plants, and not captured by microbes or bound to soil particles.

This new knowledge that levels of amino acids occur in such high quantities in the vicinity of soil organic matter hotspots brings new considerations for plant uptake and metabolism. For example, as other researchers cited herein have shown, amino acids are directly available for plant uptake, and represent a readily useable form of organic N without the need for prior mineralisation by microbes. Likewise, as cited herein, certain plants have been shown to transport common amino acid Glycine directly from roots to shoots, bypassing root metabolism; indicating that organic N forms may be immediately relevant for plant growth immediately after uptake.

The microdialysis method holds many promising applications for sampling within soil monitoring scenarios, owing to its un-invasive nature, continuous sampling capabilities, bi-directional analyte flow, and likewise its ability to be placed within close spatial proximity to the target sample or nutrient source.

However, several considerations should be noted when employing the microdialysis method for soil sampling. Microdialysis probes are expensive and incredibly prone to breakage, blocking, and membrane degradation. As probes are sold as single use, they require the utmost care to maintain and use multiple times when sampling. It may be better for other researchers

to use more robust probe models than the ones used here – though they often come at an even higher price.

Bibliography

Albornoz, F. (2016). Crop responses to nitrogen overfertilization: A review. *Scientia horticulturae*, *205*, 79-83.

Allen, M. (1991). The Ecology of mycorrhizae. 1st edition. Cambridge: Cambridge University.

Amelung, W., Zhang, X. and Flach, K. (2006). Amino acids in grassland soils: Climatic effects on concentrations and chirality. *Geoderma*, 130(3-4), pp.207-217.

Atkin, O. (1996). Reassessing the nitrogen relations of Arctic plants: a mini-review. *Plant, Cell and Environment*, 19(6), pp.695-704.

Baessler, P. (1884). Assimilation des asparagins durch die pflanze. *Landw. Versuchs. Stat.*, (33), pp.231-240.

Bardgett, R., Streeter, T. and Bol, R. (2003). Soil microbes compete effectively with plants for organic-nitrogen inputs to temperate grasslands. *Ecology*, 84(5), pp.1277-1287.

Berg, G. and Smalla, K. (2009). Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. *FEMS Microbiology Ecology*, 68(1), pp.1-13.

Bernhard, A. (2010). The Nitrogen Cycle: Processes, Players, and Human Impact. *Nature Education Knowledge*, 3(10):), p.25.

Biernath, C., Fischer, H. and Kuzyakov, Y. (2008). Root uptake of N-containing and N-free low molecular weight organic substances by maize: A 14C/15N tracer study. *Soil Biology and Biochemistry*, 40(9), pp.2237-2245.

Bremner, J. (1949). Studies on soil organic matter: Part I. The chemical nature of soil organic nitrogen. *The Journal of Agricultural Science*, 39(02), p.183.

Bremner, J. (1967). Nitrogenous compounds. *Soil Biochemistry*. 1st ed. Dekker, p.19.

Brigham, R. (1917). Assimilation of organic nitrogen by Zea mays and the influence of bacillus subtilis on such assimilation. *Soil Science*, 3(2), p.155.

Buckley, S., Brackin, R., Näsholm, T., Schmidt, S., & Jämtgård, S., (2017). Improving in situ recovery of soil nitrogen using the microdialysis technique. *Soil Biology and Biochemistry*, 114, p. 93-103.

Bush, D. (1993). Proton-coupled sugar and amino acid transporters in plants. *Annual Review of Plant Physiology and Plant Molecular Biology*, 44(1), p.513-542.

Carvalhais, L., Dennis, P., Fedoseyenko, D., Hajirezaei, M., Borriss, R. and von Wirén, N. (2011). Root exudation of sugars, amino acids, and organic acids by maize as affected by nitrogen, phosphorus, potassium, and iron deficiency. *Journal of Plant Nutrition and Soil Science*, 174, p.3–11.

Chapin, F., Moilanen, L. and Kielland, K. (1993). Preferential use of organic nitrogen for growth by a non-mycorrhizal arctic sedge. *Nature*, 361(6408), p.150-153.

Chapman, S., Langley, J., Hart, S. and Koch, G. (2006). Plants actively control nitrogen cycling: uncorking the microbial bottleneck. *New Phytologist*, 169(1), p.27-34.

Connor, D. (2008). Organic agriculture cannot feed the world. *Field Crops Research*, 106(2), p.187-190.

Crookes, W. (1899). The Wheat Problem. Based on remarks made in the presidential address to the British Association at Bristol in 1898. John Hyde. 1st ed. John Murray: London.

Dachnowski, A. and Gormley, R. (1914). The physiological water requirement and the growth of plants in glycocoll solutions. *American Journal of Botany*, 1(4), p.174.

Dakora, F. and Phillips, D. (2001). Root exudates as mediators of mineral acquisition in low-nutrient environments. *Plant and Soil*, 245, p.35–47.

De Bruijn, F. (2015). Principles of plant-microbe interactions. 1st ed. Switzerland: Springer, p.215 - 225.

Di, H. J., & Cameron, K. C. (2002). Nitrate leaching in temperate agroecosystems: sources, factors and mitigating strategies. *Nutrient cycling in agroecosystems*, *64*(3), 237-256.

De Lange, E.C.M., (2012). Recovery and calibration techniques: toward quantitative microdialysis. *Microdialysis in Drug Development, (M. Müller, ed.), AAPS Advances, Pharmaceutical Sciences Series*, pp. 13-33. Springer Nature, Switzerland.

Desimone, M., Catoni, E., Ludewig, U., Hilpert, M., Schneider, A., Kunze, R., Tegeder, M., Frommer, W. and Schumacher, K. (2002). A novel superfamily of transporters for allantoin and other oxo derivatives of nitrogen heterocyclic compounds in arabidopsis. *The Plant Cell Online*, 14(4), p.847-856.

Dixon, R. and Kahn, D. (2004). Genetic regulation of biological nitrogen fixation. *Nature Reviews Microbiology*, 2(8), p.621-631.

Dunn, R., Mikola, J., Bol, R. and Bardgett, R. (2006). Influence of microbial activity on plant–microbial competition for organic and inorganic nitrogen. *Plant and Soil*, 289(1-2), p.321-334.

Epstein, E. (1971). Mineral nutrition of plants: principles and perspectives. 1st ed. New York: Wiley.

Erisman, J., Sutton, M., Galloway, J., Klimont, Z. and Winiwarter, W. (2008). How a century of ammonia synthesis changed the world. *Nature Geoscience*, 1(10), p.636-639.

Evans, L. (1998). Feeding the ten billion. 1st ed. Cambridge, UK: Cambridge University Press.

FAO (2015). World fertilizer trends and outlook to 2018. Rome: Food and agriculture organization of the United Nations.

Farrell, M., Hill, P., Farrar, J., Bardgett, R. and Jones, D. (2011). Seasonal variation in soluble soil carbon and nitrogen across a grassland productivity gradient. *Soil Biology and Biochemistry*, 43(4), p.835-844.

Farrell, M., Hill, P., Wanniarachchi, S., Farrar, J., Bardgett, R., Jones, D., (2011). Rapid peptide metabolism: a major component of soil nitrogen cycling? *Global Biogeochemical Cycles*, 25.

Farrell, M., Prendergast-Miller, M., Jones, D., Hill, P. and Condron, L. (2014). Soil microbial organic nitrogen uptake is regulated by carbon availability. *Soil Biology and Biochemistry*, 77, p.261-267.

Fischer, W., Andre, B., Rentsh, D., Krolkiewicz, S., Tegeder, M., Breitkreuz, K. and Frommer, W. (1998). Amino acid transport in plants. *Trends in Plant Science*, 3(5), p.188-195.

Fischer, W., Kwart, M., Hummel, S. and Frommer, W. (1995). Substrate specificity and expression profile of amino acid transporters (AAPs) in arabidopsis. *Journal of Biological Chemistry*, 270(27), p.16315-16320.

Fischer, W., Loo, D., Koch, A., Ludewig, U., Boorer, K., Tegeder, M., Rentsch, D., Wright, E. and Frommer, W. (2002). Low and high affinity amino acid H+-cotransporters for cellular import of neutral and charged amino acids. *The Plant Journal*, 29(6), p.717-731.

Fowler, D., Coyle, M., Skiba, U., Sutton, M., Cape, J., Reis, S., Sheppard, L., Jenkins, A., Grizzetti, B., Galloway, J., Vitousek, P., Leach, A., Bouwman, A., Butterbach-Bahl, K., Dentener, F., Stevenson, D., Amann, M. and Voss, M. (2013). The global nitrogen cycle in the twenty-first century. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 368(1621), p.20130164-20130164.

Franklin, O., Cambui, C., Gruffman, L., Palmroth, S., Oren, R. and Näsholm, T. (2016). The carbon bonus of organic nitrogen enhances nitrogen use efficiency of plants. *Plant, Cell & Environment*, 40(1), p.25-35.

Galloway, J. (1998). The global nitrogen cycle: changes and consequences. *Environmental Pollution*, 102(1), p.15-24.

Galloway, J., Leach, A., Bleeker, A. and Erisman, J. (2013). A chronology of human understanding of the nitrogen cycle. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 368(1621).

Galloway, J., Townsend, A., Erisman, J., Bekunda, M., Cai, Z., Freney, J., Martinelli, L., Seitzinger, S. and Sutton, M. (2008). Transformation of the nitrogen cycle: recent trends, questions, and potential solutions. *Science*, 320(5878), p.889-892.

Galloway, J., Winiwarter, W., Leip, A., Leach, A., Bleeker, A. and Erisman, J. (2014). Nitrogen footprints: past, present and future. *Environmental Research Letters*, 9(11), p.115003.

Ghosh, B. and Burris, R. (1950). Utilization of nitrogenous compounds by plants. *Soil Science*, 70(3), p.187-204.

Gruber, N. and Galloway, J. (2008). An Earth-system perspective of the global nitrogen cycle. *Nature*, 451(7176), p.293-296.

Haichar, F., Marol, C., Berge, O., Rangel-Castro, J., Prosser, J., Balesdent, J., Heulin, T. and Achouak, W. (2008). Plant host habitat and root exudates shape soil bacterial community structure. *The International Society Microbial Ecology Journal*, 2(12), p.1221-1230.

Hamilton, E. and Frank, D. (2001). Can plants stimulate soil microbes and their own nutrient supply? Evidence from a grazing tolerant grass. *Ecology*, 82(9), p.2397.

Hartmann, A., Rothballer, M. and Schmid, M. (2007). Lorenz Hiltner, a pioneer in rhizosphere microbial ecology and soil bacteriology research. *Plant and Soil*, 312(1-2), p.7-14.

Hawkins, H., Johansen, A. and George, E. (2000). Uptake and transport of organic and inorganic nitrogen by arbuscular mycorrhizal fungi. *Plant and Soil*, 226: p.275–285.

Heijden, M., Martin, F., Selosse, M. and Sanders, I. (2015). Mycorrhizal ecology and evolution: the past, the present, and the future. *New Phytologist*, 205: p.1406–1423.

Hill, P., Farrar, J., Roberts, P., Farrell, M., Grant, H., Newsham, K., Hopkins, D., Bardgett, R. and Jones, D. (2011). Vascular plant success in a warming Antarctic may be due to efficient nitrogen acquisition. *Nature Climate Change*, 1(1), pp.50-53.

Hill, P.W., Jones, D.L., (2018). Plant-microbe competition: does injection of isotopes of C and N into the rhizosphere effectively characterise plant use of soil N? *New Phytologist, In Press.*

Hill, P., Marsden, K. and Jones, D. (2013). How significant to plant N nutrition is the direct consumption of soil microbes by roots?. *New Phytologist*, 199(4), pp.948-955.

Hill, P., Quilliam, R., DeLuca, T., Farrar, J., Farrell, M., Roberts, P., Newsham, K., Hopkins, D., Bardgett, R., Jones, D., (2011). Acquisition and assimilation of nitrogen as peptide-bound and D-enantiomers of amino acids by wheat. *PLoS ONE 6*.

Hodge, A., Campbell, C. and Fitter, A. (2017). An arbuscular mycorrhizal fungus accelerates decomposition and acquires nitrogen directly from organic material. *Letters To Nature*, VOL 413, p.297 - 299.

Hodge, A., Robinson, D. and Fitter, A. (2000). Are microorganisms more effective than plants at competing for nitrogen? *Trends in Plant Science*, 5(7), p.304-308.

Hodges, S. (2010). *Soil fertility basics*. 1st ed. Soil Science Extension, North Carolina State University.

Hutchinson, H. and Miller, N. (1912). The direct assimilation of inorganic and organic forms of nitrogen by higher plants. *The Journal of Agricultural Science*, 4(03), p.282.

Inselsbacher, E., Öhlund, J., Jämtgård, S., Huss-Danell, K. and Näsholm, T., (2011). The potential of microdialysis to monitor organic and inorganic nitrogen compounds in soil. *Soil Biology and Biochemistry* 43, 1321-1332.

Inselsbacher, E., and Näsholm, T., (2012). A novel method to measure the effect of temperature on diffusion of plant-available nitrogen in soil. *Plant and Soil*, 354(1-2), 251-257.

International Fertilizer Industry Association (IFA) (2013). Assessment of Fertilizer Use by Crop at the Global Level 2010 - 2010/11. Paris: International Fertilizer Industry Association.

Jackson, L., Burger, M. and Cavagnaro, T. (2008). Roots, nitrogen transformations, and ecosystem services. *Annual Review of Plant Biology*, 59(1), pp.341-363.

Jan, M., Roberts, P., Tonheim, S., Jones, D.L., (2009). Protein breakdown represents a major bottleneck in nitrogen cycling in grassland soils. *Soil Biology and Biochemistry*, 41, 2272-2282.

Jiang, L., Wang, S., Luo, C., Zhu, X., Kardol, P., Zhang, Z., Li, Y., Wang, C., Wang, Y. and Jones, D. (2016). Effects of warming and grazing on dissolved organic nitrogen in a Tibetan alpine meadow ecosystem. *Soil and Tillage Research*, 158, pp.156-164.

John, T., Coleman, D. and Reid, C. (1983). Growth and spatial distribution of nutrient-absorbing organs: selective exploitation of soil heterogeneity. *Plant and Soil*, 71(1-3), pp.487-493.

Jones, D. (1998). Organic acids in the rhizosphere – a critical review. *Plant and Soil*, 205:, pp.25–44.

Jones, D. (1999). Amino acid biodegradation and its potential effects on organic nitrogen capture by plants. *Soil Biology and Biochemistry*, 31(4), pp.613-622.

Jones, D. (1999). Biodegradation kinetics and sorption reactions of three differently charged amino acids in soil and their effects on plant organic nitrogen availability. *Soil Biology and Biochemistry*, 31(9), pp.1331-1342.

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

Jones, D. and Darrah, P. (1994). Role of root derived organic acids in the mobilization of nutrients from the rhizosphere. *Plant and Soil*, 166(2), pp.247-257.

Jones, D. and Murphy, D. (2007). Microbial response time to sugar and amino acid additions to soil. *Soil Biology and Biochemistry*, 39(8), pp.2178-2182.

Jones, D. and Willett, V. (2006). Experimental evaluation of methods to quantify dissolved organic nitrogen (DON) and dissolved organic carbon (DOC) in soil. *Soil Biology and Biochemistry*, 38(5), pp.991-999.

Jones, D., Clode, P., Kilburn, M., Stockdale, E. and Murphy, D. (2013). Competition between plant and bacterial cells at the microscale regulates the dynamics of nitrogen acquisition in wheat (Triticum aestivum). *New Phytologist*, 200(3), pp.796-807.

Jones, D., Healey, J., Willett, V., Farrar, J. and Hodge, A. (2005). Dissolved organic nitrogen uptake by plants—an important N uptake pathway? *Soil Biology and Biochemistry*, 37(3), pp.413-423.

Jones, D.L., Kielland, K., (2002). Soil amino acid turnover dominates the nitrogen flux in permafrost-dominated taiga forest soils. *Soil Biology and Biochemistry*, 34, 209 - 219.

Jones, D., Kielland, K., Sinclair, F., Dahlgren, R., Newsham, K., Farrar, J. and Murphy, D. (2009). Soil organic nitrogen mineralization across a global latitudinal gradient. *Global Biogeochemical Cycles*, 23(1).

Jones, D.L., Owen, A., Farrar, J., (2002). Simple method to enable the high resolution determination of total free amino acids in soil solutions and soil extracts. *Soil Biology and Biochemistry*, 34, 1893-1902.

Jones, D., Shannon, D., Junvee-Fortune, T. and Farrar, J. (2005). Plant capture of free amino acids is maximized under high soil amino acid concentrations. *Soil Biology and Biochemistry*, 37(1), pp.179-181.

Jones, D., Shannon, D., V. Murphy, D. and Farrar, J. (2004). Role of dissolved organic nitrogen (DON) in soil N cycling in grassland soils. *Soil Biology and Biochemistry*, 36(5), pp.749-756.

Kandemir, T., Schuster, M., Senyshyn, A., Behrens, M. and Schlögl, R. (2013). The Haber - Bosch process revisited: on the real structure and stability of “ammonia iron” under

working conditions. *Angewandte Chemie International Edition*, 52(48), pp.12723-12726.

Kardol, P., Veen, G., Teste, F. and Perring, M. (2015). Peeking into the black box: a trait-based approach to predicting plant-soil feedback. *New Phytologist*, 206(1), pp.1-4.

Kaye, J. and Hart, S. (1997). Competition for nitrogen between plants and soil microorganisms. *Trends in Ecology & Evolution*, 12(4), pp.139-143.

Kielland, K. (1994). Amino acid absorption by Arctic plants: implications for plant nutrition and nitrogen cycling. *Ecology*, 75(8), p.2373.

Kim, J. and Rees, D. (1994). Nitrogenase and biological nitrogen fixation. *Biochemistry*, 33(2), pp.389-397.

Kinraide, T. (1981). Inter-amino acid inhibition of transport in higher plants: evidence for two transport channels with ascertainable affinities for amino acids. *Plant physiology*, 68(6), pp.1327-1333.

Klein, G. and Tauböck, K. (1932). Argininstoffwechsel und Harnstoffgenese bei höheren Pflanzen. II. Abbau von Arginin intermediar zu Harnstoff in Pflanzenzellen. *Biochemistry*, (255), p.278.

Knicker, H. (2000). Biogenic nitrogen in soils as revealed by solid-state carbon-13 and nitrogen-15 nuclear magnetic resonance spectroscopy. *Journal of Environment Quality*, 29(3), p.715.

Kojima, R. (1947). Soil organic nitrogen. *Soil Science*, 64(2), p.157.

Kuzyakov, Y. (2002). Review: Factors affecting rhizosphere priming effects. *Journal of Plant Nutrition and Soil Science*, 165(4), p.382.

Kuzyakov, Y., and Blagodatskaya, E., (2015). Microbial hotspots and hot moments in soil: Concept and Review. *Soil Biology and Biochemistry* 83, 184-199.

Kuzyakov, Y., Hill, P. and Jones, D. (2006). Root exudate components change litter decomposition in a simulated rhizosphere depending on temperature. *Plant and Soil*, 290(1-2), pp.293-305.

Ladd, J. and Jackson, R. (1982). Biochemistry of Ammonification. 1st ed. Glen Osmond, South Australia, Australia: Division of Soils, CSIRO, p.173.

Ladha, J., Tirol-Padre, A., Reddy, C., Cassman, K., Verma, S., Powlson, D., van Kessel, C., de B. Richter, D., Chakraborty, D. and Pathak, H. (2016). Global nitrogen budgets in cereals: A 50-year assessment for maize, rice, and wheat production systems. *Sci. Rep.*, 6, p.1935.

Lambers, H., Chapin, F. and Pons, T. (2008). Plant physiological ecology. 2nd ed. New York, NY: Springer New York.

Landeweert, R., Hoffland, E., Finlay, R., Kuyper, T. and van Breemen, N. (2001). Linking plants to rocks: ectomycorrhizal fungi mobilize nutrients from minerals. *Trends in Ecology & Evolution*, 16(5), pp.248-254.

Lee, C., Ribbe, M. and Hu, Y. (2014). *Met. Ions Life Sci*. 14th ed. pp.147–176.

Li, Z. and Bush, D. (1990). pH-dependent amino acid transport into plasma membrane vesicles isolated from sugar beet leaves: I. Evidence for carrier-mediated, electrogenic flux through multiple transport systems. *Plant Physiology*, 94(1), pp.268-277.

Lipson, D. and Näsholm, T. (2001). The unexpected versatility of plants: organic nitrogen use and availability in terrestrial ecosystems. *Oecologia*, 128(3), pp.305-316.

Lipson, D., Schadt, C., Schmidt, S. and Monson, R. (1999). Ectomycorrhizal transfer of amino acid-nitrogen to the alpine sedge Kobresia myosuroides. *New Phytologist*, 142(1), pp.163-167.

Lutz, M. (1898). Recherches sur la nutrition des vegetaux a l'aide de substances azotecs de nature organique. *Ann. Sci. Bot.*, 7, pp.1 - 98.

Malloch, D., Pirozynski, K. and Raven, P. (1980). Ecological and evolutionary significance of mycorrhizal symbioses in vascular plants (A Review). *Proceedings of the National Academy of Sciences*, 77(4), pp.2113-2118.

Marella, V.S., Roberts, P., Hill, P., Jones, D.L., (2017). Different ways in which CO_2 can be released during the turnover of roots in soil. *Biology and Fertility of Soils* 53, 369-374.

Marschner, P., Marhan, S., Kandeler, E., (2012). Microscale distribution and function of soil microorganisms in the interface between rhizosphere and detritusphere. *Soil Biology and Biochemistry* 49, 174-183.

Marschner, P., Yang, C., Lieberei, R. and Crowley, D. (2001). Soil and plant specific effects on bacterial community composition in the rhizosphere. *Soil Biology and Biochemistry*, 33(11), pp.1437-1445.

Martens, D. and Frankenberger, W. (1994). Assimilation of exogenous 2?-14C-indole-3-acetic acid and 3?-14C-tryptophan exposed to the roots of three wheat varieties. *Plant and Soil*, 166(2), pp.281-290.

McClain, M., Boyer, E., Dent, C., Gergel, S., Grimm, N., Groffman, P., Hart, S., Harvey, J., Johnston, C., Mayorga, E., McDowell, W., Pinay, G., (2003). Biogeochemical hot spots and hot moments at the interface of terrestrial and aquatic ecosystems. *Ecosystems*, 6, 301-312.

McKane, R., Johnson, L., Shaver, G., Nadelhoffer, K., Rastetter, E., Fry, B., Giblin, A., Kielland, K., Kwiatkowski, B., Laundre, J. and Murray, G. (2002). Resource-based niches provide a basis for plant species diversity and dominance in arctic tundra. *Nature*, 415(6867), pp.68-71.

McKee, H. (1962). Nitrogen metabolism in plants. 1st ed. Oxford: Clarendon Press.

Miller, A. and Bowman, W. (2002). Variation in nitrogen-15 natural abundance and nitrogen uptake traits among co-occurring alpine species: do species partition by nitrogen form? *Oecologia*, 130(4), pp.609-616.

Miller, A. and Bowman, W. (2003). Alpine plants show species-level differences in the uptake of organic and inorganic nitrogen. *Plant and Soil*, 250(2), pp.283-292.

Miller, A. and Cramer, M. (2005). Root nitrogen acquisition and assimilation. *Plant and Soil*, 274(1-2), pp.1-36.

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

Miro M, Frenzel W., (2004). Implantable flow-through capillary-type microdialyzers for continuous in situ monitoring of environmentally relevant parameters. *Analytical Chemistry* 76: 5974–5981.

Molliard, M. (1910). Recherches sur l'utilisation par les plantes supérieures de diverses substances organiques azotées. *Bulletin de la Société Botanique de France*, 57(7), pp.541-547.

Moran-Zuloaga, D., Dippold, M., Glaser, B., Kuzyakov, Y., (2015). Organic nitrogen uptake by plants: re-evaluation by position-specific labelling of amino acids. *Biogeochemistry* 125, 359-374.

Mulder, E. (1975). Physiology and ecology of free-living, nitrogen fixing bacteria IN nitrogen fixation by free-living microorganisms. 1st ed. Cambridge: Cambridge University press, pp.3 - 28.

Mulvaney, R., (1996). Nitrogen—inorganic forms. In: Sparks, D.L. (Ed.). Methods of Soil Analysis. Part 3. Chemical Methods. Soil Science Society of America, Madison, WI, USA, pp. 1123–1184.

Nakamura, T. (1897). On the relative value of asparagin as a nutrient for phanerogams and fungi. *Bull. Col. Agr. Tokyo Imperial University*, 2, pp.465 - 470.

Nasholm, T., Ekblad, A., Nordin, A., Giesler, R., Hogberg, M. and Hogberg, P. (1997). Boreal forest plants take up organic nitrogen. *Nature*, 392, pp.914 - 916.

Nasholm, T., Huss-Danell, K. and Hogberg, P. (2000). Uptake of organic nitrogen in the field by four agriculturally important plant species. *Ecology*, 81(4), p.1155.

Näsholm, T., Kielland, K. and Ganeteg, U. (2009). Uptake of organic nitrogen by plants. *New Phytologist*, 182(1), pp.31-48.

Neff, J., III, F. and Vitousek, P. (2003). Breaks in the cycle: dissolved organic nitrogen in terrestrial ecosystems. *Frontiers in Ecology and the Environment*, 1(4), p.205.

Nehls, U., Göhringer, F., Wittulsky, S. and Dietz, S. (2009). Fungal carbohydrate support in the ectomycorrhizal symbiosis: a review. *Plant Biology*, 12, pp.292 - 301.

Nightingale, G. (1937). The nitrogen nutrition of green plants. *The Botanical Review*, 3(3), pp.85-174.

Nordin, A., Högberg, P. and Näsholm, T. (2001). Soil nitrogen form and plant nitrogen uptake along a boreal forest productivity gradient. *Oecologia*, 129(1), pp.125-132.

Nordin, A., Schmidt, I. and Shaver, G. (2004). Nitrogen uptake by Arctic soil microbes and plants in relation to soil nitrogen supply. *Ecology*, 85(4), pp.955-962.

Oliver, D. (1994). The glycine decarboxylase complex from plant mitochondria. *Annual Review of Plant Physiology and Plant Molecular Biology*, 45(1), pp.323-337.

Ortiz-Lopez, A., Chang, H. and Bush, D. (1999). Amino acid transporters in plants. *Biochimica et Biophysica Acta*, 1465, pp.275-280.

Owen, A. and Jones, D. (2001). Competition for amino acids between wheat roots and rhizosphere microorganisms and the role of amino acids in plant N acquisition. *Soil Biology and Biochemistry*, 33(4-5), pp.651-657.

Postgate, J. (1998). Nitrogen fixation. 1st ed. Cambridge, U.K.: Cambridge University Press.

Raghavan, V. and Torrey, J. (1964). Inorganic Nitrogen Nutrition of the Seedlings of the Orchid, Cattleya. *American Journal of Botany*, 51(3), p.264.

Raymond, J. (2003). The natural history of nitrogen fixation. *Molecular Biology and Evolution*, 21(3), pp.541-554.

Reinhold, L. and Kaplan, A. (1984). Membrane transport of sugars and amino acids. *Annual Review of Plant Physiology and Plant Molecular Biology*, 35(1), pp.45-83.

Rentsch, D., Schmidt, S. and Tegeder, M. (2007). Transporters for uptake and allocation of organic nitrogen compounds in plants. *FEBS Letters*, 581(12), pp.2281-2289.

Richardson, A., Barea, J., McNeill, A. and Prigent-Combaret, C. (2009). Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. *Plant and Soil*, 321(1-2), pp.305-339.

Rothstein, D. (2009). Soil amino-acid availability across a temperate-forest fertility gradient. *Biogeochemistry*, 92(3), pp.201-215.

Roy, J., Caldwell, M. and Pearce, R. (1994). Exploitation of Environmental Heterogeneity by Plants. 1st ed. Oxford: Elsevier Science.

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

Sauheitl, L., Glaser, B., Weigelt, A., (2009). Uptake of intact amino acids by plants depends on soil amino acid concentrations. *Environmental and Experimental Botany* 66, 145-152.

Schimel, J., Bennett, J., (2004). Nitrogen mineralization: challenges of a changing paradigm. *Ecology* 85, 591-602.

Schippers, B., Bakker, A. and Bakker, P. (1987). Interactions of deleterious and beneficial rhizosphere microorganisms and the effect of cropping practices. *Annual Review of Phytopathology*, 25(1), pp.339-358.

Schmidt, S., Mason, M., Sangtiean, T. and Stewart, G. (2003). Do cluster roots of Hakea actities (Proteaceae) acquire complex organic nitrogen? *Plant and Soil*, 248(1/2), pp.157-165.

Schnitzer, M. and Khan, S. (1989). Soil organic matter. 4th ed. Amsterdam: Elsevier, pp.100 - 130.

Schreiner, O. (1912). The organic constituents of soils. *Science*, 36(931), pp.577-587.

Schreiner, O. and Skinner, J. (1912). Nitrogenous Soil Constituents and their Bearing on Soil Fertility. *US Department of Agriculture, Soils Bulletin*, 87.

Schulten, H. and Schnitzer, M. (1998). The chemistry of soil organic nitrogen: a review. *Biology and Fertility of Soils*, 26(1), pp.1-15.

Schulze, E. (1901). Can leucin and tyrosin be used as plant nutrients? *Landw. Vers. Stat.*, 56, pp.97-106.

Shibata, H., Galloway, J., Leach, A., Cattaneo, L., Cattell Noll, L., Erisman, J., Gu, B., Liang, X., Hayashi, K., Ma, L., Dalgaard, T., Graversgaard, M., Chen, D., Nansai, K., Shindo, J., Matsubae, K., Oita, A., Su, M., Mishima, S. and Bleeker, A. (2016). Nitrogen footprints: regional realities and options to reduce nitrogen loss to the environment. *Ambio*.

Singh, B., Millard, P., Whiteley, A. and Murrell, J. (2004). Unravelling rhizosphere–microbial interactions: opportunities and limitations. *Trends in Microbiology*, 12(8), pp.386-393.

Smith, V. H., & Schindler, D. W. (2009). Eutrophication science: where do we go from here?. *Trends in ecology & evolution*, *24*(4), 201-207.

Sowden, F., Chen, Y. and Schnitzer, M. (1977). The nitrogen distribution in soils formed under widely differing climatic conditions. *Geochimica et Cosmochimica Acta*, 41(10), pp.1524-1526.

Sprent, J. and Sprent, P. (1990). Nitrogen fixing organisms. 1st ed. London: Chapman and Hall.

Stevenson, F. (1982). Organic Forms of Soil Nitrogen. In: Nitrogen in Agricultural Soils. 1st ed. Illinois: University of Illinois, pp.67 - 68.

Streeter, T., Bol, R. and Bardgett, R. (2000). Amino acids as a nitrogen source in temperate upland grasslands: the use of dual labelled (13C, 15N) glycine to test for direct uptake by dominant grasses. *Rapid Communications in Mass Spectrometry*, 14(15), pp.1351-1355.

Sulyok, M., Miró, M., Stingeder, G., & Koellensperger, G. (2005). The potential of flow-through microdialysis for probing low-molecular weight organic anions in rhizosphere soil solution. *Analytica Chimica Acta*, 546(1), 1-10.

Suzuki, U. (1897). On the formation of asparagine in plants under different conditions. *Bull. Coll. Agric. Tokyo*, (2), p.409.

Tanaka, I. (1931). Studien über die Ernährung der hoheren Pflanzen mit den organischen Verbindungen. *Japanese Journal of Botany*, (5), p.323.

Tartowski, S. and Howarth, R. (2013). *Nitrogen, Nitrogen Cycle*. [online] Available at: http://dx.doi.org/10.1016/B978-0-12-384719-5.00098-8 [Accessed 17 Nov. 2016].

Tegeder, M. and Rentsch, D. (2010). Uptake and partitioning of amino acids and peptides. *Molecular Plant*, 3(6), pp.997-1011.

Thompson, A. (1899). Die kulturpflanzen and organischen stickstoffverbindungen. *Naturf. Gesell. Univ. Sitzber. Jurjew.*, 12, pp.307 - 322.

Tisdale, S., Nelson, W. and Beaton, J. (1990). Soil fertility and fertilizers. 1st ed. New York, N.Y.: Macmillan.

UN (2015). World Population Prospects The 2015 Revision. New York: Department of Economic and Social Affairs - United Nations.

Uscola, M., Villar-Salvador, P., Oliet, J. and Warren, C. (2017). Root uptake of inorganic and organic N chemical forms in two coexisting Mediterranean forest trees. *Plant and Soil*.

van Kessel, C., Clough, T. and van Groenigen, J. (2009). Dissolved Organic Nitrogen: An Overlooked Pathway of Nitrogen Loss from Agricultural Systems?. *Journal of Environment Quality*, 38(2), p.393.

Virtanen, A. and Linkola, H. (1946). Organic Nitrogen Compounds as Nitrogen Nutrition for Higher Plants. *Nature*, 158(4015), pp.515-515.

Vitousek, P., Aber, J., Howarth, R., Likens, G., Matson, P., Schindler, D., Schlesinger, W. and Tilman, D. (1997). Technical Report: Human Alteration of the Global Nitrogen Cycle: Sources and Consequences. *Ecological Applications*, 7(3), p.737.

Vojvodic, A., Medford, A., Studt, F., Abild-Pedersen, F., Khan, T., Bligaard, T. and Nørskov, J. (2014). Exploring the limits: a low-pressure, low-temperature Haber–Bosch process. *Chemical Physics Letters*, 598, pp.108-112.

Vonk, J., Middelburg, J., Stapel, J. and Bouma, T. (2008). Dissolved organic nitrogen uptake by seagrasses. *Limnology and Oceanography*, 53(2), pp.542-548.

Wallenstein, M., McMahon, S. and Schimel, J. (2009). Seasonal variation in enzyme activities and temperature sensitivities in Arctic tundra soils. *Global Change Biology*, 15(7), pp.1631-1639.

Ward, B. (2012). Fundamentals of Geobiology. 1st ed. Blackwell Publishing, pp.36 - 48.

Warren, C. (2014). Organic N molecules in the soil solution: what is known, what is unknown and the path forwards. *Plant and Soil*, 375(1-2), pp.1-19.

Weigelt, A., Bol, R. and Bardgett, R. (2004). Preferential uptake of soil nitrogen forms by grassland plant species. *Oecologia*, 142(4), pp.627-635.

Weigelt, A., King, R., Bol, R. and Bardgett, R. (2003). Inter-specific variability in organic nitrogen uptake of three temperate grassland species. *Journal of Plant Nutrition and Soil Science*, 166(5), pp.606-611.

Weston, K., Hall, J. and Williams, L. (1995). Characterization of amino-acid transport in Ricinus communis roots using isolated membrane vesicles. *Planta*, 196(1).

White, P. (1937). Amino acids in the nutrition of excised tomato roots. *Plant physiology*, 12(3), pp.793-802.

White, P. (1939). Glycine in the nutrition of excised tomato roots. *Plant physiology*, 14(3), pp.527-538.

White, P. (1943). Further Evidence on the Significance of Glycine, Pyridoxine and Nicotinic Acid in the Nutrition of Excised Tomato Roots. *American Journal of Botany*, 30(1), p.33.

Wolf, W. (1868). Das Tyrosin als stickstofflieferndes Nahrungsmittel bei der Vegetation der Roggenpflanze in wassriger Losung. *Landw. Vers. Sta.*, (10), p.13.

Yamaguchi, S. (1930). Studies on the resorption of urea by root of Zea mays seedlings in sterile culture. *J. Fac. Sci. Hokkaido University. Botany*, 5(1), p.37.

Appendix A – Short Communication Supplementary Material

Soil moisture (%): Soil samples from the collection site (n = 4) were analysed gravimetrically in porcelain crucibles before and after drying at 105 ºC for 16 hours. Moisture content was calculated following the equation:

$$\text{MC\%} = \frac{W2-W3}{W3-W1} \times 100$$

Where:

W1 = Weight of crucible (g)

W2 = Weight of moist soil + crucible (g)

W3 = Weight of dried soil + crucible (g)

pH: Soil samples from the collection site (n = 4) were extracted using DI water at a 1:5 ratio and shaken at room temperature for 15 minutes, after which they were analysed using a standard electrode for pH (Hanna Instruments, pH209, pH meter).

EC: Soil samples from the collection site (n = 4) were extracted using DI water at a 1:5 ratio and shaken at room temperature for 15 minutes, after which they were analysed for EC using a standard electrode (Hannah Instruments, EC215, Conductivity meter).

Total Carbon (%) & Nitrogen (%): Total C & N for soil samples (n = 4) were analysed using an elemental analyser (Leco TruSpec Micro CHNS analyser, Leco Corp., MI, USA), taken from oven dried and ground samples**.**

Nitrate (N mg kg^{-1} oven dry soil): Nitrate samples (n = 4) derived from homogenised bulk soil, and extracted using deionised water, were analysed spectrophotometrically (BioTek PowerWave XS microplate spectrophotometer, BioTek Instruments Inc., VT, USA); following methods described in Mulvaney, (1996). The same methods were employed to analyse microdialysis samples.

Ammonium (N mg kg^{-1} oven dry soil): Ammonium samples (n = 4) derived from homogenised bulk soil and extracted using deionised water, were analysed spectrophotometrically (BioTek PowerWave XS microplate spectrophotometer, BioTek

Instruments Inc., VT, USA); following methods described in Mulvaney, (1996). The same methods were employed to analyse microdialysis samples.

Total C & N for each protein source supplied was analysed using an elemental analyser (Leco TruSpec Micro CHNS analyser, Leco Corp., MI, USA), taken from oven dried and ground samples.

3. Contamination of Samples at Hour 30

This was simply a result of expanding volume forcing the lids off faulty Eppendorf vials used during freezing at hour 30. Upon defrosting, liquid leaked from all replicates and homogenised within the zip-lock bag used during freezing. It was therefore impossible to discern true concentrations of any one sample and subsequently hour 30 samples were discarded and omitted from analysis.

4. Electroconductivity and pH

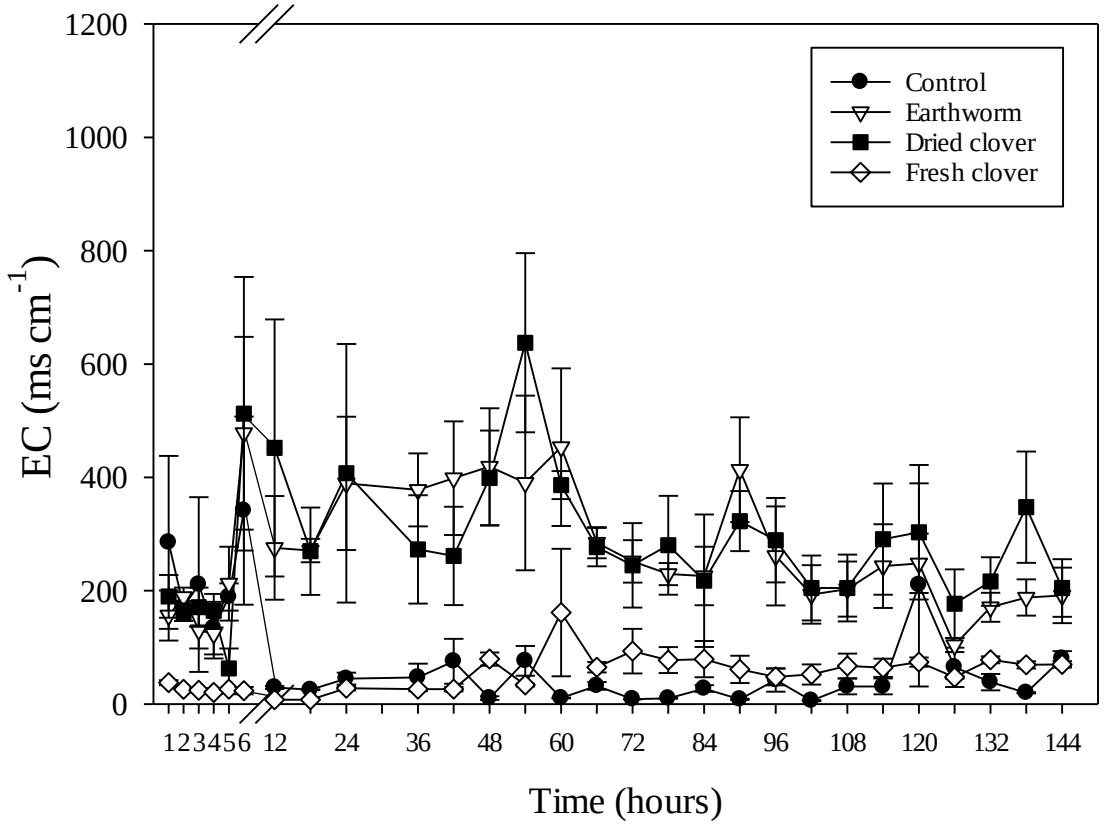

Figure A.1. Electroconductivity in all treatments (1 – 144 hours). Values represent means ± SEM (*n* = 4).

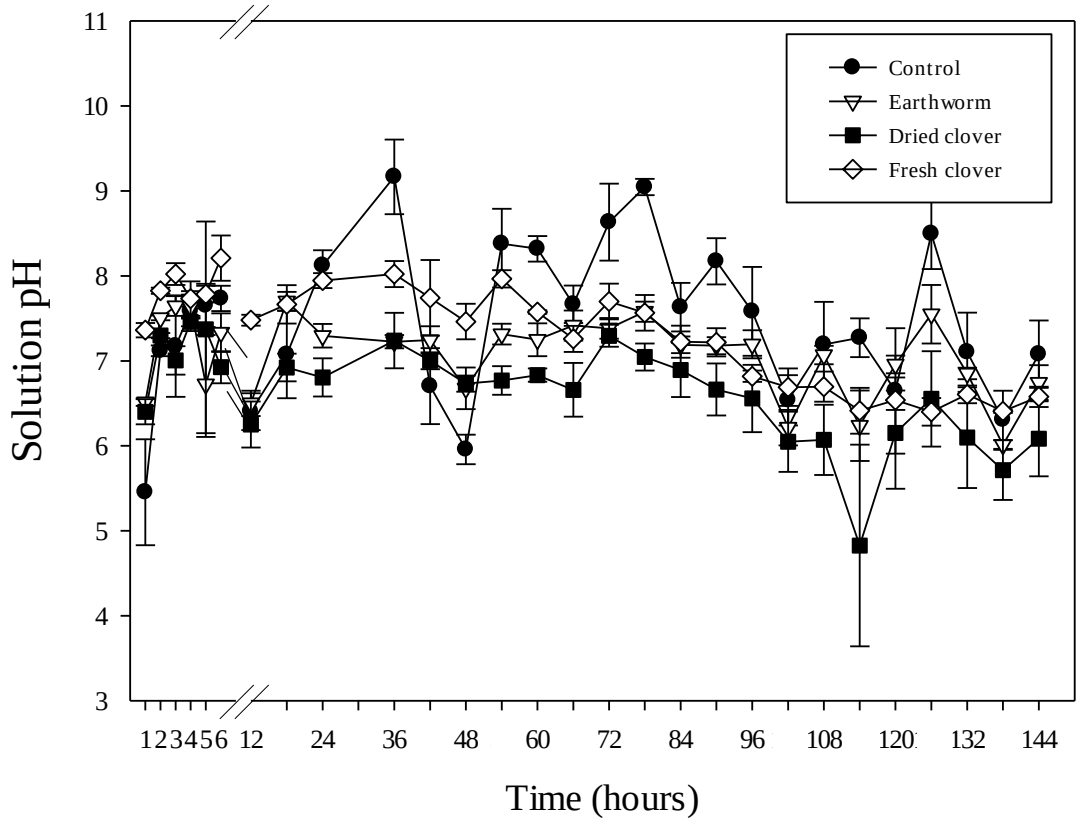

Figure A.2. pH in all treatments (1 - 144 hours). Values represent means ± SEM (*n* = 4).

References

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

Mulvaney, R., 1996. Nitrogen—inorganic forms. In: Sparks, D.L. (Ed.). Methods of Soil Analysis. Part 3. Chemical Methods. Soil Science Society of America, Madison, WI, USA, pp. 1123–1184.