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A microdialysis perspective of soil nitrogen availability

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Abstract

Soil nitrogen (N) availability is a critical component of ecosystem function and a main driver of below and above ground productivity. As such, the topic is of interest to ecologists exploring the constraints on soil processes as they relate to plant and microbial productivity, as well as agriculturalists seeking to maximise crop N uptake whilst minimising N losses from soil. Soil N encompasses a diversity of compounds spanning from organic to inorganic. Quantifying the bioavailability of different N forms is difficult and likely affected by factors that include climate and water, and the physical, chemical and biological characteristics of soils, all of which can vary significantly at the microsite level.

Much of what we know about N availability is derived from destructive soil sampling techniques, especially extractions, which introduce considerable artefacts that alter N availability and may not reflect *in situ* availability. Microdialysis is an *in situ* sampling technique, developed originally for biomedical research, which has shown potential to provide a plant-relevant perspective of N availability in near undisturbed soil. By deploying small probes (~0.5 × 10 mm) fitted with a semi-permeable membrane, soil solutes are sampled *via* diffusion alone, mimicking some of the spatial N movement and encounters experienced by plant roots. Some discoveries with soil microdialysis have contrasted soil extractions; e.g. revealing a much higher contribution of amino acids than soil extractions which in turn were dominated by ammonium. This disparity warrants investigation. Furthermore, there is potential for microdialysis to explore other aspects of nutrient cycling and soil function, which requires optimisation of the technique to advance understanding and performance.

This thesis aims to advance microdialysis as a tool for soil N research, and existing knowledge of its application in soil environments is synthesised in Chapter 2.

Chapter 3 explores microdialysis and extractions in the context of litter decomposition showing that patterns of total N release over 100 days were similar between methods. However, microdialysis sampled little ammonium in comparison to soil extractions, suggesting that the available N pool *in situ* is dominated by amino acids and nitrate. This finding highlights unanswered questions about the true fate of ammonium in soils, whether it be rapid utilisation by soil microbes, or adsorption to soil surfaces.

Chapter 4 explores the effect of microdialysis and soil extractions on inorganic N availability in 24 sugarcane soils with diverse soil texture (4.6 to 54 % clay content) and chemistry (e.g. 0.68 to 3.3 % organic carbon content). Again, ammonium contributed least to microdialysis fluxes, likely due the minimal disturbance of adsorbed N fractions during deployment. Mobilisation of adsorbed N from higher clay soils may have contributed to a higher prevalence of ammonium in KCl extractions. Both

methods provide complimentary views of soils, revealing soluble and adsorbed N fractions that help contextualise the roles of soil environments on N availability.

Chapter 5 deploys microdialysis to investigate enzyme dynamics in soil environments. Many techniques that quantify soil enzyme activity suffer similar methodological problems as soil N extraction – notably, large extraction-based disturbances during processing. Few tools allow for quantification of enzyme activity in undisturbed soils, and even fewer can differentiate free enzymes in soil solution from those stabilised to soil surfaces. Microdialysis shows much potential here as a tool to sample enzymes from soil solution, with enzymes subjected to a modified functional assay. Microdialysis uncovers sensitive changes in enzyme production induced by the addition of soybean litter, and free enzymes contributed a greater proportion of total enzyme activity in litter-amended soil; however, soil water availability emerges as a likely constraint on enzyme recovery.

Chapter 6 explores ways of optimising microdialysis for soil N sampling. Three probe designs, including membranes of greater length and pore size, were used to sample organic and inorganic N from artificial solution, laboratory soil and field soil of a boreal heath forest. Longer membranes were successfully improved recovery and precision of amino acids and ammonium in field soils, with a potential bias towards more mobile amino acids. Pore sizes did not affect N recoveries, indicating that membrane length had greater control in complex soil environments.

Taken together, this thesis presents microdialysis as a holistic technique for exploring N cycling and N availability. Future research should consider how the method can be deployed to sample a wider range of soil solutes related to nutrient cycling and rhizosphere processes, and how it could be best used to maximise standardisation and comparability between studies.

Declaration by author

This thesis *is composed of my original work, and contains* no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Publications included in this thesis

Chapter 6 is represented by the following published work:

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Conference abstracts

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Contributions by others to the thesis

Guidance in experimental design, analysis and data interpretation, and minor editing was provided by advisors Prof. Susanne Schmidt, Dr. Richard Brackin and Dr. Paul Dennis. Further contributions are provided below:

Chapter 3:

Assistance in carrying out the experimental soil harvests was provided by Dr. Richard Brackin, Dr. Maren Westermann, Lisa Xian, João Carlos de Freitas Junior, Shelby Berg and Jay Anderson.

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Statement of parts of the thesis submitted to qualify for the award of another degree

No works submitted towards another degree have been included in this thesis.

Research Involving Human or Animal Subjects

No animal or human subjects were involved in this research.

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Microdialysis, soil nitrogen availability, organic nitrogen, inorganic N, amino acids, soil enzymes, flux, soil extraction, diffusion, plant nutrition.

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FoR code: 0503, Soil Sciences, 70%

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List of Abbreviations

AA	Amino acid
Ala	Alanine
A_m	Surface area of a microdialysis membrane
Arg	Arginine
Asp	Aspartic Acid
Asn	Asparagine
°C	Celsius
C	Carbon
Ca^{2+}	Calcium ion
CaCl2	Calcium Chloride
C_{dial}	Concentration of a solute in a dialysate
$CHCL_3$	Chloroform
cm	Centimetre
CO_2	Carbon dioxide
$CuSO_4$	Cupric Sulphate
Cys	Cysteine
D	Diffusive Flux
dH_2O	Deionised water
DIN	Dissolved inorganic nitrogen
DNA	Deoxyribonucleic acid
DON	Dissolved organic nitrogen
DW	Dry weight
EC	Electro-conductivity
E_d	Relative recovery of a solute
EEA	Exoenzyme activity
FDA	Fluorescein di-acetate
g	Grams
GBR	Great Barrier Reef
Gln	Glutamine
Glu	Glutamic Acid
Gly	Glycine
h	Hour
H_2O	Water
Ha	Hectare
His	Histidine
Ile	Isoleucine
K^+	Potassium ion
K_2SO	Potassium sulphate
KCl	Potassium chloride
KNO_3	Potassium Nitrate
kDa	Kilodalton
L	Litre
Leu	Leucine
LMW-N	Low molecular weight nitrogen
Lys	Lysine
MB-C	Microbial biomass carbon
MB-N	Microbial biomass nitrogen
Met	Methionine

min	Minute
Mg	Magnesium
mL	Millilitre
mm	Millimetre
mM	Millimole
MPa	Megapascal
mRNA	messenger ribonucleic acid
MWCO	Molecular weight cut-off
N	Nitrogen
N_2	Di-nitrogen
N_2O	Nitrous oxide
Na	Sodium
Na_2CO_3	Sodium carbonate
$NaNO_3$	Sodium nitrate
NaOH	Sodium hydoxide
NH_3	Ammonia
NH_4	Ammonium ion
nmol	Nanomole
NO_2^-	Nitrite ion
NO_3	Nitrate ion
OC	Organic carbon
P	Phosphorus
P_i	Inorganic phosphorus
PAES	Polyarylethersulphone
PES	Polyethersulphone
Phe	Phenylalanine
PF	Permeability factor
PMN	Potentially-mineralisable nitrogen
Pro	Proline
Q_p	Flow rate
R_d	Resistances to solute movement imposed by dialysate
R_{ext}	Resistances to solute movement imposed by an external environment
R_m	Resistances to solute movement imposed by a membrane
RPM	Revolutions per minute
SDS-PAGE	Sodium dodecyl sulphate – polyacrylamide gel electrophoresis
SEM	Standard error of the mean
Ser	Serine
SOM	Soil organic matter
t	Sampling time
t	Tonne
TCA	Trichloroacetic acid
THAM	Tris-hydroxymethylmethane
Thr	Threonine
Tyr	Tyrosine
Val	Valine
VCL_3	Vanadium (III) chloride
UPLC	Ultra-pressure liquid chromatography
WHC	Water-holding capacity
µL	Microlitre
µm	Micrometre
µM	Micromole

1 Introduction

Soil nitrogen (N) availability is vitally important for plant nutrition and ecosystem function. As a significant macronutrient for biological growth and activity, N availability is closely linked with ecosystem productivity, and can strongly influence above and below ground ecological processes (Chapman et al., 2006, Lambers et al., 2008).

The topic of 'availability' received much consideration in recent years. Nitrogen management practices in agricultural industries worldwide have gained attention after extensive use of Haber-Bosch N fertiliser in cropping systems having resulted in an N pollution crisis that is recognised as one of our greatest environmental challenges (Rockstrom et al., 2009, Erisman et al., 2008). However, plants can acquire a surprising diversity of N forms from the soil (Näsholm et al., 2009, Schmidt et al., 2013, Selosse et al., 2017), questioning long-standing paradigms about plant nutrition and N management in cropping systems (Schimel and Bennett, 2004).

Knowledge of N availability, including the forms and rates at which plants and microbes may encounter N compounds, is crucial to improving N management practices in agricultural systems, and to better predict ecosystem responses to increasing amounts of anthropogenic N deposition in natural systems. In Australia, sugarcane agriculture is an example of a high-risk system with potentially adverse environmental consequences – mostly due to the proximity of production areas to the Great Barrier Reef (GBR). Sugarcane cropping features high inputs of N (160kg N ha-1, or more; Schroeder et al., 2005, Calcino et al., 2018), of which large proportions can be leached out of soils into adjacent waterways or groundwater, and ultimately into the GBR lagoon. Declining health in of the GBR is linked to terrestrial releases of pollutants such as N (Brodie et al., 2001, Bainbridge et al., 2009, Benn et al., 2010, Brodie et al., 2010, Thorburn et al., 2011). It is thought that a reduction in N runoff has occurred (by as much as 16%; Great Barrier Reef Marine Park, 2014) as N fertiliser application rates have been lowered compared to the 1990s; but runoff targets set by the Queensland Government – calling for reductions of dissolved inorganic N loads from rivers by as much as 80% (Commonwealth of Australia, 2015) – requires drastic and long-lasting N management changes in the Australian sugar industry.

1.1 Defining N availability

In this thesis, 'N availability' and 'bio-available N' is defined as the proportion of total soil N that is *accessible* by plants or soil microbes; that is, the amount and types of N encounterable *before* uptake. Additionally, 'plant-available N' will refer to proportion of total soil N potentially encounterable

specifically by a plant *before* uptake. Although uptake processes are important for understanding the utilisation efficiency of encountered N forms by specific plants and microbial communities, N availability is used here as a specific term to encompass the suite of N forms biologically accessible in a particular soil, and are measurable in soil solution.

1.2 Complexities of soil nitrogen research

Much of what limits research in this area is the sheer complexity of soil N cycling. The dynamic process that culminates in 'N availability' is in fact many specific, cryptic processes, all of which are affected by climate, vegetation, and the physical, chemical and biological properties of soils (Lambers et al., 2008, Hinsinger et al., 2009). Throughout the cycle, reactive N can exist in soil in numerous forms; as organic N within a diverse pool of compounds including proteins, peptides, heterocyclic compounds, amino sugars and amino acids, quaternary ammonium compounds, nucleic acids and others (Schulten and Schnitzer, 1997, Warren, 2014), as inorganic ammonium (NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-), and as gases (e.g. NO_x, NH_3). Nitrogen can exist freely in soil solution as dissolved organic N (DON), or dissolved inorganic N (DIN); alternatively, many forms can exist in a bound state, adsorbed to soil particles, contained within living microorganisms, invertebrates and roots, and in necromass. Much N occurs as positively charged compounds, including many proteins, amino acids, quaternary ammonium compounds and NH_4^+ (Quiquampoix et al., 1995, Rothstein, 2010, Nieder et al., 2011, Yu et al., 2013). Such bound states may have consequences for bioavailability, and mobility through the soil via diffusion and mass flow (Oyewole et al., 2014, Tinker and Nye, 2000).

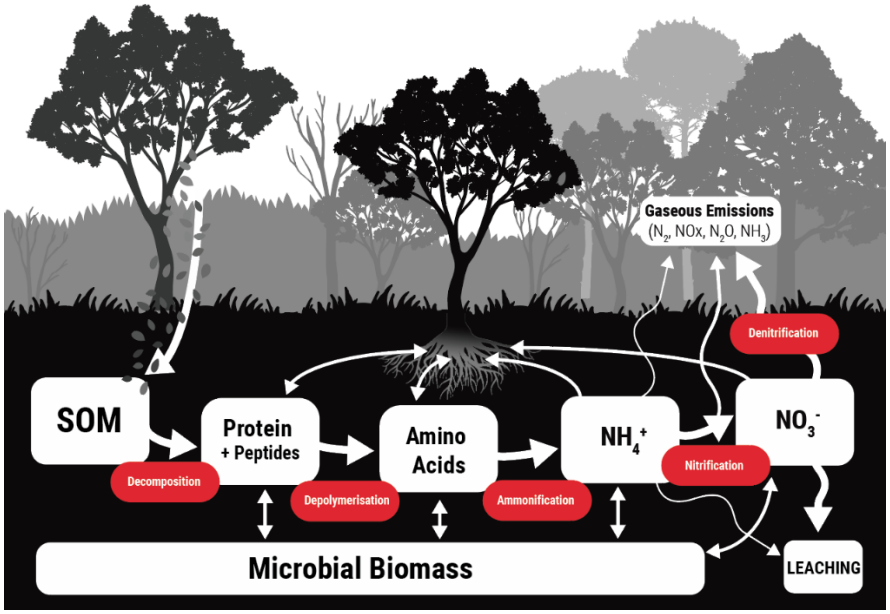

Figure 1-1. A simplified representation of the N cycle, with main soil N pools and soil biological transformations that contribute (in red).

1.3 Decomposition, and the dominance of organic N in soil

Although the inert atmospheric gas dinitrogen (N_2) constitutes 78% of Earth's atmosphere, it is inaccessible to the vast majority of organisms. However, reactive N can enter the soil N cycle through several means. Firstly, reactive N derived from lightning and rainfall can deposit a certain amount of N into ecosystems (Galloway et al., 2004). Biological N fixation (performed by N-fixing microbes) contributes a much larger proportion of reactive N; most notably, by rhizobia living symbiotically with legume plants, but also by free-living bacteria and cyanobacteria (Vitousek et al., 2002). However, the predominant pathway for the entry of N into the soil is through the reclamation of already fixed forms through the decomposition of soil organic matter (SOM) – a heterogeneous distribution of dead biomass from plants, animals and soil microbes. Anthropogenic activities have also resulted in a third and fourth route that including deposition of reactive N from air pollution that can exceed 50 kg N ha^{-1} y^{-1} (Liu et al., 2013) and input into agricultural soils as mineral fertiliser or organic materials such as composts, manures or biosolids.

Irrespective of the N input, ultimately the decomposition process is mediated by a succession of soil fauna and microorganisms which transform large, complex biological materials (e.g. proteinaceous compounds, structural cell components) into smaller polymers, oligomers and monomers (such as peptides and amino acids). Further microbial transformation can mineralise organic N to NH_4^+ and NO_3^-. However, this N pathway is by no means a one-way street. Nitrogen can be assimilated by microbes or plants at any stage of the cycle, transformed and returned to the soil as SOM, only to be reassimilated as another form (Schimel and Bennett, 2004, Geisseler et al., 2010). Protein and peptides consistently constitute the majority of soil N (Abe and Watanabe, 2004, Schulten and Schnitzer, 1997), and size-based fractionation of DON in grassland soils found most featured molecular weights > 100 kDa (Farrell et al., 2011a). Low molecular weight N (LMW-N) such as amino acids, small peptides, and quaternary ammonium compounds (all < 1 kDa) also significantly contribute in many soils (Warren, 2013, Farrell et al., 2011a). Organic N can often dominate dissolved N fluxes in a variety of natural and agricultural soils (Brackin et al., 2015, Farrell et al., 2011a, Shaw et al., 2014, Inselsbacher et al., 2014, Farrell et al., 2011b, Prendergast-Miller et al., 2015), although high turnover rates may account for their relatively small contribution to total N compared to protein (Farrell et al., 2011b, Jones et al., 2005, Jones and Kielland, 2002, Jones and Kielland, 2012, Näsholm et al., 2009, Warren, 2018).

Given such high proportions of organic N in soil, can we consider this pool 'available' to plants? During much of the 20th century, the consensus view of plant nutrition placed inorganic N such as NH_4^+ and NO_3^- at the centre of plant N uptake, reliant on soil microorganisms (both mycorrhizal and

free-living) to mineralise organic N (Schimel and Bennett, 2004). It has been argued that this greater importance placed upon inorganic N was established after the development, and application of Haber-Bosch-derived N to agriculture post World War II, which did indeed provide significant increases in crop production, and thus greater interest in its use (Paungfoo-Lonhienne et al., 2012, Näsholm et al., 2009). However, the notion that organic N is abundant in soil, and that plants could potentially access these fractions (independent of mycorrhizal pathways) is not new (e.g. Hutchinson and Miller, 1912, Lathrop, 1917). There is now significant evidence that plants can access a variety of soil organic N, including protein (Paungfoo-Lonhienne et al., 2008), DNA (Paungfoo-Lonhienne et al., 2010), peptides (Soper et al., 2011, Komarova et al., 2008) and amino acids (Wright, 1962, Kielland, 1994, Näsholm et al., 1998, Jamtgard et al., 2008, Warren and Adams, 2007, Vinall et al., 2012, Ganeteg et al., 2017). This understanding of the diversity of N forms potentially available to plants must change our approach to the relationship between N cycling and plant uptake. Our understanding of the bottleneck to plant N availability is no longer simply the mineralisation of organic N to inorganic, but the decomposition of SOM via depolymerisation (Figure 1-2) (Schimel and Bennett, 2004, Jan et al., 2009).

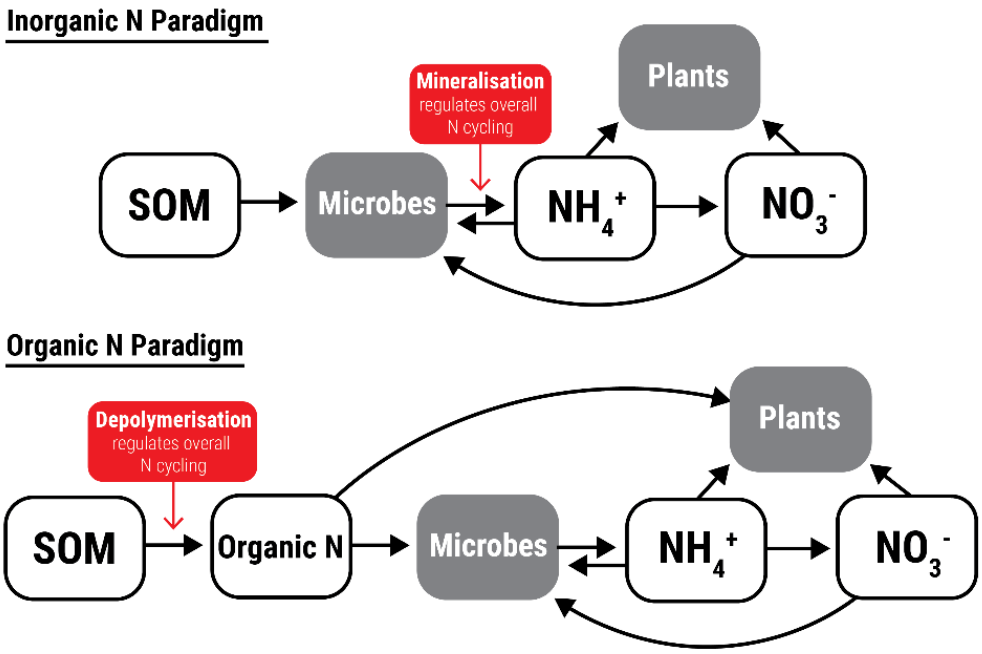

Figure 1-2. An overview of our changing view of N availability in soils. In the inorganic N paradigm, mineralisation of organic N by microbes is responsible for regulating N cycling and availability of inorganic N (ammonium, NH_4^+; nitrate, NO_3^-) for plants. In the organic N paradigm, depolymerisation of soil organic matter (SOM) regulates cycling and availability, allowing plants to access both organic and inorganic N forms. Modified from Schimel and Bennett (2004).

However, to better understand this new paradigm, we need methods of sampling and analysing soil N which represent a plant's perspective of the soil environment. In my thesis, I argue that methodological bias towards inorganic N may have also played a role in our historical interpretations of plant-available N. Much of what is known about soil N availability is built upon methods such as soil extractions (discussed below) known to introduce significant artefacts during processing. *In situ* methods may provide a more relevant picture of bioavailable N, and this thesis will particularly focus on *microdialysis* – a technique recently introduced to environmental sciences (discussed further in 1.5).

1.4 Towards better methods of quantifying soil N availability

1.4.1 Soil Nitrogen Availability

Many studies of soil N rely on long-used methods of aqueous soil extraction, which samples dissolved and exchangeable N from soil samples. Although some variation exists (Ros et al., 2009), methods follow the following pattern (**Figure 1-3**): 1) destructive sampling of soils from field sites; 2) sieving of samples to remove large organic particulates and homogenise samples; 3) samples are shaken in a slurry with an extractant – either deionised water (dH_2O), or a salt solution (e.g. KCl, K_2SO_4), with liquids collected via filtration or centrifugation for N analysis.

Figure 1-3. A simplified example of a soil extraction method. In the final step, salt extractant concentrations can vary, as can the type (KCl and K_2SO_4 are both commonly used).

Although soil extractions are cheap to implement, each step introduces increasing experimental error that can bias estimates of plant-available N. For instance, the destructive harvest of soil from a field site can destroy fine roots, microbial cells and tissues, releasing cell contents and thus increasing organic N concentrations (Hobbie and Hobbie, 2013), with the same actions also possible during sieving and shaking. Additionally, DON present in soils can be made increasingly available to living

microbes, promoting mineralisation of DON, overestimating inorganic N and underestimating organic N (Rousk and Jones, 2010, Inselsbacher, 2014). Confounding factors arising from extractant choice, storage, drying, shaking time, and soil-to-extractant ratios can also greatly affect N yields within extracts (Jones and Willett, 2006). Ros et al. (2009) found that methodology among 127 studies of soil N accounted for much of the variation in extractable organic N estimates – far greater than environmental or management factors. This highlights the need for alternative methods of sampling soil N capable of minimal disturbance to soil environments. Only then can we achieve N estimates which better reflect *in situ* conditions. We must also ask whether measures of absolute N are relevant to bioavailability, given the unavoidable situation of dynamic microbially-mediated turnover of N in soil.

An appropriate *in situ* sampling method must therefore provide minimal disturbance to soil structures and minimise N transformations by extant soil microbes. Weihermuller et al. (2007) outlines several methods which minimise soil disturbances to various degrees, with a focus on sampling soil water – among them, tension lysimeters have gained some use (Andersson, 2003, Jämtgård et al., 2010). Variable in size and design, tension lysimeters sample soil water under negative pressure through application of a partial vacuum (Warren, 2014), and can be installed *in situ*. Microlysimeter designs can feature small probes (2.5 mm diameter) which can assist in further minimising disturbance in soils. Additionally, small pore membrane sizes (0.1 µm diameter) allows for exclusion of microbial cells, and thus sterile collection of soil water – minimising N mineralisation before analysis. However, the use of lysimeters require high soil moisture contents (at least 70% water holding capacity) for successful sample collection (Warren, 2014, Miró and Frenzel, 2005). As their mode sampling requires a negative pressure to generate a mass flow of soil water over the membrane, estimates may be biased towards the largest water-filled pore spaces, given that such spaces tend to empty in order of largest to smallest (Miró and Frenzel, 2005). Membrane selectivity may also present difficulties in interpretation (Wessel-Bothe et al., 2000), with absolute measurements likely requiring calibration by testing recovery of target compounds (Warren, 2014).

1.4.2 Enzyme activity, and the proteolysis 'bottleneck'

Given the 'bottleneck' role of depolymerisation in N supply, active enzymes related to the release of N from complex polymers can be considered key controllers of N availability within soils. A number of enzyme families exist that fulfil this ecological role, such as protease (cleaving protein), aminopeptidase (peptides), urease (urea), amidase (non-protein amine groups) and chitinase (chitin) (Caldwell, 2005, Sinsabaugh and Follstad Shah, 2012). As protein generally dominates organic N pools in soil, protease activity is often viewed as the most important gateway for N release into soil systems (Schimel and Bennett, 2004, Sinsabaugh and Follstad Shah, 2012).

Many methods of examining soil enzyme activity suffer from similar problems associated with soil N extractions – notably, a high degree of disturbance to soil environments during sampling and processing (Wallenstein and Weintraub, 2008). Because of this, such enzyme techniques are widely recognised as providing only a 'potential' measure of activity under optimum incubation conditions (Burns et al., 2013). *In situ* approaches that can quantify enzyme activity in intact soils are highly sought after, and has led to the development of method such as zymography (Dong et al., 2007, Spohn et al., 2013, Spohn and Kuzyakov, 2014, Hofmann et al., 2016), as well as genetic / transcriptomic (Garoutte et al., 2016) and proteomic approaches (Schulze et al., 2004, Alessi et al., 2017). Although each method has benefits and weaknesses (discussed further in Chapter 5), new methods which can investigate the dynamics of enzyme production and activity at microscale environments are highly desirable, as are methods which can differentiate between distinct soil enzyme locations – those free in soil solution, and those which are bound to soil surfaces (Wallenstein and Weintraub, 2008). Such efforts may provide clearer pictures of the roles of microbial and plant-derived enzymes and exudates have on nutrient availability in critical soil zones, such as a rhizosphere (Hinsinger et al., 2009, van Dam and Bouwmeester, 2016).

1.5 Microdialysis: an *in situ* method for exploring soil N dynamics

This thesis focuses primarily on 'microdialysis' – an alternative *in situ* technique which minimises soil disturbances, and samples soil solutes through diffusion alone. The technique is introduced in greater detail in Chapter 2. Briefly, microdialysis is centred around small probes (24 mm × 0.5 mm) which can be inserted into soil with minimal disturbance, with each probe fitted with a semi-permeable membrane (10mm length) with a designated molecular weight cut-off (MWCO). Although microdialysis and small microfiltering tension lysimeters may share some similar functions, one key difference is the method of sampling. Whilst tension lysimeters rely on negative pressure to 'suck' soil solution (soil water + solutes) surrounding the probe, microdialysis relies on passive diffusion of compounds, driven by a concentration gradient across the semi-permeable membrane (Miró and Frenzel, 2005), with the only exchange between the external solution and the internal sample being the solutes themselves. It is this reason that has allowed the technique to flourish in its original domain of biomedical research, allowing continuous monitoring of biological processes without disruption (Nandi and Lunte, 2009), and there is great potential for similar successes in soil research. As the technique measures a 'diffusive flux' of solutes in soil solution under near-undisturbed conditions, using probe dimensions similar to a plant root, we hypothesise that the technique provides a more relevant perspective of N availability in soil environments. The technique has already provided

surprising results, particularly the greater relative contribution of organic N to total N fluxes, compared to soil extractions that generally are dominated by inorganic N (Brackin et al., 2015, Ganeteg et al., 2017, Inselsbacher and Näsholm, 2012a, Inselsbacher et al., 2014, Oyewole et al., 2014, Oyewole et al., 2016). The greater presence of organic N in fluxes is thought to be a product of the minimal disruption of the soil environment which reduces mineralisation and artificial solubilisation of N (Inselsbacher, 2014, Rousk and Jones, 2010).

Figure 1-4. A microdialysis system being deployed *in situ* within the O horizon of a heath forest soil, near Umeå, Sweden.

However, further work is required to clarify these disparities between methods, and evaluate whether extractions and microdialysis provide information about the same N pools in soils. In Chapter 3, we compare both sampling techniques within the framework of a litter decomposition experiment with expected outcomes for N cycling and general availability – allowing for a comprehensive view of how extractions (KCl and H_2O) and microdialysis sample organic and inorganic N pools over a 100-day period. In Chapter 4, we explore whether soil type can influence yields of inorganic N, as sampled by both methods across 24 agricultural soils.

There is also interest in understanding how microdialysis can be applied to investigate a larger diversity of soluble molecules, and whether its *in situ* nature can exploited to explore dynamic soil processes mediated by soil microbes and plants. In Chapter 5, microdialysis is used to directly sample soil enzymes, and explores how the technique can be used to quantify *in situ* enzyme activity.

Given its laboratory origins, there is also a need to better optimise microdialysis for soil sampling – particularly to improve the concentration of N compounds in collected samples for more precise analysis of fluxes. In Chapter 6, we explore modifications to microdialysis membranes which may assist in increasing the amount of soil N we sample using the technique.

1.6 Research Objectives

1.6.1 Research Questions

My thesis investigates the following research questions:

1. Do relative estimates of N availability differ between microdialysis and extraction methods? **(addressed in Chapters 3 & 4)**

2. Is the microdialysis technique sensitive to N released during plant litter decomposition? **(addressed in Chapter 3)**

3. Can microdialysis be used to directly sample free enzymes in soil solution? **(addressed in Chapter 5)**

4. Can microdialysis be optimised to improve recovery of N compounds from soil environments? **(addressed in Chapter 6)**

1.6.2 Chapter Objectives

Chapter 2 provides background on theoretical and experimental considerations regarding the microdialysis technique, and reviews of the current state of microdialysis use in soil environments.

Chapter 3 compares the microdialysis and soil extractions to investigate N release during litter decomposition, with expected outcomes for N availability providing a baseline for comparison between methods.

Chapter 4 compares inorganic N availability in 24 sugarcane soils as quantified by microdialysis and soil extractions, and considers the role of soil parameters on measurements acquired by each method.

Chapter 5 explores the potential of microdialysis to sample soil enzymes, and whether the technique can differentiate between stabilised enzymes bound to soil surfaces, and free enzymes in soil solution.

Chapter 6 examines potential modifications to microdialysis probe characteristics that could help improve N concentrations in dialysates for improved analytical detection.

2 Microdialysis in soil environments

2.1 Introduction

Microdialysis is a passive sampling technique initially developed for biomedical research and used extensively in neurology, pharmokinetics and pathology (Bourne, 2003, Hammarlund-Udenaes, 2017, Miró and Frenzel, 2005, Nandi and Lunte, 2009). It remains as one of the only tools capable of high-resolution sampling of target solutes *in vivo*, in part due to the minimal disruption imparted to surrounding tissue structures, and the passive nature of the sampling process (Nandi and Lunte, 2009).

Over the past two decades, there has been increased interest in applying the microdialysis technique to the environmental sciences. It has been deployed successfully in both laboratory and field settings to sample a variety of compounds from waste water and soil solution, including saccharides (Torto et al., 2000), metal ions (Torto et al., 2002, Mogopodi and Torto, 2003, Miró et al., 2005), organic acids (Sulyok et al., 2005), and organic and inorganic nitrogen (N) (Inselsbacher et al., 2011, Inselsbacher and Näsholm, 2012b, Inselsbacher et al., 2014, Oyewole et al., 2014, Shaw et al., 2014, Brackin et al., 2015). The use of microdialysis is driven by a desire to circumvent many of the drawbacks associated with conventional sampling methods, such as single-time point manual sampling, sample clean-up, and sample degradation or transformation through extended handling (Miró and Frenzel, 2005, Rousk and Jones, 2010, Inselsbacher, 2014). Recent research – particularly investigations of soil nutrients such as N and phosphorus – have focused more closely on the potential for the technique to sample solutes under near-undisturbed soil conditions. Microdialysis of soils is used as a predictor of solute bio-availability at small spatial scales relevant to plant and microbial nutrition (Inselsbacher et al., 2011, Oyewole et al., 2014, Brackin et al., 2015, Demand et al., 2017).

The technique allows sampling solutes through the passive process of diffusion (Figure 2-1). This is facilitated by small microdialysis probes (often 0.5 mm in diameter), each fitted with a semi-permeable membrane, which can be installed into most soil environments with minimal disturbance to surrounding soil structures. Each membrane has a designated molecular weight cut-off (MWCO); 20 kDa MWCO membranes are often used for sampling soil N, but both smaller and larger cut-offs are available. Solutes within the soil solution diffuse across the membrane along a concentration gradient, induced by the perfusion of a solution behind the membrane (termed 'perfusate') using high-precision pumps. After the solute crosses the membrane, the constant flow of perfusate pushes the combined perfusate/solute solution (termed 'dialysate') out of the probe for offline or online analysis. The rate at which target solutes diffuse across the membrane is commonly termed a 'diffusive flux',

shown as the amount of solute collected per unit surface area of membrane, per unit time (for instance, nmol N cm^{-2} h^{-1}).

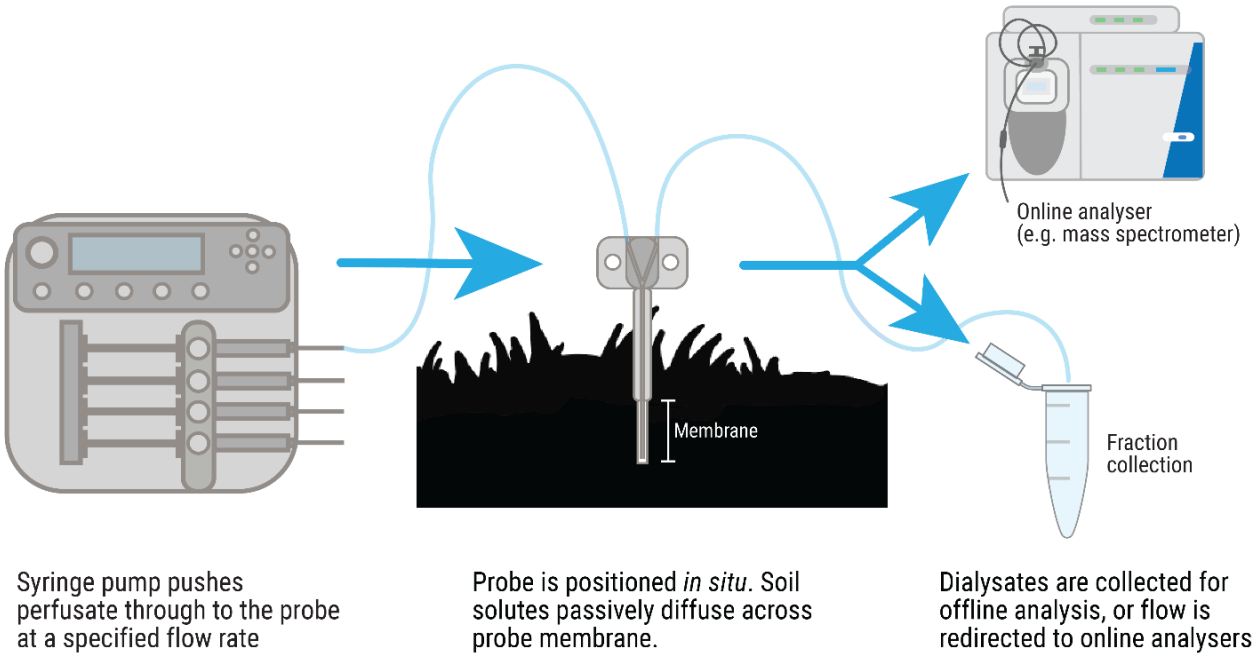

Figure 2-1. A typical microdialysis setup where dialysate is either immediately analysed (e.g. via mass spectrometer) or collected for later analysis.

Error! Reference source not found. Soil environments present unique challenges for microdialysis sampling, given the many attributes which are, in most part, absent from the biological applications for which the technique was initially developed for. Extra-cellular matrices of mammalian tissues, the original target of microdialysis, are relatively homogenous; in contrast, soils are highly heterogeneous with regards to temperature, moisture, mineral and organic matter content, texture, porosity and microbial activity, with differences between locations and soil types. Additionally, the very small scale at which the technique operates may exacerbate this variability. Much of the interest in microdialysis for sampling soils lies in the ability to integrate all these factors into a single measure of solute availability, under prevailing soil conditions.

With the use of microdialysis gaining momentum in the soil and plant sciences, there is a need to discuss its standardised use and interpretation of measurements. Therefore, the focus of this chapter is to explore the development of microdialysis as it relates to soil research and current state of use. Potential directions for future research and application are discussed in Chapter 6.

2.2 Theoretical and experimental considerations

As microdialysis relies on diffusion to sample target solutes, the effectiveness of the technique can depend on any number of biological, chemical and physical factors which modify the diffusion of a solute. This is a particular concern in a complex environment such as a soil matrix. As many soil solutes exist at low concentrations, an understanding of how these factors contribute to the sampling efficiency of the technique is important to choosing an appropriate experimental setup to balance sensitivity with the practicality of deployment.

2.2.1 Defining the term 'recovery'

In microdialysis studies, the term 'recover' is in many ways synonymous with 'sample' (verb) but can be interpreted in several ways depending on the context. In biomedical research, the term 'recovery' is derived from *in vitro* studies sampling solutes from a solution of known concentration, where recoveries are often reported as a proportion of the external concentration, as a 'relative recovery' (%). Studies that report an absolute mass transport of a solute across the membrane can sometimes be described as an 'absolute recovery' – however more recent soil studies have chosen to use the term 'diffusive flux' or 'influx' to describe the absolute recovery (e.g. Inselsbacher et al., 2011). In this chapter, we use 'recovery' to refer to absolute recovery, unless relative recovery is indicated.

2.2.2 Theoretical model of solute diffusion and microdialysis recovery

Diffusion is a process of molecular movement driven entirely by the stochastic thermal motion through a medium. Fick's first law outlines a general model for diffusion, where the diffusive flux of a solute (F) is primarily driven by the magnitude of its concentration gradient across an x-axis of a two-dimensional cross-section of a medium (dC/dx), and the molecular properties of the solute (ionic charge, size etc.) as determined by its diffusion coefficient (D), as shown in the following equation (Tinker and Nye, 2000):

$$F = -D\ \mathrm{d}C/\mathrm{d}x \qquad (1)$$

where the minus sign signals that solutes move from regions of high to low concentration. Diffusion coefficients can vary by orders of magnitude depending on the solute in question, and the medium it is moving through – for instance, although nitrate (NO_3^-) and potassium (K^+) have similar self-diffusion coefficients in water at 25 °C (NO_3^- = 19.0 × 10^{-6} cm^{-2} sec^{-1}; K^+ = 19.0 × 10^{-6} cm^{-2} sec^{-1}) (Li and Gregory, 1974), NO_3^- is an order of magnitude more mobile in soil solution at field capacity (NO_3^- = 1× 10^{-6} cm^{-2} sec^{-1}; K^+ = 1 × 10^{-7} cm^{-2} sec^{-1}) (Nielsen, 2017). As most soil studies are concerned with sampling ionic solutes, characteristics such as electrical charge and size impact on

the interactions with the solid phase of a soil matrix – a solute may bind to soil particles (such as clay or organic matter), slowing or halting lateral diffusion, only to be later released back into soil solution through ionic exchange. Additionally, solutes may be affected by the presence of concomitant ions which enhance or counteract diffusion, and can be further influenced by environmental factors such as water availability and soil temperature. Because of these interactions, more complex variations of Eqn. 1 can been generated to account for the resistive properties of soil environments (Tinker and Nye, 2000).

Although microdialysis sampling is driven by diffusion, there are several considerations which can subsequently affect the mass transport of solutes across the membrane. Bungay et al. (1990) has outlined a general theoretical framework for the microdialysis technique, outlining the contributing resistances to solute recoveries in biological tissues. The framework describes the (relative) recovery of a solute (E_d) as a function of several resistances to solute movement imposed by the sampling environment (R_{ext}), the physical characteristics of the membrane (R_m), and interaction with the dialysate (R_d), in addition to the perfusate flow rate (Q_p), as outlined in the following equation:

$$E_d = 1 - \exp\left(-\frac{1}{Q_p(R_d+R_m+R_{ext})}\right) \qquad (2)$$

In the context of soil environments, R_{ext} can include any factor affecting solute diffusion through the external environment such as water availability, interactions with soil particles and organic matter (Tinker and Nye, 2000), or microbial interactions which can immobilise or remove solutes from the solution (Schimel and Bennett, 2004). R_d includes resistances introduced by the perfusate, such as viscosity, temperature and solutes already present in the perfusate. *Rm* includes physical characteristics of the membrane – primarily, surface area – but also the membrane's effect on a solute's diffusion coefficient which is an integrated measure of a membrane's porosity and path tortuosity, and the degree of molecular interaction between the membrane polymer and external solutes (both target and non-target). These interaction effects are sometimes described as membrane 'fouling' and can lead to the partial or complete blockage of membrane pores which limits or prohibits diffusion of other compounds through the membrane.

When sampling from an aqueous solution it is expected that $R_{ext} = 0$, as barriers to mass transport of a solute are largely removed. This simplifies Eqn. 2 somewhat, allowing for the calculation of the combined effect of the membrane and dialysate on recovery, referred to as the permeability factor (*PF*), as follows:

$$PF = 1/(R_m + R_d) \qquad (3)$$

and can be calculated by a linear regression of -ln(1-E_d) against 1/Q_d. This has previously been used to estimate the efficiency of different membrane types on recovery (Torto et al., 1998, Buckley et al., 2017) and to predict the relative recovery of metal ions from solution (Miró et al., 2010).

2.2.3 Soil matrix resistances

For low molecular weight compounds sampled from complex environments (such as a soil solution), $R_{ext} >> R_m >> R_d$; that is, the environmental resistances to solute movement have a much greater influence than those imposed by the membrane and dialysate (Hsiao et al., 1990, Miró et al., 2010). In soil environments, this will likely be a combination of any of the following factors (Figure 2-2):

- Molecular interactions between a solute and the solid phase of the soil (mineral particles; organic matter);
- Moisture content (which may increase soil interactions and path tortuosity under drier conditions);
- Microbial (and enzymatic) interactions that immobilise or transform a solute in solution.

The degree of molecular interaction between solutes and the soil's solid phase is dependent solute characteristics – such as electrical charge and molecular weight – and the local physicochemical nature of the soil matrix. For instance, anions may have reduced soil interactions compared to cations, which are more likely to be adsorbed to negatively-charged soil sites. Larger macromolecules (such as proteins) can also be affected by increased drag resistances through solution, and may be hindered by smaller soil pores (Tinker and Nye, 2000); however even low molecular weight solutes have a non-linear relationship between their molecular weight and recovery via microdialysis (Inselsbacher et al., 2011, Jämtgård et al., 2018). Moisture content can affect the tortuosity of a solute's path through a soil matrix, reduce localised concentration gradients and increase the likelihood of adsorption to soil surfaces. Dryer soil conditions can also increase the viscosity of water layers surrounding soil particles, modifying the liquid-phase diffusion of all soil solutes (Tinker and Nye, 2000).

In addition to the physical soil matrix, soil microbes influence resistance by acting as both sinks and sources of solutes. Through consumption, microbes remove a solute from solution, and subsequently transform it through metabolic processes before re-release back into solution (Geisseler et al., 2010). Similarly, exoenzymes (enzymes released into soil solution by microbes) can act as transformational agents, degrading solutes (particularly macromolecules) and modifying soil concentrations (Sinsabaugh and Follstad Shah, 2012).

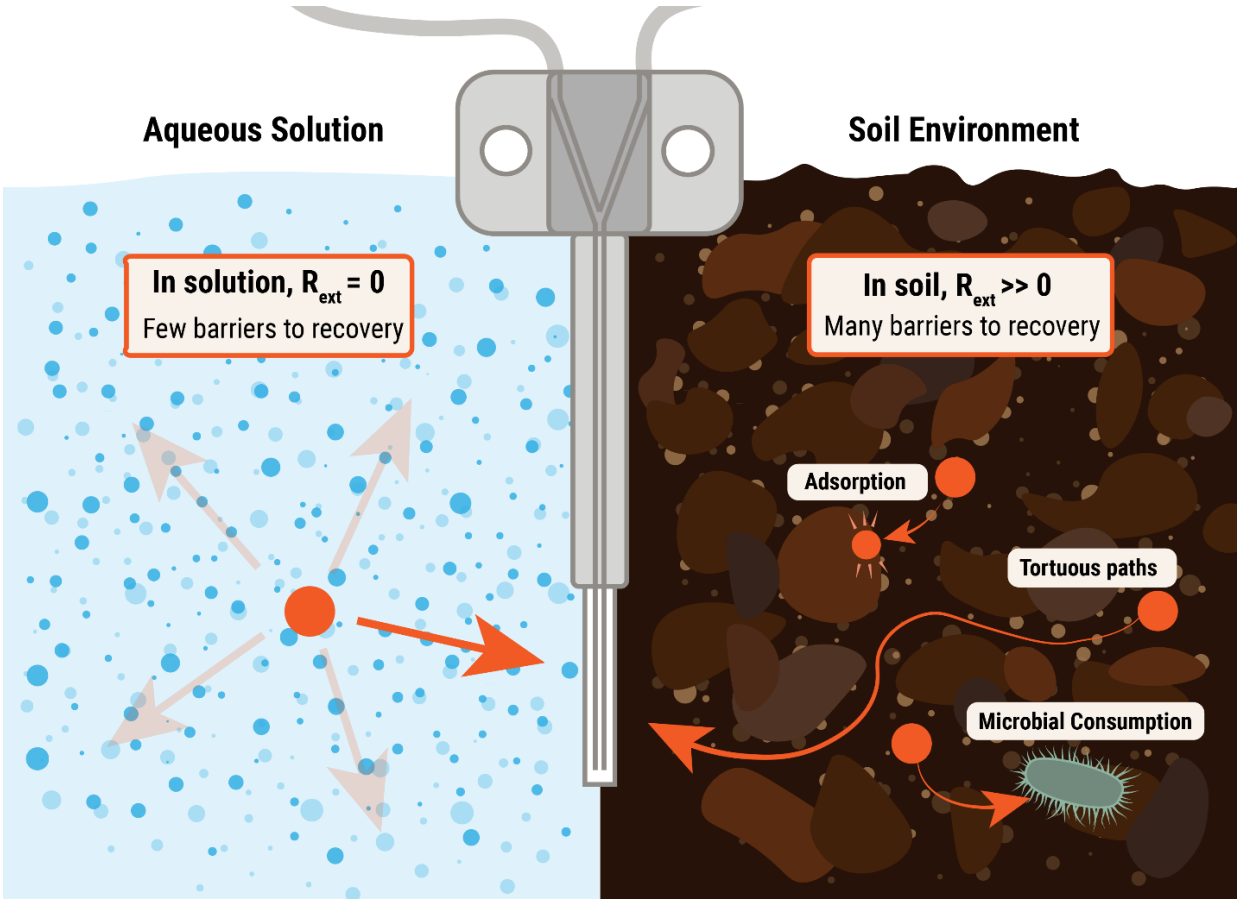

Figure 2-2. Resistances to solute movement in aqueous solutions and soil environments.

2.3 Optimising recovery with microdialysis

2.3.1 R_{ext} – the soil environment

By modifying the soil matrix to favour greater solute movement, it may be possible to improve solute recoveries from a soil environment. This could be achieved by increasing water availability to reduce solid phase interactions (Tinker and Nye, 2000), increasing soil temperatures to improve diffusion rates (Inselsbacher and Näsholm, 2012b), or sterilising soils to remove microbial interactions (Warren, 2018). However, for most *in situ* applications, significant soil manipulation is undesirable and negates much of the benefit of using the microdialysis technique.

2.3.2 Q_p – flow rate

Flow rate of the perfusate (Q_p) is often a primary consideration when attempting to optimise recoveries – as slower flow rates can significantly increase relative recoveries (Miró and Frenzel, 2004, Miró et al., 2005, Inselsbacher et al., 2011, Demand et al., 2017). As the endpoint of diffusion is an equilibrium (or steady-state) of solutes between the external solution and the inner membrane,

slow flow rates (approaching 0 µL min^{-1}) allow for maximum solute exchange times, and subsequently recovered concentrations will approach equilibrium with the external solution (Figure 2-3). However, extremely slow flow rates (< 1 µL min^{-1}) can be impractical for many experimental applications, simply because of the significant time required to acquire enough sample volume for analysis. In most microdialysis studies of soil N relations, a flow rate of 5 µL min^{-1} has been used as a compromise between sensitive recovery and optimal sampling time and volume (Table 2-2) – particularly for field sampling (Inselsbacher and Näsholm, 2012a, Inselsbacher et al., 2014, Brackin et al., 2015). However, slower flow rates (1 – 2 µL min^{-1}) have been used in more recent studies to increase sensitivity in relation to other environmental fluxes (Leitner et al., 2017a,b), or to optimise sensitivity and flow volumes for online analyses (Warren, 2018).

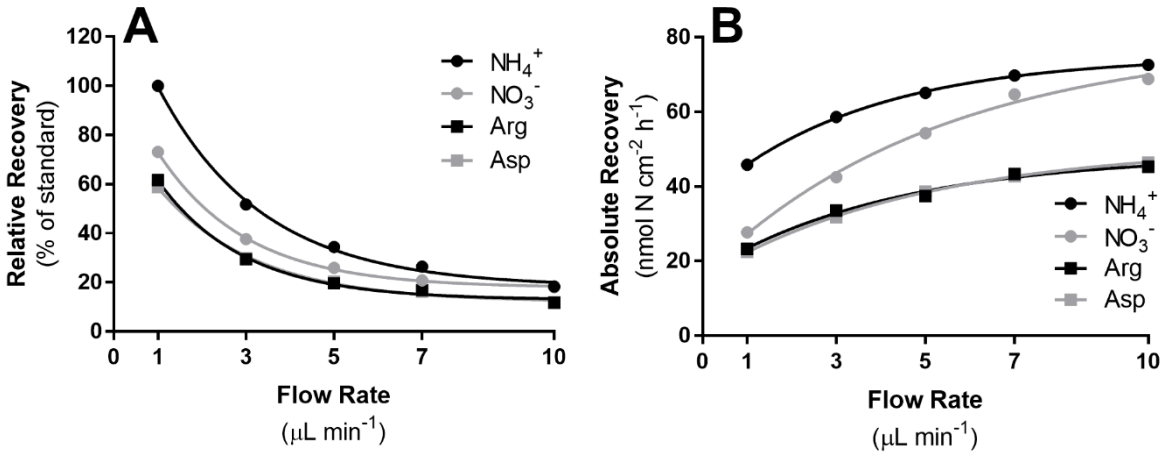

Figure 2-3. Effect of flow rate on the relative recovery (**A**) and absolute recovery (**B**) of ammonium (NH_4^+), nitrate (NO_3^-), arginine (Arg) and aspartic acid (Asp) from a stirred standard solution containing 100 µM of each N compound. Modified from Inselsbacher et al. (2011).

2.3.3 R_d – perfusate composition

Although perfusate resistances (R_d) may only have minor effects on recovery, studies across both biomedical and environmental fields have used a variety of perfusate compositions. They are chosen for their similarity to the ionic composition of the external medium, to minimise influences of osmotic pressure on the surrounding environment, and to reduce perfusate loss across the membrane which can subsequently affect dialysate concentrations (Stenken, 1999). Soils studies have used a range of perfusates, from simple electrolyte solutions such as $NaNO_3$ (Sulyok et al., 2005), KNO_3 (Miró and Frenzel, 2004, Demand et al., 2017) and $CaCl_2$ (Miró et al., 2005, Miró et al., 2010), to more complex artificial soil solutions (Warren, 2018), although many recent soil studies have used high-purity deionised water.

This has raised the question of whether fluxes of target compounds in these studies are significantly influenced by using water, as the recovery of some ions can be significantly modified by the presence (or absence) of other concomitant ions in soil solution and the perfusate (Miró and Frenzel, 2004, Demand et al., 2017). However, Warren (2018) found no differences in relative recovery of organic N compounds (glycine, arginine and betaine) using either water or an artificial soil solution. Tinker and Nye (2000) suggest that the diffusive flux of an ion may be close to its self-diffusion coefficient (i.e. rates of diffusion independent of concomitant ions) when the ion is a small proportion of the total solute pool, and when most of the solutes present have similar mobilities. Given that in most soil environments, many compounds such as amino acids or phosphate occur in low soluble concentration and have low mobilities, choosing water as a perfusate may have only a small influence, although this may be dependent on site and solute characteristics. However, perfusate choice may be of greater influence in recently-fertilised soils where the proportions of a few inorganic ions are artificially increased (Inselsbacher et al., 2014, Brackin et al., 2015) – but more work is needed to clarify the magnitude of these interactions in empirical measurements.

2.3.4 R_m – membrane characteristics

Membrane resistance (R_m) has been a target of several studies seeking to optimise recoveries by choosing optimal membrane characteristics. Microdialysis membranes are generally classed by their molecular-weight cut-off (MWCO, e.g. 20 kDa; or 100 kDa); that is, their ability to prohibit diffusion of 90% of molecules of a specific molecular weight (Drioli et al., 2015). The MWCO is often misinterpreted as a hard limit, however molecules at and above the rated weight can diffuse through the membrane, but due to physical hindrances rates of diffusion will be significantly slower rates than those below the MWCO (Ao and Stenken, 2006). However, this imparts a degree of size discrimination that makes the method attractive for excluding unwanted interfering compounds such as humic acids (Miró and Frenzel, 2004), or enzymes and microbes which can degrade solutes in dialysates. For most low molecular weight compounds, a larger MWCO will likely have little effect on recovery (Buttler et al., 1996, Torto et al., 1998), but will affect larger molecules such as proteins (Rosenbloom et al., 2005). However, larger MWCO membranes are subject to water loss, which can affect subsequent quantification of solute concentrations. Water loss from membranes can be negated by using additional push/pull set-ups, or negative hydrostatic pressures that maintain water pressure inside the membrane (Kjellström et al., 2000, Rosenbloom et al., 2005, Demand et al., 2017).

Membrane materials may also affect solute recoveries, given each are likely to have different resistive properties. A variety of polymers have been used for membrane construction (Hammarlund-Udenaes, 2017), although more recent soil studies have used commercially-available membranes constructed of polyarylethersulphone (PAES). The effect of membrane material on solute recoveries in soil

environments remains unclear, but it is known that membranes can be subject to 'fouling' – the binding of solutes to the physical structure of the membrane, which can decrease the permeability of a membrane through blockage of pores. Some studies refer to this decreased performance through fouling as 'Andrade effects' (Torto et al., 1998). These effects are difficult to quantify directly, but can be estimated by sampling from a standard solution of a known concentration before and after the fouling has occurred. Some performance differences between membrane materials – including the degree of fouling – have been noted within aqueous bioreactor environments with high temperature variability and high protein concentrations (Buttler et al., 1996, Torto et al., 1998). However, Hsiao et al. (1990) found that the resistive properties of the external environment, in this case biological tissues, were far more significant than those imposed by different membrane materials – which suggests that membrane composition may have little overall influence on recovery in complex environments such as a soil matrix.

Membrane length has been previously shown to increase solute recoveries in biomedical contexts (Tossman and Ungerstedt, 1986, Hsiao et al., 1990), by increasing the surface area in contact with the exterior environment. Several membrane lengths are used in soil (Table 2-2), although little is known about their contribution to improving solute recoveries. We explore the effect of increased membrane length on N recoveries in Chapter 6.

2.4 Quantifying solute recovery

The quantification of microdialysis-derived fluxes represents a point of divergence between soil and biomedical studies, with the latter primarily using microdialysis to determine the extracellular concentration of a target solute *in vivo* (Stenken, 1999, Kho et al., 2017). In contrast, soil studies have focused generally on absolute fluxes, with exception to studies that evaluate the technique for the relative recovery of a solute (Miró et al., 2010, Inselsbacher et al., 2011, Jämtgård et al., 2018, Buckley et al., 2017).

Both means of quantifying fluxes require some level of calibration – that is, an evaluation of solute recovery to ensure measurements are accurate. In biomedical studies, calibration is used to derive *in vivo* solute concentrations – that is, the external concentration of a solute in the extra-cellular matrix. Several methods of calibration have been explored during the development of the technique (reviewed by Kho et al., 2017). At the core of most methods is an evaluation of relative recovery (or loss) from a standard solution of known solute concentration, which is used to estimate an absolute concentration *in vivo* (Table 2-1). The *in vitro* methods represent the easiest pathway to calibration, its use has declined given that *in vitro* recoveries are not representative of *in vivo* conditions (Kho et al., 2017).

Table 2-1. Microdialysis calibration methods used in biomedical studies to estimate solute concentrations *in vivo* (Kho et al., 2017).

Calibration Method	Description	Assumptions & Requirements
In vitro	Relative recovery is estimated from a stirred aqueous solution of known solute concentration	*In vitro* recoveries are assumed to be identical to *in vivo* recoveries
Low flow rate	Low flow rates can provide near 100% recoveries. Extrapolation to zero-flow is used to estimate external concentration	Challenges occur with low volume yields and requires highly sensitive analytical instruments
No-Net-Flux	Probes are perfused with a series of different concentrations of solute, concentration where zero net flux occurs represents the external concentration	Time-consuming approach and changes to perfusate concentration may also change existing *in vivo* concentrations at sampling site
Retrodialysis	Probes are perfused with a known concentration of solute; external concentration is calculated from the relative loss of solute into external solution	Requires target solute to be absent from external solution, hence not suitable for studying endogenous compounds; also requires sufficient diffusion into surrounding matrix.

In contrast, most soil studies have used calibration for quality control of probes, to ensure comparable performance between probes during an experiment. *In vitro* calibration is commonly used, with a variation of *in vivo* calibration that involves 'spiking' a soil with standard solution to observe the resistive influence of the soil matrix on solute recovery (Inselsbacher et al., 2011, Jämtgård et al., 2018). This means that both *in vitro* and *in vivo* calibrations are independent of final flux measurements; instead, diffusive flux of a solute (D) is generally calculated as follows:

$$D = C_{dial} / A_m \times t \qquad (4)$$

where C_{dial} is the concentration of solute in the dialysate, A_m is the surface area of the membrane, and t is the sampling time.

2.5 Applications of microdialysis in soil environments

2.5.1 Diversity of compounds

2.5.1.1 *Chloride, metal ions and organic acids*

Miró and Frenzel (2004) first introduced the microdialysis technique to soil environments, exploring the recovery of chloride ions from a compost and potting mix. The authors explored several relevant aspects of the technique and showed that relative recovery decreased with increasing flow rate, and

that humic compounds were excluded (in solutions as high as 5 g/L humic acids). The technique was therefore suitable for *in situ* sampling of target solutes as well as a clean-up step that removes interfering compounds such as humic acids for downstream analysis.

Research on metal ions in soil found that microdialysis could discriminate between free and organically-bound metal fractions, and was sensitive to temporal changes in availability when extraction reagents were added to free the bound ions (Miró and Frenzel, 2005). Additionally, by combining estimates of soil diffusion coefficients (Olesen et al., 2001) with models of microdialysis recovery (Bungay et al., 1990), estimates of *in situ* concentrations of soil metal ions could be calculated (Miró et al., 2010). A further benefit of microdialysis is its effectiveness at lower water-holding capacities (WHC) than suction cups, which are another *in situ* sampling method with microdialysis working at 50% WHC compared to suction cups at 70% WHC (Miró et al., 2010).

Research exploring organic acid dynamics in soils found that spikes of oxalic and citric acid to soils, which mimic pulses of root exudation, were recoverable in time fractions, but the magnitude of recovery was likely influenced by adsorption processes in soils with higher calcium and organic matter content (Sulyok et al., 2005).

2.5.1.2 *Nitrogen*

Studies exploring soil N relations have comprised the largest component of research using the microdialysis technique in soil. Inselsbacher et al. (2011) first explored its potential to sample soluble N from soil solution including NO_3^-, NH_4^+, and amino acids, and this study has influenced subsequent research through choices of flow rate, probe type and methods of flux measurement and analysis. Microdialysis use in laboratory and field settings has explored aspects of N bioavailability in soils of natural and agricultural ecosystems.

As research on soil N has traditionally relied on aqueous soil extractions to estimate N pools, there is discussion regarding how microdialysis relates to these measures. It is generally accepted that conventional extractions can modify N pools within a soil sample by disturbing soil structures such as fine roots and hyphae (Hobbie and Hobbie, 2013), mineralising organic N, and increasing the proportion of inorganic N, particularly NH_4^+ (Rousk and Jones, 2010, Inselsbacher, 2014). This is of relevance when estimating the plant availability of different N forms in soil, as soil extracts may bias our perspective towards inorganic N (Paungfoo-Lonhienne et al., 2012).

Indeed microdialysis studies have shown that low molecular weight (LMW) organic N (amino acids have been the focus) contribute much to total N fluxes in forest and agricultural soils with up to 82% of the LMW N pool (Inselsbacher and Näsholm, 2012a, Inselsbacher et al., 2014, Oyewole et al., 2014, 2016, Brackin et al., 2015). The proportion of organic N contributes to total N fluxes depends

however on soil type and land management (Shaw et al., 2014, Brackin et al., 2015). Given the low-disturbance nature of the microdialysis technique, diffusive fluxes are potentially a more relevant measure of plant-available N than soil extraction. Following on from this notion, LMW organic N may be a dominant source of plant available N in many soils, with the notable exception of soils that have recently received mineral N fertiliser.

2.5.2 Relating diffusive fluxes to other ecologically-relevant fluxes

Microdialysis-derived diffusive fluxes are measured in similar units as nutrient uptake rates of roots, which allows exploring the balance between N and P supply to the root and plant uptake. Comparing N fluxes in sugarcane soils and the potential uptake rates of sugarcane roots, Brackin et al. (2015) and found that in urea-fertilised soils, inorganic N fluxes exceeded root uptake capacities 2 to 30-times for nitrate and ammonium, respectively, indicative of an oversupply of N. In contrast, organic-fertilised or unfertilised soils featured fluxes of inorganic and organic N that more closely matched the roots' potential uptake capacity, with the exception of nitrate fluxes in organic-fertilised soil which exceeded root uptake 5-fold. Chin et al. (2018) compared NH_4^+ fluxes released from organic wastes and estimated root uptake capacities, finding N released from poultry manures exceeded root uptake capacities for both sugarcane and sorghum up to ~300-fold. However, waste amended with clinoptilolite (a naturally-occurring zeolite) had significantly lower NH_4^+ fluxes that approached root uptake capacities. Oyewole et al. (2016) explored N fluxes in boreal forest soils, comparing diffusive fluxes with the potential N uptake of Scots Pine roots, and found that plant uptake is likely constrained by diffusive fluxes of N, rather than the uptake capacities of roots, with the exception of nitrate. Lastly, Demand et al. (2017) found that soil inorganic P (P_i) fluxes measured with microdialysis spanned similar P root uptake rates reported in the literature.

Leitner et al. (2017a) has shown how diffusive fluxes can be related to greenhouse gas emission (NO, N_2O) with quantification of gaseous releases simultaneously with diffusive fluxes after soil drying and rewetting, with high temporal sensitivity. Spatial sensitivity of microdialysis was also exploited by combining the technique with microCT imaging to observe NO_3^- accumulation, and depletion of NH_4^+ near the roots of maize plants when compared to bulk soil (Brackin et al., 2017). Both studies highlight the potential of applying microdialysis with other techniques capable of monitoring multiple processes non-destructively.

2.5.3 Microdialysis as a root simulator

Perhaps some of the most interesting applications of microdialysis have capitalised on unique aspects of the technique – given microdialysis probes share similar physical dimensions, and modes of nutrient exposure as plant roots. Oyewole et al. (2014) investigated the role of transpiration-driven

mass flow on the supply of N towards plant roots, primarily by using a perfusate containing dextran that modifies the osmotic potential of the probes. By adjusting the dextran concentration, a water potential of -0.1 MPa induced a mass flow of water over the membrane, which increased soil N fluxes, particularly NO_3^-. Further research applying this technique in the field has highlighted the likely importance of transpiration and mass flow in N acquisition by plants (Oyewole et al., 2017), revealing the potential of microdialysis as a tool to study interconnected soil-plant processes.

Another promising application of the microdialysis technique is retrodialysis – that is, the delivery of a compound to the soil solution by its inclusion in the perfusate. This approach exploits the diffusion of solutes both inwards and outwards across the membrane (Kho et al., 2017). As many rhizosphere processes are driven by the exudation of diverse organic compounds from roots, retrodialysis has great potential for exploring the effects of root exudation on nutrient availability and microbial interaction. In the first published study to explore retrodialysis in soils environments, Demand et al. (2017) delivered citrate to soils in an attempt to mobilise P_i, which generally has low soil mobility due to strong adsorption to positively-charged soil minerals (Hinsinger, 2001). Higher concentrations of citrate (1 mM) increased P_i fluxes in one of three soils, but it was noted that delivery of citrate produced greater variability in P_i fluxes overall – which may highlight the effectiveness of microdialysis to quantify soil heterogeneity, and the roles of nutrient-rich microsites in plant nutrition. Retrodialysis enables elaborate studies of soils, and many areas of plant and microbial research may benefit from application of the technique to explore exudate compositions, and their roles in shaping the soil environment.

2.5.4 Online analyses

Microdialysis is also capable of being paired with online analytical instruments such as separation columns (Kjellström et al., 1999, e.g. Jen et al., 2001, Jen and Liu, 2006), spectrometers (Miró et al., 2005, Warren, 2018) and flow-through detectors (Miró and Frenzel, 2004, 2005), allowing for continuous monitoring of solute fluxes at high temporal resolution. Coupling microdialysis with online analysers has become common in biomedical studies (Jin et al., 2008), and there are potential applications in soil research to study the rapid production and turnover of metabolites by both plants and microbes in important soil microsites such as the rhizosphere (van Dam and Bouwmeester, 2016).

Initial investigations of soil fluxes employed online setups with a potentiometric detector (for chloride ions) and electrothermal atomic-adsorption spectrometry (for metal ions), and demonstrated the potential for microdialysis to simplify analyses by acting as a clean-up stage to remove humic compounds which can interfere with some online instruments (Miró et al., 2005). For the first time in soil, Warren (2018) combined microdialysis with simultaneous mass spectrometry to explore the

dynamics of amino acid turnover in soils, allowing for precise measurements of ^{15}N-^{13}C L-alanine at one-minute increments, compared to 20+ minutes using conventional fraction collection (Inselsbacher et al., 2011, Leitner et al., 2017a,b, Ganeteg et al., 2017). Detectable concentrations of an added alanine standard decreased to below detection limits within 5 – 20 minutes; in contrast to sterilised soils, which still maintained detectable concentrations after three hours, graphically demonstrating the role of microbial consumption in rapid amino acid turnover in soils.

2.6 Conclusions

Microdialysis is a promising new technique for sampling solute fluxes in soil environments. The technique is not without its challenges in both deployment and interpretation but provides a tool with unrivalled spatial and temporal precision that could open doors to new areas of soil research previously too challenging to investigate with existing methods. Further discussion surrounding standardisation and interpretation of measurements are provided in Chapter 7.

Table 2-2. Scientific literature exploring the application of microdialysis in soil environments, by year of publication. Membrane materials, PAES = Polyarylethersulphone; PES = Polyethersulphone.

Author (Year)	Target Solutes	Environment	Flow Rate ($\mu L\ min^{-1}$)	Membrane Material	Probe Construction	MWCO	Membrane length	Perfusate	Remarks
Miró and Frenzel (2004)	Chloride	Potting mix; compost	2	Regenerated cellulose	Linear	15-25 kDa	30 mm	Water, KNO_3	Recoveries can be modified by use of electrolyte in perfusate (KNO_3) (from solution). Microdialysis can act as a clean-up stage, excluding even high concentrations of humic compounds from sample. First study to sample solutes from a soil-like environment - with ~100% recovery rates of chloride ions.
Miró et al. (2005)	Metal ions (Pb, Fe)	Soil	2	Regenerated cellulose	Linear	5 kDa	40 mm	0.01M $CaCl_2$	Microdialysis can discriminate between free and organically-bound metal fractions. Also sensitive to changes in availability after addition of extraction reagents (over a time-course of 220mins).
Sulyok et al. (2005)	Organic Acids (oxalic, citric)	Soil	2	Regenerated cellulose	Linear	5 kDa	30 mm	0.1M $NaNO_3$	Spikes of 100 µM oxalic and citric acids added to soils were detectable but differed between soil type and organic acid. Some soils (with higher calcium and organic matter) likely had increased adsorption/immobilisation of organic acids.
Miró et al. (2010)	Metal Ions (Cd, Pb, Cu, Ni)	Soil	2	Regenerated cellulose	Linear	5 kDa	30 mm	0.01M $CaCl_2$	Microdialysis can effectively sample solutes at water holding capacities as low as 50% (compared to suction cups, 70%). Application of Bungay model with constant slope model of Olesen allowed for prediction of solute concentrations from dialysate concentrations.
Inselsbacher et al. (2011)	Nitrogen (NO_3^-, NH_4^+, Amino Acids)	Solution, Forest and Agricultural soil	5 (soil); 1-10 (solution)	PAES	Concentric	20 kDa	10 mm	Water	First study investigating soil N fluxes with the technique. Versus lysimeters, MD showed higher proportions of amino acids, less inorganic N. Spatial variability across a 13 x 7 cm grid was highest with amino acids. Absolute depletions over time were greatest with amino acids, likely due to diffusive properties and molecular weight. Technique showed good sensitivity between two varying soil types.
Inselsbacher and Näsholm (2012a)	Nitrogen (NO_3^-, NH_4^+, Amino Acids)	Forest soils (fertilised, unfertilised)	5	PAES	Concentric	20 kDa	10 mm	Water	Fluxes estimate significantly more amino acids than extractions - 80% of plant-available soil N; NO_3^- and NH_4^+ only 10% each. Conclude that organic N dominates N supply in boreal forest soils.
Inselsbacher and Näsholm (2012b)	Nitrogen (NO_3^-, NH_4^+, Amino Acids)	Forest soil	5	PAES	Concentric	20 kDa	10 mm	Water	Soil temperature has a significant effect on subsequent N fluxes, and disproportionately affects compounds of with greater molecular weights.
Inselsbacher et al. (2014)	Nitrogen (NO_3^-, NH_4^+, Amino Acids)	Forest soils (fertilised, unfertilised)	5	PAES	Concentric	20 kDa	10 mm	Water	Short-term (hours) and long-term (weeks) N fluxes investigated. Some amino acid fluxes remained stable (contrary to hypothesised depletion) suggesting stability within some N pools. N fertilisation with NO_3^- was observable as a spike in fluxes within first 2 days after application - but quickly disappeared, suggesting rapid immobilisation or leaching.

Author (Year)	Target Solutes	Environment	Flow Rate ($\mu L\ min^{-1}$)	Membrane Material	Probe Construction	MWCO	Membrane length	Perfusate	Remarks
Oyewole et al. (2014)	Nitrogen (NO_3^-, NH_4^+, Amino Acids)	Solution, Forest Soil	1	PAES	Concentric	20 kDa	10 mm	Water, Dextran	Use of dextran to lower osmotic potential of perfusate - allowing for estimates of plant-available N supplied by mass flow, which substantially increased N recoveries in soils (particularly inorganic N); suggests mass flow has a significant role in N supply in boreal forest soils.
Shaw et al. (2014)	Nitrogen (NO_3^-, NH_4^+, Amino Acids)	Grassland soils	5	PES	Concentric	100 kDa	4 mm	Water	Fluxes and extractions (water, K_2SO_4) compared; fluxes were similar to water extractions, but magnitude of fluxes did not always reflect pool size estimated by extractions. Concluded diffusion is decoupled from concentration; i.e., affected by resistances to movement through soil.
Brackin et al. (2015)	Nitrogen (NO_3^-, NH_4^+, Amino Acids)	Agricultural soils (fertilised, organic-fertilised, unfertilised)	5	PAES	Concentric	20 kDa	10 mm	Water	Diffusive fluxes were compared with potential N uptake rates by sugarcane roots; NH_4^+ fluxes in fertilised soils were substantially larger than uptake rates, suggesting an N surplus vulnerable to loss. In unfertilised soils, amino acids dominate fluxes.
Oyewole et al. (2016)	Nitrogen (NO_3^-, NH_4^+, Amino Acids)	Forest soils (fertilised, unfertilised)	5	PAES	Concentric	20 kDa	10 mm	Water	Amino acids dominate plant-available N in boreal forest soils, regardless of fertiliser management. Uptake of N appears more constrained by the diffusive fluxes of N compounds rather than root uptake capacity, except for NO_3^-.
Brackin et al. (2017)	Nitrogen (NO_3^-, $NH_4^{+)}$	Agricultural soil	5	PAES	Concentric	20 kDa	10 mm	Water	MicroCT used to position probes adjacent to maize roots. NO_3^- was found to accumulate near roots, whereas NH_4^+ was depleted - suggesting soil processes such as transpiration-induced mass flow, and microbial transformations may affect spatial availability of N.
Buckley et al. (2017)	Nitrogen (NO_3^-, NH_4^+, Amino Acids)	Solution, forest soil	1-10 (solution, soil microcosms); 5 (field soil)	PAES (20 kDa); PES (100 kDa)	Concentric	20 kDa, 100 kDa	10 mm, 30 mm	Water	Increasing the surface area of membranes significantly increases recovery of N compounds from soil, particularly amino acids, with a potential bias towards negatively charged, and uncharged compounds.
Demand et al. (2017)	Phosphate	Solution, soil (forest, grassland, agricultural)	0.5 -4 (solution); 2 (soil)	PAES	Concentric	20 kDa	4 mm	KNO_3; KNO_3 + citric acid	First study investigating soil phosphate fluxes with the technique. Recoveries of P were independent of bulk concentrations - concluding the prominence of P hotspots. High concentrations of citrate (1 mM) in the perfusate increased mobilisation of phosphate, but not in all soils tested.

Author (Year)	Target Solutes	Environment	Flow Rate (µL min^{-1})	Membrane Material	Probe Construction	MWCO	Membrane length	Perfusate	Remarks
Leitner et al. (2017a)	Nitrogen (NO_2^-, NO_3^-, NH_4^+, Amino Acids)	Grassland soil	1 - 5	PAES	Concentric	20 kDa	10 mm	Water	Simultaneous measurements of soil N fluxes with gas (NO, N_2O) fluxes, investigating dynamics after soil drying and rewetting. N compounds were found to accumulate during soil drying, and their mobilisation after rewetting was linked to increased gas emissions.
Leitner et al. (2017b)	Nitrogen (NO_3^-, NH_4^+, Amino Acids)	Forest soil	1 - 5	PAES	Concentric	20 kDa	10 mm	Water	Technique showed sensitivity to changes in N fluxes in soils wetted after prolonged drought and demonstrated sensitivity to differences in N availability between drought treatments; in contrast to water extracts, which showed no differences.
Oyewole et al. (2017)	Nitrogen (NO_3^-, NH_4^+, Amino Acids)	Forest Soil (nutrient poor vs rich)	1	PAES	Concentric	20 kDa	10 mm	Water, Dextran	Amino acids dominated diffusive fluxes in both soils, but with mass flow (induced by dextran in perfusate) N flux increased by 9 times, saw an increased proportion of NO_3^-; concluded that transpiration-induced mass flow is a key component of N acquisition in boreal forest trees.
Ganeteg et al. (2017)	Nitrogen (NO_3^-, NH_4^+, Amino Acids)	Agricultural soil	5	PAES	Concentric	20 kDa	10 mm	Water	Used to demonstrate an increase in glutamine availability after the addition of a labelled standard solution to soil as part of a plant root uptake experiment.
Jämtgård et al. (2018)	Peptides	Forest and agricultural soil	1 - 10	PAES	Concentric	20 kDa	10 mm	Water	First study sampling peptides from soil solution using the technique. Peptides display similar diffusive properties to amino acids - depletion over time, and dependent on molecular weight. Technique can be paired with UPLC-MS for sensitive analysis.
Warren (2018)	Alanine	Forest soil	2	PAES	Concentric	20 kDa	10 mm	Water, artificial soil solution	Continuous sampling paired with online amino acid analysis via mass spectrometry. Artificial soil solution used as perfusate had no significant on amino acid recoveries. Method allowed for real-time measurements of $^{13}C/^{15}N$-alanine, undetectable 5-20mins after addition to soil, suggesting rapid turnover by soil microbes.
Chin et al. (2018)	Nitrogen (NH_4^+ and NO_3^-)	Organic wastes amended with sorbents	5	PAES	Concentric	20 kDa	10 mm	Water	Used technique to quantify dissolved inorganic N released from organic wastes amended with sorbents. Some sorbent materials showed high N adsorption capacity, significantly reducing N fluxes.

3 Microdialysis is a sensitive method for investigating nitrogen fluxes during litter decomposition

3.1 Introduction

Soil N availability is an important parameter for microbial function and plant nutrition. Knowledge of bioavailable N forms in soils, and the rates at which soil organisms and plants encounter them, is crucial to understanding soil function in natural ecosystems and improving soil N management in bioproduction systems. The factors that influence the bioavailability of N in soils are complex and dynamic. They include abiotic (e.g. temperature, water availability, soil physio-chemical properties) and biotic factors (e.g. microbial community, decomposition rates, N conversion by microbes and plants), with each affecting others in numerous ways (Vitousek and Howarth, 1991, Tinker and Nye, 2000, Schimel and Bennett, 2004). Determining N availability in soils is challenging when considering the diversity of N pools - a heterogeneous mixture of organic and inorganic N compounds of high to low molecular weight; some free in soil solution, and others bound to soil particles through ionic interactions and in exchange with the soil solution (Tinker and Nye, 2000).

Aqueous soil extraction is often the method of choice to determine N availability in soils. Water extractions quantify dissolved N in the soil solution, while salt extractions (e.g. KCl, K_2SO_4) detect additional N that is bound to soil particles through ionic interactions (Ros et al., 2009). A drawback of any extraction is their severe disruption of the soil environment during sampling and processing, releasing N from previously protected soil components such as fine roots and hyphae (Hobbie and Hobbie, 2013), and facilitating the transformation of N including mineralisation and nitrification (Rousk and Jones, 2010, Inselsbacher, 2014). Therefore, the accuracy of soil extractions for estimating bioavailable N could be improved, and *in situ* methods – such as tension lysimeters and ion exchange membranes and resins – are used to sample soil N with minimal disturbance (Weihermuller et al., 2007, Jämtgård et al., 2010).

As discussed in Chapter 2, microdialysis is an alternative *in situ* solute sampling technique that has recently been adapted from medical applications to environmental research (Miró and Frenzel, 2004, Öhlund and Näsholm, 2004, Sulyok et al., 2005, Miró and Frenzel, 2005, Inselsbacher et al., 2011). The microdialysis technique centres on thin probes featuring a semi-permeable membrane inserted into an intact soil with minimal disturbance to surrounding structures. Sampling of soil solutes occurs *via* passive diffusion, induced by the slow perfusion of water behind the membrane that allows solutes

surrounding the probe to move across the membrane along a concentration gradient. The sampled solute (termed 'dialysate') is collected for analysis and expressed as diffusive flux; the amount of solute that crosses the membrane over the sampling period (e.g. nmol N cm^{-2} h^{-1}). By minimising soil disruption, temporally integrating sampling and mimicking root function, microdialysis has potential to investigate fine-scale processes related to soil function, microbial activities and plant nutrition.

Previous investigators have proposed that microdialysis provides better estimates of plant-available N than soil extractions because diffusive fluxes relate directly to root surface area and uptake capacities (Oyewole et al., 2014, 2017, Brackin et al., 2015, 2017). Data generated *via* soil extractions and microdialysis can contrast, with the latter detecting a much higher prevalence of amino acids, particularly in unfertilised soils (Inselsbacher et al., 2011, Buckley et al., 2017, Jämtgård et al., 2018). As much of what we know about soil N cycling and availability has been estimated using various soil extractions (Lipson and Näsholm, 2001, Ros et al., 2009), this disparity warrants further evaluation.

Here we compared microdialysis and extractions to sample available N from a sugarcane soil amended with increasing rates of soybean litter (C/N: 12.6) and sugarcane litter (C/N: 29), incubated over a 100-day period. This experimental setup exposes both techniques to a framework of predictable N release with expected N immobilisation in soil amended with sugarcane litter, and N mineralisation in soybean-amended soil (Cabrera et al., 2005, Geisseler et al., 2010, Brackin, 2013). This allows comparing estimates of total N release, and the relative availability of N pools as detected by both techniques. These litter types represent a common input into Australian sugarcane soils. Legume break-crops are planted between sugarcane ratoon cycles, and under optimal conditions can supply as much as 250 - 300 kg N ha^{-1} from biological N fixation (Garside and Bell, 2001, Crews and Peoples, 2004). The wide adoption of green cane trash blanketing in the Australian sugarcane industry sees much of the post-harvest sugarcane residues left on fields and represents considerable C and N inputs with on average 5 t C ha^{-1} and as much as 50 kg N ha^{-1} (Robertson and Thorburn, 2007). Legume and sugarcane litter will therefore have impacts on short- and long-term N cycling in these systems, and exploring these dynamics with high temporal resolution may assist with management decisions to increase nitrogen use efficiency of sugarcane systems. As microbial activity acts as a gatekeeper to N release during litter decomposition (Schimel and Bennett, 2004, Geisseler et al., 2010), we also quantified microbial biomass, soil respiration and protease activity to contextualise N measurements.

Few studies have explored the effectiveness of the microdialysis technique for time-series analyses, exploring changes in N fluxes over extended sampling times. Recent work has demonstrated that continuous microdialysis sampling is sensitive to changes in N fluxes over short to medium timescales (hours to days) (Leitner et al., 2017a, b). Longer periods (days to weeks and months) of continuous sampling are likely to be subject to confounding issues surrounding the formation of

depletion zones around microdialysis probes, particularly for larger, or less mobile solutes (Inselsbacher et al., 2011). However, Inselsbacher et al. (2014) successfully sampled NO_3^- at discrete intervals over a 25-day period. Here, we extended the sampling period to 100 days and compared the presence of low molecular weight N compounds detected with microdialysis and conventional soil extractions.

3.2 Materials and methods

3.2.1 Soil and litter collection

Soil was collected from a long-term agricultural field under sugarcane monoculture at Maroochy River, Queensland **(**26°34'S, 153°00'E). The soil is classified as a sulfidic hydrosol (pH_{H2O} = 5.2; EC 71.8 µS/m)**.** Soil was collected from the top 10 cm, from the shoulder of a sugarcane row of a site that did not receive N fertiliser. Prior to commencing the experiment, soil was stored at 4°C for one month, sealed in a storage bucket to prevent water evaporation – periodically opened to limit anaerobic processes.

Fresh sugarcane leaf litter and whole soybean plants were collected from the same field, from rows under sugarcane-soybean intercropping. Litter was ground to 2 mm by centrifugal grinding mill (Rentsch ZM, 2000; Ultra Centrifugal Mill, Haan, Germany), and stored in airtight containers until use.

Table 3-1. Carbon and nitrogen properties of litter and soil. Numbers in brackets represent ± 1 SE.

Litter Type	**Soybean**	**Sugarcane**	**Soil**
% C	40.0 (3.17)	40.6 (0.32)	1.99
% N	3.17 (0.03)	1.4 (0.03)	0.22
C/N ratio	12.62	29	9.05

3.2.2 Experimental Design

Unsieved soil (50 g dry soil equivalent) was placed into a custom-made microcosm (Inselsbacher et al., 2009). Microcosms were pre-incubated at 27°C for one week at 70% water-holding capacity (WHC). Soils were mixed with litter at three rates of N with 0.02, 0.2 and 1 mg N g^{-1} dry soil, and control soils receiving no litter.

Table 3-2. Amount of C and N added to soil microcosms per litter treatment.

	Litter-N Addition (mg N g^{-1} soil DW)	Litter-C (Soybean) (mg C g^{-1} soil DW)	Litter-C (Sugarcane) (mg C g^{-1} soil DW)
Control	0	0	0
Low	0.02	0.25	0.58
Medium	0.2	2.5	5.8
High	1.0	12.6	29.0

Microcosms were arranged into a randomised block design in plastic tube racks and incubated at 27 °C and ~ 80% humidity for 100 days, with watering every 1-2 days to maintain 70% WHC.

3.2.3 Soil Sampling

For the soil extractions, eight microcosms of each litter treatment were destructively harvested at days 3, 7, 11, 17, 30, 60 and 100 after start of incubation. At time of harvest, soils were emptied into a clean plastic tub and hand-mixed to homogenise each replicate before sampling. Day 0 samples were taken immediately prior to litter addition at the beginning of the experiment, but were subject to the same mixing (without litter) before sampling. Five grams of unsieved soil was shaken with 10 mL of 1M KCl, or distilled H_2O for one hour on an orbital shaker. Extracts were then centrifuged at 4500 rpm for three minutes, with supernatants collected and frozen at -80 °C until analysis.

Over the duration of the experiment, 56 intact microcosms were sampled periodically using a microdialysis system. This setup consisted of a syringe pump (CMA 4004, CMA Microdialysis AB, Kista, Sweden) equipped with four micro-syringes (2.5 mL, SGE Analytical Sciences, Ringwood, Australia) connected to four microdialysis probes (CMA 20). These were connected to a refrigerated fraction collector (CMA 470) kept at 6°C. The probes each featured a polyarylethersulphone (PAES) membrane, 10 mm long, 0.5 mm diameter, and with a molecular weight cut-off of 20 kDa. Probes were inserted into the top of each microcosm and perfused with ultra-pure water (Milli-Q, Millipore Corporation) for one hour at a flow rate of 5 µL min^{-1} as per Inselsbacher et al. (2011). After sampling, dialysates were immediately frozen at -80 °C until N analysis, and probes were removed from soil microcosm until the next sampling day.

3.2.4 Measurements of microbial activity

Microbial biomass was measured using a $CHCl_3$ fumigation method (see Joergensen and Brookes, 1990), using 5 g of soil, and 24 h fumigation exposed to a $CHCl_3$ atmosphere, followed by extraction of N and C with K_2SO_4. Microbial biomass-N was measured using a ninhydrin-reactive N assay (Joergensen and Brookes, 1990). Microbial biomass-C was measured using a Shimadzu TOC-VCSH,

where total organic C was calculated by subtracting total inorganic C (carbonates and bicarbonates) from total C. A K_{EN} of 0.54, and a K_{EC} of 0.45 was used to estimate the fraction of microbial biomass-N and biomass-C mineralised, respectively (Brookes et al., 1985, Vance et al., 1987).

Protease activity was determined as per Kandeler (1996), using 0.5 g of unsieved soil, and with reaction volumes modified to fit within the well of a 300 µL 96-well microtiter plate.

CO_2 respiration was measured following the protocol of Brackin et al. (2013) with eight microcosms per litter treatment measured using a cresol red indicator in 1% agar (Rowell, 1995, Campbell et al., 2003), set in wells of a breakable 96-well microtitre plate (Costar EIA/RIA 1 X 8 Stripwell Plate, Corning, USA). Individual wells were fixed with temporary adhesive into the headspace of the microcosm tubes and sealed for 1-2 hours. Wells were replaced into the 96-well plate arrangement and read at 520 nm using a spectrophotometer. Measurements were made daily between days 0 and 20, every 2 to 4 days until day 57, and every 4 to 7 days until day 100.

3.2.5 Analysis of nitrate, ammonium and amino acids

Nitrate concentration of microdialysis samples and soil extractions (KCl and H_2O) were determined via the reduction of nitrate to nitrite with vanadium (III) chloride (VCl_3), followed by the Griess reaction (Miranda et al., 2001). Ammonium and amino acids were determined as per Holst et al. (2012), using an Ultra Pressure Liquid Chromatography (UPLC) Unit (Waters, Milford, USA), equipped with a BEC C18 1.7 µm 2.1 mm × 50 mm analytical column and a tunable UV detector set at 254 nm. Samples were derivatised using AccQ-Tag derivatisation kits (Waters, Milford, USA) and filtered (0.2 µm GHP AcroPrep TM 96 Filter Plates; Pall Life Sciences, Ann Arbor, USA) via centrifugation. Sample separation was achieved through a solvent gradient of a H_2O-based solution to 55% acetonitrile over the course of a 10 min run.

3.2.6 Statistical Analysis

Data from microbial activity assays and N measurements were analysed using one-way ANOVA, followed by Tukey's HSD post hoc test to determine significant differences between treatments (GraphPad Prism 6, Graphpad Software, Inc.). N measurements from litter treatments across all time were also analysed using a repeated measures one-way ANOVA, followed by Tukey's HSD post hoc test to determine significant differences between treatments (GraphPad Prism 6, Graphpad Software, Inc.). Pearson moment correlations were used to determine relationships within microbial activity measurements, and between microbial activity and N availability. Before each test, normality of distributions was tested using a D'Agostino-Pearson normality omnibus test (GraphPad Prism 6, Graphpad Software, Inc.); data that did not meet assumptions of normality were $\log_{10}$-transformed.

3.3 Results

3.3.1 Total nitrogen as estimated by soil extraction and microdialysis

Total N concentrations from KCl and H_2O extractions, and total N fluxes from microdialysis shared similar patterns across the 100-day incubation (Figure 3-1). Mean N concentrations and fluxes generally peaked at day 100 in all treatments. Sugarcane treatments had N concentrations and fluxes similar to, or significantly less than the no-litter control (Figure 3-1, top row). In contrast, all N sampling methods detected significant increases in N fluxes and concentrations in the soybean treatments over time, although this was most apparent with Soy Medium and High treatments (Figure 3-1, bottom row).

There were notable differences between sampling methods. The timing of N mineralisation differed in soybean treatments: N levels in KCl extractions were significantly elevated over the controls in the Soybean treatments from day 3 – 7, whereas N fluxes and H_2O extraction concentrations increased from day 11. Both fluxes and concentrations for Soy Medium and High treatments increased significantly between days 17 and 30, levelling to a plateau from days 30 through 100 (fluxes and H_2O extractions), or steadily increased (KCl and H_2O extractions).

3.3.2 Absolute N pools estimated by extractions and microdialysis

Absolute N fluxes and concentrations of inorganic N (NO_3^-, NH_4^+) and organic N (amino acids, AAs) are shown in Tables 3-5 to 3-7. Both concentrations and fluxes of AAs were generally greatest at the beginning of the experiment (days 3 – 17) and decreased between days 30 – 100. There were some differences in AAs between treatments; most notably, concentrations in KCl extractions were greater than controls in Cane High and Soy High treatments at day 3 ($p < 0.05$) (Table 3-6). In contrast, fluxes in Cane Medium treatments were significantly smaller than control soils at day 3, and by day 7 fluxes in all cane treatments were significantly smaller than controls and soybean treatments ($p < 0.05$) (

Table 3-7).

NH_4^+ was highest in the Soy High treatment and was often significantly greater than other treatments ($p < 0.05$) at most time points regardless of sampling method. KCl extractions sampled more NH_4^+

than H_2O extractions, which sampled very little with exception to the Soy High treatment (Table 3-5). NO_3^- concentrations and fluxes increased in all methods and treatments from day 30, but NO_3^- in soybean treatments was significantly greater than controls by day 11 with KCl extractions and microdialysis, and by day 17 in H_2O extractions.

Combined analyses of N pools across all time-points (using repeated measure one-way ANOVA) showed no differences between treatments for amino acids for any sampling method, but the Soy High treatment was significantly different from controls ($p < 0.05$) for NH4+ (KCl, microdialysis), NO3- (KCl, microdialysis) and Total N (all sampling methods).

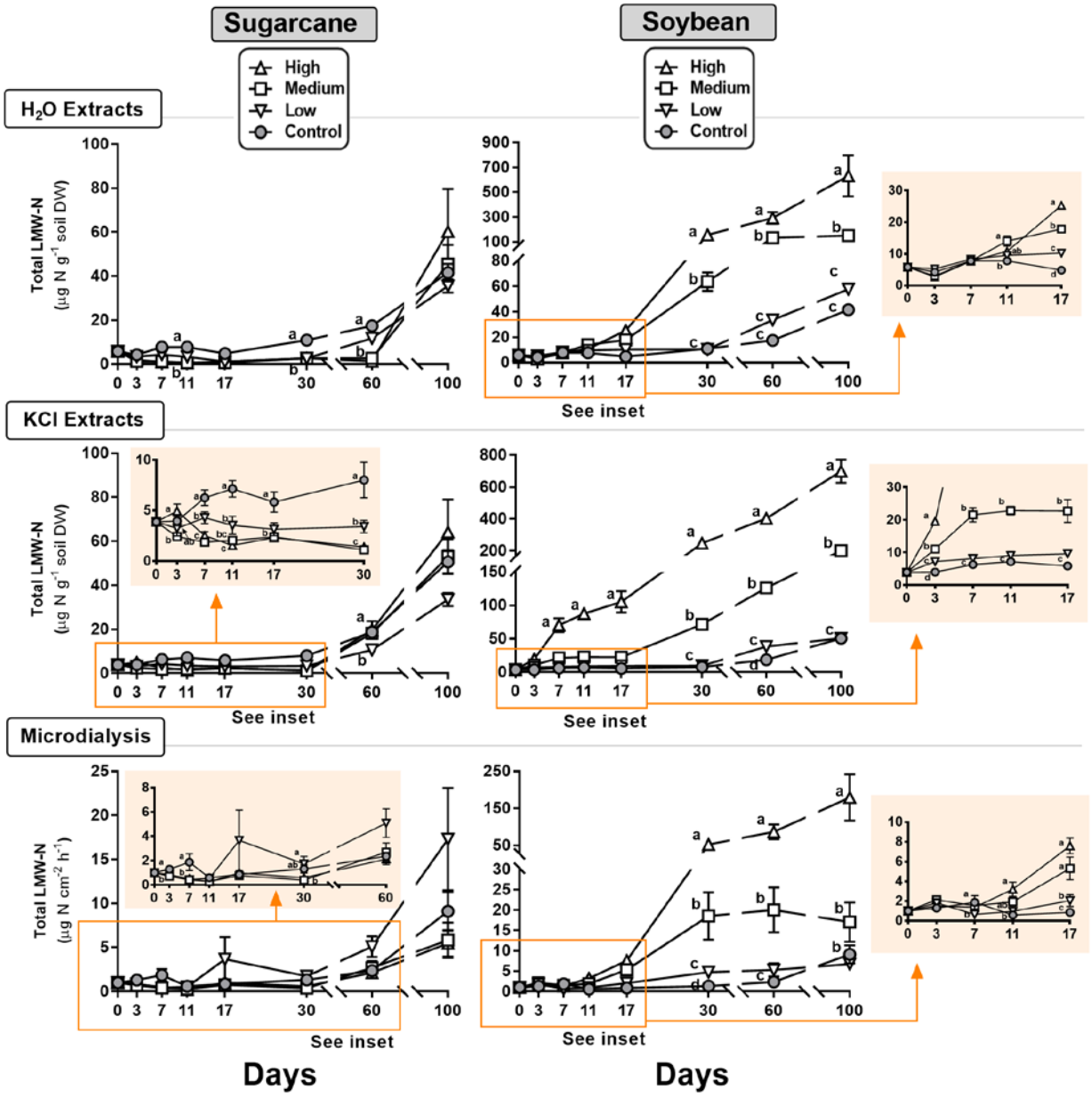

Figure 3-1. Total N concentrations (H_2O and KCl extracts) and Total N fluxes (microdialysis) as measured by each sampling method, from soils amended with three rates of sugarcane litter (left column) and soybean litter (right column), incubated for 100 days. Error bars represent ± 1 SEM; for each data point, n = 8. Letters denote statistical differences between groups at each time point.

3.3.3 Relative nitrogen pools estimated by extractions and microdialysis

The proportions of total N as inorganic N (NO_3^-, NH_4^+) and as organic N (amino acids, AAs) are shown in Figure 3-2 (additional detail for each treatment is presented in Figures 3-7 to 3-9). Some generalities were observed across methods. For instance, by day 100 NO_3^- was observed to be the most dominant N form regardless of treatment or sampling method. Additionally, Cane medium and high treatments observed greater proportions of AAs in the first 7 days after amendment. All methods also observed significant proportions of NH_4^+ in the Soy High treatment, however the magnitude of the contribution was much less pronounced in microdialysis fluxes.

However, each method generally sampled distinct patterns in N pool across the experiment. In H_2O extractions, NO_3^- was a dominant N form for many treatments, with exception to earlier timepoints of Cane Medium and High (dominated by AAs), and Soy High (dominated by NH_4^+). In Soy Medium and High treatments, KCl extracts were dominated by inorganic N, first as large pools of NH4 between days 3 and 17, and then as NO_3^- until day 100.

Fluxes uniformly observed high proportions of AAs during the first week of sampling, regardless of litter treatment (days 0 to 17, Figure 3-2, right column). However, when Soy Medium an High was quickly dominated by NO_3^- by day 11, AAs still dominated N pools until at least day 17, (day 30 in Cane Low), when N pools were then dominated by NO_3^- until day 100. NH_4^+ fluxes generally contributed very little to overall fluxes – although at its greatest, briefly contributed 43.9% of total N fluxes on day 30 in the Cane Medium treatment (Figure 3-12).

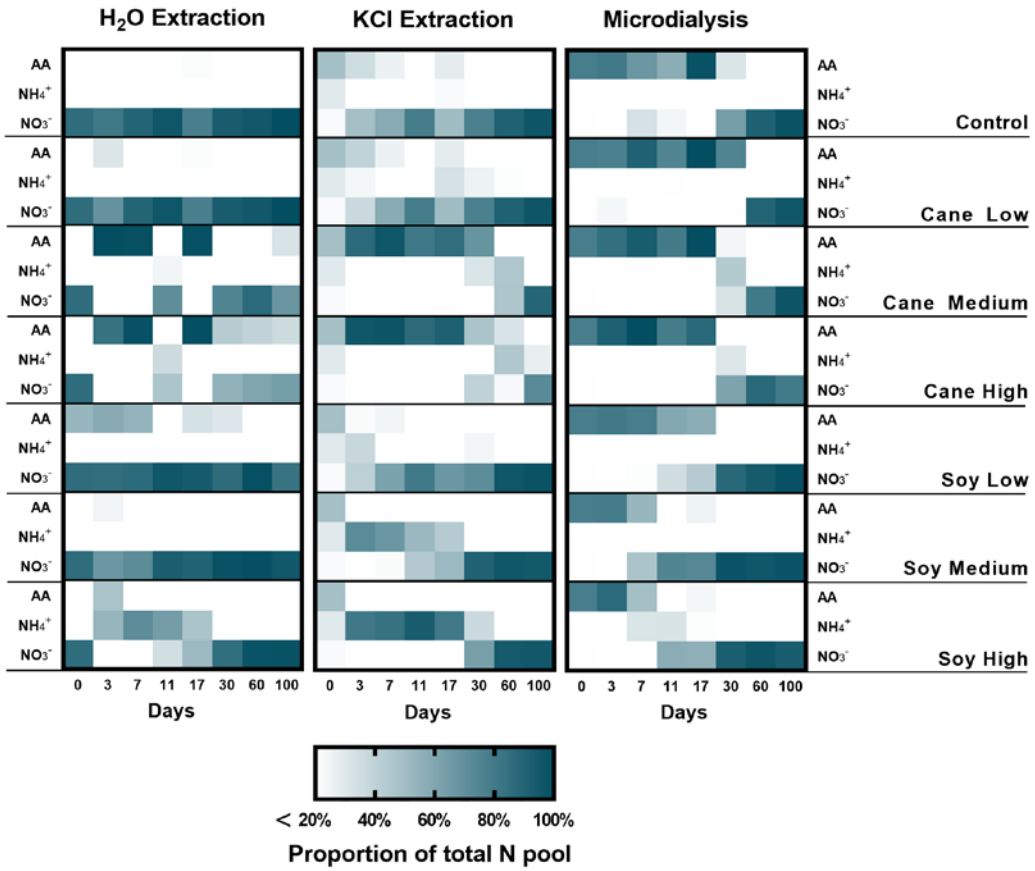

Figure 3-2. Heat map with proportions of nitrate (NO_3^-) ammonium (NH_4^+) and amino acid-N (AA) to total N (in %) in soils incubated with increasing rates of soybean and sugarcane litter over 100 days. White and lighter shades represent proportions closer to 0%, darker shades represent proportions closer to 100%.

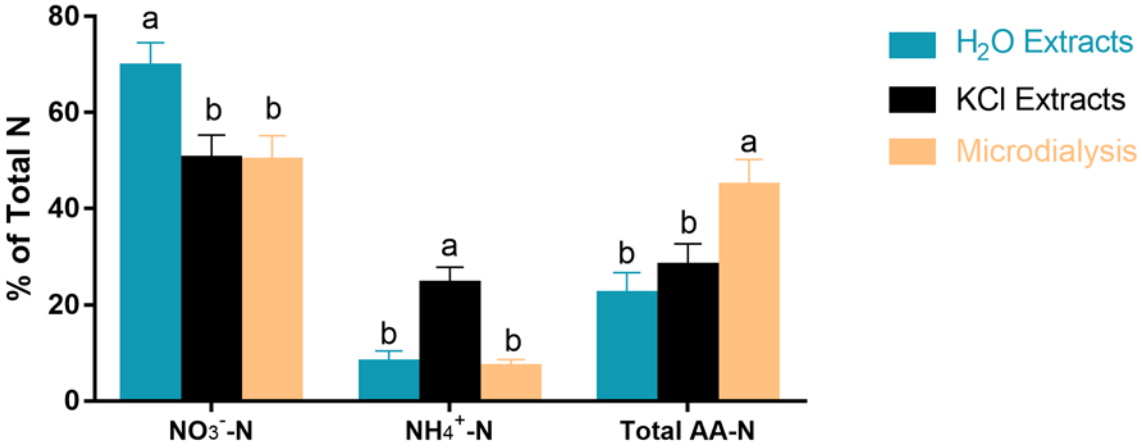

Figure 3-3. Average proportions (%) of total N represented by nitrate (NO_3^--N), ammonium (NH_4^+-N) and total amino acids (Total AA-N) across the entire experiment, as estimated by extraction methods (KCl, in black; H_2O, in blue) and microdialysis (in orange). Letters denote significant differences between treatments ($p < 0.05$), n = 50. Error bars represent ± SEM.

When pooling relative estimates of total N pools from each method, pairing values across methods for each time point, significant differences were found in estimates of total amino acids (Figure 3-3), where microdialysis were significantly greater than KCl and H_2O extracts (ANOVA, $F_{2,98} = 5.87$, $p < 0.034$; Tukey's Post-Hoc test – H_2O vs. KCl, $p > 0.05$; microdialysis vs. KCl, $p = 0.04$).

NH_4^+ proportions were also significantly different between methods, where KCl extractions were greater than H_2O extractions and microdialysis (ANOVA, $F_{2,98} = 14.52$, $p < 0.001$; Tukey's Post-Hoc test – H_2O vs. KCl, $p < 0.001$; microdialysis vs. KCl, $p < 0.001$; H_2O vs microdialysis, $p > 0.05$).

Finally, NO_3^- proportions in H_2O extractions,were significantly greater than other methods (ANOVA, $F_{2,98} = 5.023$, $p = 0.0078$; Tukey's Post-Hoc test – H_2O vs. KCl, $p = 0.02$, microdialysis vs. KCl, $p = p > 0.05$, microdialysis vs. H_2O, $p = 0.018$.

As differences in total N proportions may be dependent on day of sampling, we plotted the proportions of N pools as estimated by microdialysis, against values from the same time points and treatments in H_2O extractions (Figure 3-4) and KCl extractions (Figure 3-5), to observe whether any time or treatment were more prominent than others. NH_4^+ was generally estimated to be greater in KCl extractions, with the most consistent differences being in the Soy High and Medium treatments at days 3 to 17 (Figure 3-5, NH_4^+). In H_2O extracts, NO_3^- proportions were estimated to be greater for control, low and medium litter treatments between days 3 and 30. Total AAs generally contributed less to extractions than in microdialysis fluxes, but this was largely confined to soybean treatments (Figures 4 and 5, AAs). In contrast, Cane Medium and High treatments between days 3 and 17 were similar between methods, at close to 100% of total N. Additionally, at most time points towards the end of the experiment (particularly days 60 to 100), all methods estimated AAs approaching 0%. This is oppositely reflected in NO_3^- proportions, which approached 100% at the same time points (Figures 3-4 and 3-5, NO_3^-).

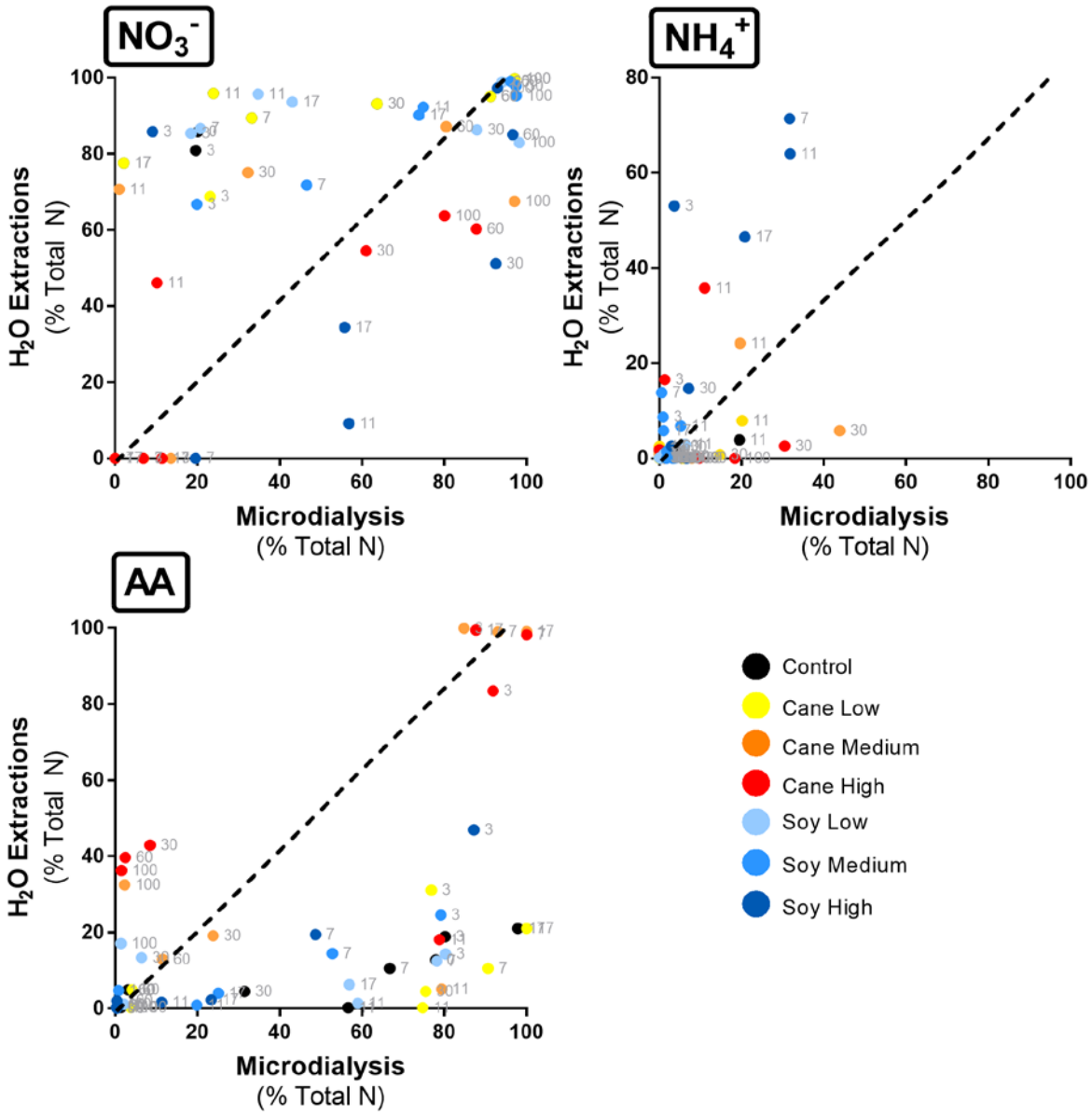

Figure 3-4. X-Y plots of total N proportions (% Total N) represented by nitrate (NO_3^-), ammonium (NH_4^+) and amino acids (AA) estimated by **microdialysis (x-axis) versus H_2O extraction (y-axis)** for the same litter treatments and harvest time points. Values representing cane treatments are shown in yellow-red; soybean treatments in light blue to dark blue, and controls in black. Numbers next to points indicate sample day. The dashed line represents equal representation of N by both sampling methods.

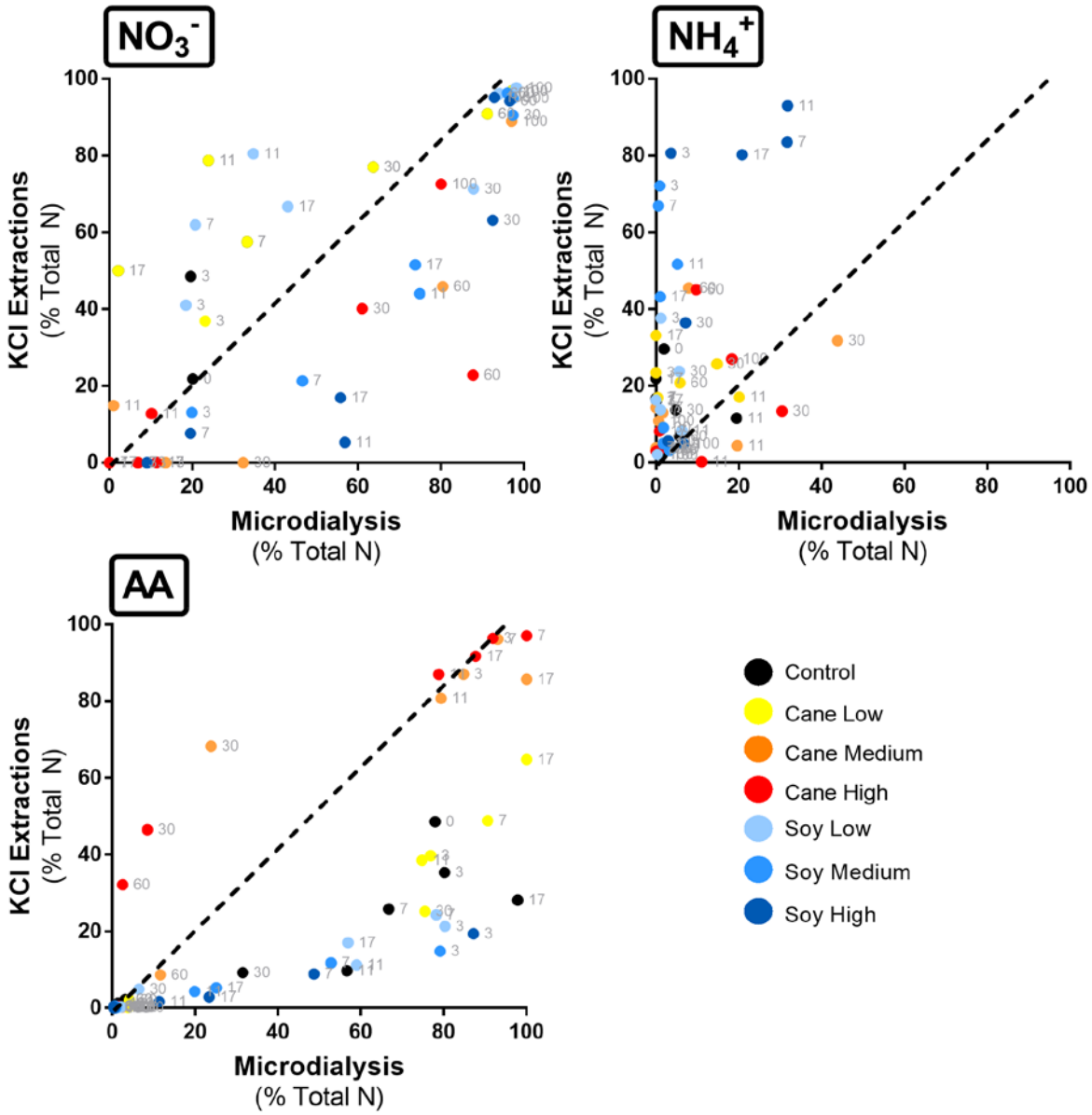

Figure 3-5. X-Y plots of total N proportions represented by nitrate (NO_3^-), ammonium (NH_4^+) and amino acids (AA) estimated by **microdialysis (x-axis) versus KCl extraction (y-axis)** for the same litter treatments and harvest time points. Values representing cane treatments are shown in yellow-red; soybean treatments in light blue to dark blue, and controls in black. Numbers next to points indicate sample day. The dashed line represents equal representation of N by both sampling methods.

3.3.4 *Individual amino acids as sampled by extractions and microdialysis*

To determine differences in individual amino acid sampling between microdialysis and extraction methods, we pooled amino acid fluxes and concentration values from all treatments on day 7, chosen as a day in which all sampling methods had high contributions of amino acids in samples (Figure 3-6). Notable differences include greater proportions of arginine, lysine (both positively-charged) and glutamine (uncharged) in KCl extractions, and greater proportions of threonine, proline and cysteine (uncharged), histidine (positively-charged) as well as aspartic acid and glutamic acid (negatively-charged) in H_2O extractions. Dialysates had greater proportions of valine, tyrosine and glycine (uncharged).

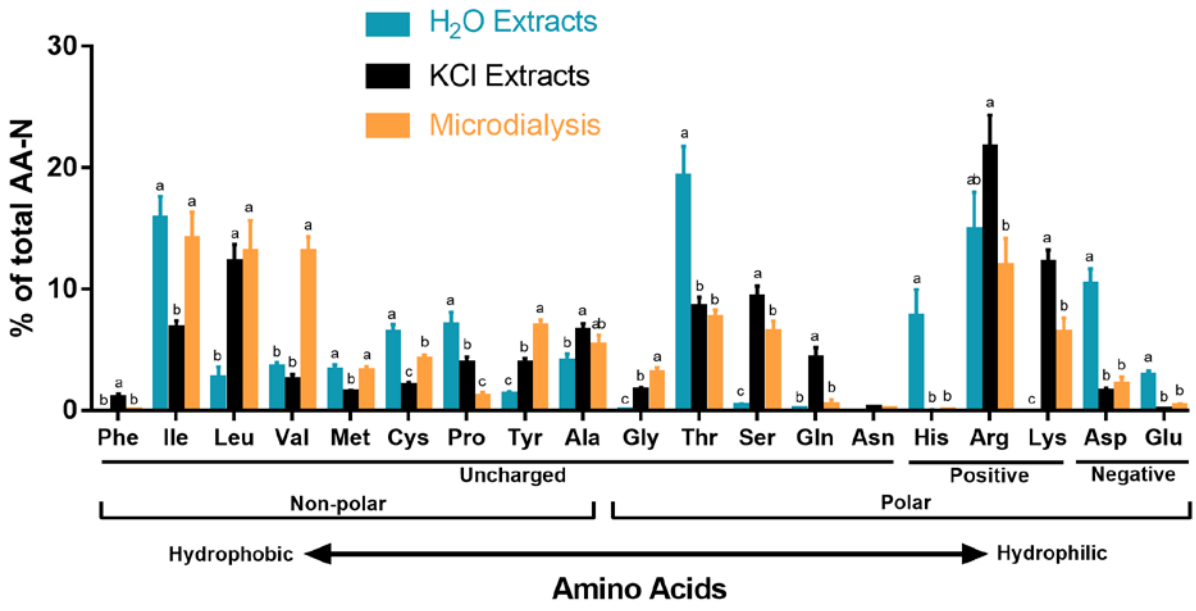

Figure 3-6. Mean proportions of total amino acids (total AA-N) attributed to 19 amino acids, as sampled by H_2O extractions (in blue), KCl extractions (in black) and microdialysis (in orange), sampled from soils 7 days after the addition of sugarcane and soybean litter. Means represent the pooled values across all treatments for each sampling method (n = 56). Error bars represent ± 1 SEM. Letters denote statistically significant differences between sampling methods ($p < 0.05$).

3.3.5 *Contribution of leucine to total amino acid fluxes and concentrations*

To compare the significance of a leucine-based signal observed in previous research with soybean litter (Buckley et al., 2016), we compared the contribution of leucine to total AA fluxes and concentrations (Figure 3-7). We observed a consistent contribution of leucine to fluxes in soybean litter treated soils (Figure 3-7, Microdialysis), which was significantly greater than controls days 3, 11 and 17, except for Soy High which decreased to control soil levels at day 11, but significantly

increased again by day 17. In contrast, cane treatments were to control during this timeframe. Beyond day 17, no consistent pattern of elevated leucine fluxes was observed. Extractions showed some differences in leucine proportions; for instance, H_2O extractions (Figure 3-7, H_2O Extracts) sampled greater leucine at day 3 in the Soy Medium treatment, and both sugarcane and soybean soils had a significant spike at day 30. Likewise, KCl extractions (Figure 3-7, KCl Extracts) had elevated leucine at with both litter types at days 3 and 7. For both extractions, no obvious sustained trend in leucine was observed.

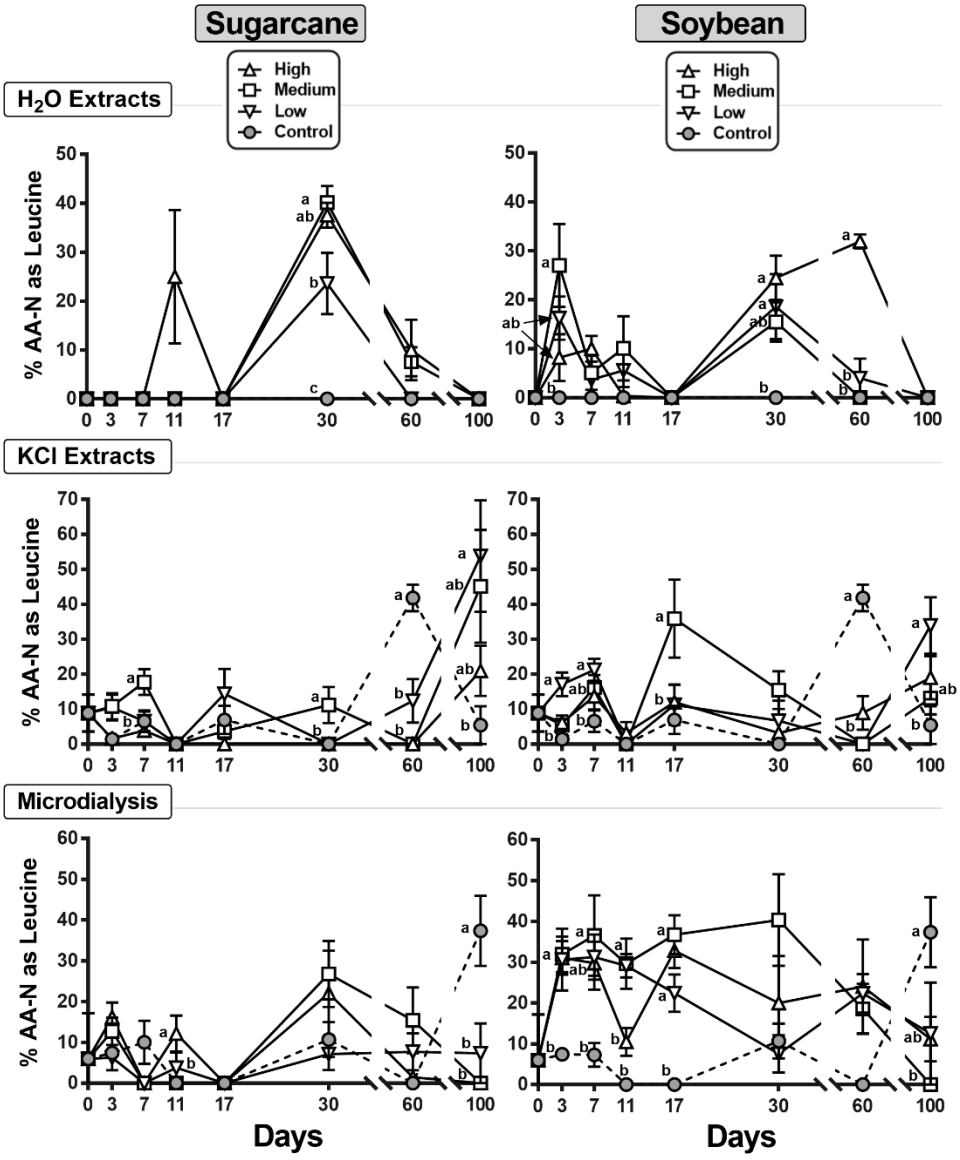

Figure 3-7. Contribution of leucine to total AA concentrations (H_2O Extracts, KCl Extracts) and total AA fluxes (Microdialysis), in soils amended with three rates of sugarcane litter (left column) and soybean litter (right column), incubated for 100 days. For each data point, error bars represent ± 1 SEM, with letters denoting significant differences ($p \leq 0.05$) at each time point.

3.3.6 Microbial biomass and activity

Measures of microbial activity were generally greatest during the first 17 days of incubation, followed by decreased activity from days 30 – 60 (Figure 3-8). The greatest increases in microbial activity were observed in Cane High and Soy High treatments, often followed by the Medium treatments.

Microbial biomass-C (Figure 3-8, MB-C) rapidly increased in the early stages of incubation, generally peaking at day 11, where the greatest biomass was observed in Cane High (520 ± 84 µg C g^{-1} soil DW). Soy High peaked at day 7 (473 ± 40 µg C g^{-1} soil DW) and dropped to control levels ($p = 0.06$) at day 11. Biomass-C in Cane High soils remained significantly greater than controls for much of the experiment, and only was similar to the control at day 100.

Microbial biomass-N (Figure 3-8, MB-N) also increased in most treatments during early stages of incubation, peaking at day 11, where Cane High reached 53 ± 5 µg N g^{-1} soil DW. At most time points (except day 60) biomass-N in Cane High was significantly greater than controls ($p < 0.05$), and at days 3 and 17, Soy High was also significantly greater than controls ($p < 0.05$).

Protease activity rapid increased in all high and medium litter treatments, reaching a peak at day 3 (Figure 3-8, Protease). At this point, Soy High featured the greatest activity (1474 ± 220 µg tyrosine g^{-1} soil DW h^{-1}) but did not peak until day 17 (at 1666 ± 55 µg tyrosine g^{-1} soil DW h^{-1}). Between days 3 and 30, protease activity Cane High and Soy High was significantly greater than controls (ANOVA – Tukey's Post-Hoc test, $p < 0.05$); and at times Soy Medium and Cane Medium also had significantly greater activity (Soy Medium – days 3 to 11; Cane Medium, days 11 to 17).

Respiration rapidly increased at the beginning of the experiment, with rates peaking at day 1 with the highest rates recorded in high litter treatments (Soy High – 5.37 ± 0.28 µg CO_2-C g^{-1} soil DW h^{-1}; Cane High – 4.6 ± 1 µg CO_2-C g^{-1} soil DW h^{-1}). Respiration rates in Cane High soils remained significantly higher than control soils ($p < 0.05$), except for day 100 where levels were no different. Soy High soils also featured significantly higher respiration rates, although significance varied from day 8 onwards – dropping consistently to control soil rates by day 29.

3.3.7 Relationships between microbial activity and N fluxes

All measures of microbial activity positively correlated with each other (

Table **3-3**), with the greatest relationships found between microbial biomass-C and biomass-N (r = 0.73, $p < 0.001$), and protease activity and CO_2 respiration (r = 0.65, $p < 0.001$). Additionally, protease activity correlated with Total AA-N estimated by extraction methods (Table 3-4), and these

relationships improved when values were restricted to days 0 to 7 which had substantial microbial activity. In contrast, protease activity did not correlate with AA-N fluxes estimated by microdialysis ($p > 0.05$). Additionally, microbial biomass-C and biomass-N did not correlate with N fluxes or N concentrations quantified by extractions ($p > 0.05$).

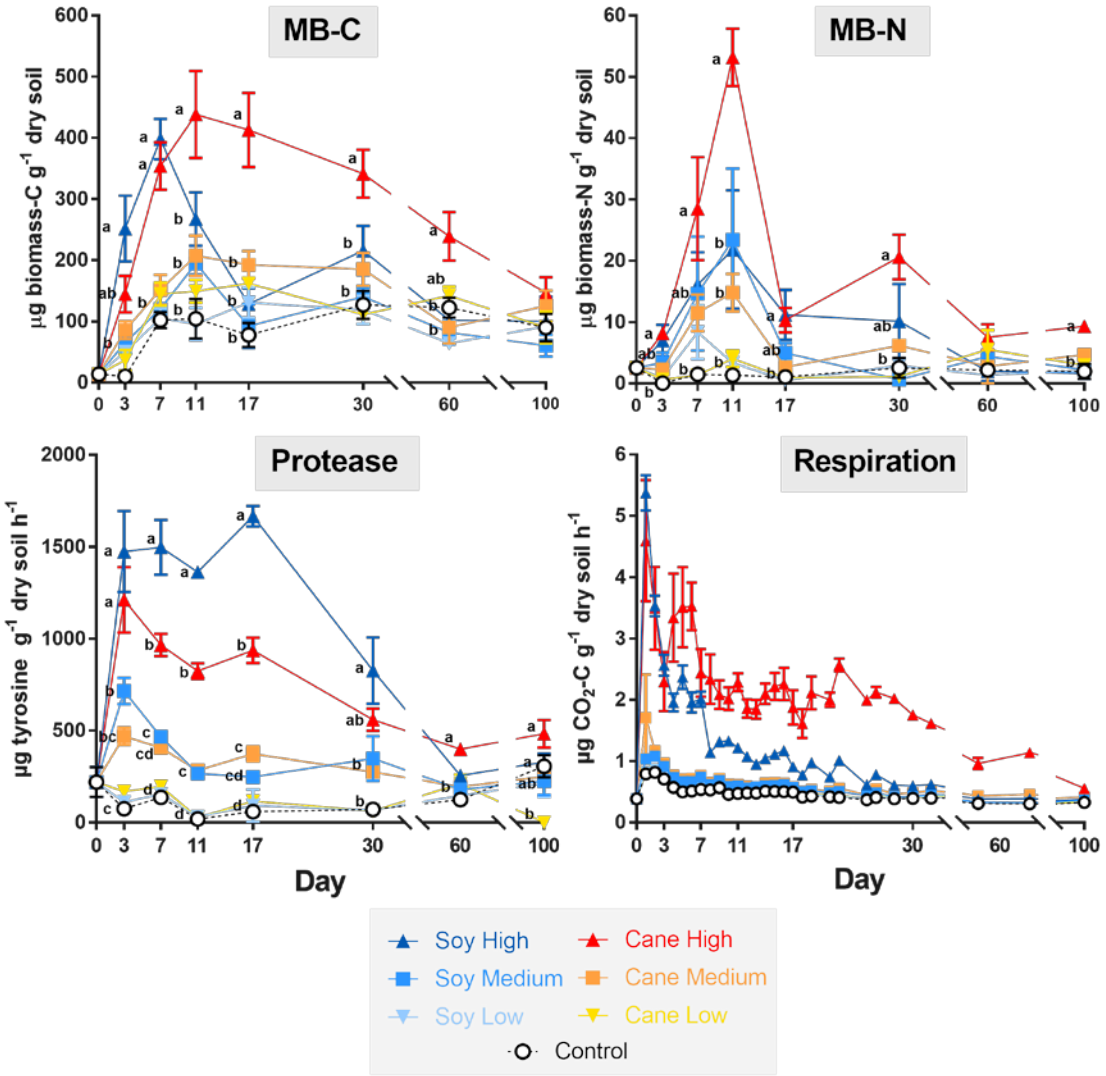

Figure 3-8. Measures of microbial biomass and activity in soils amended with three rates of soybean litter (soy; light to dark blue) and sugarcane litter (cane; orange to red), incubated over 100 days. Shown are microbial biomass-C (MB-C), microbial biomass-N (MB-N), potential protease activity (Protease) and CO_2 respiration (Respiration). For each data point, n = 8; error bars represent ± 1 SEM; letters denote statistical significance between treatments ($p < 0.05$) at each time point.

Table 3-3. Pearson product-moment correlations of microbial activity measurements. Numbers represent correlation (r) values for each comparison; for each, $p < 0.001$.

	log MB-N	**Respiration**	**log Protease**
log MB-C	0.73	0.53	0.47
log MB-N		0.53	0.61
Respiration			0.65

Table 3-4. Pearson product-moment correlations for protease activity measurements *versus* total amino acid-N (AA-N) estimated by extraction methods (KCl, H_2O) and microdialysis (MD), at three stages of the experiment (7 days, 30 days and 100 days). Values represent r-values for each correlation, asterisk denote significance * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

	Method	**log Protease**
log AA-N (7 Days)	KCl	0.76**
n = 15	H_2O	0.61*
	MD	0.34
log AA-N (30 Days)	KCl	0.54***
n = 36	H_2O	0.42**
	MD	0.18
log AA-N (100 Days)	KCl	0.32*
n = 49	H_2O	0.35*
	MD	0.15

3.4 Discussion

The questions of how much and what forms of soil N are available to plants and microbes is central to ecosystem function, and for the design of efficient agricultural systems that maximise N use and minimise N losses. To improve knowledge about which soil N pools are truly plant-available, sensitive tools must provide relevant measures, because widely used soil extractions have been criticised for artefacts and inaccuracies (Rousk and Jones, 2010, Hobbie and Hobbie, 2013, Inselsbacher, 2014). Microdialysis has been advocated as a sensitive tool that allows *in situ* passive sampling with minimal soil disturbance, using small probes that mimic a plant root in size (and partly in function). To advance understanding of the microdialysis technique, we used it alongside conventional soil extractions to sample the N released from the decomposition of two litter types with contrasting C/N ratio (low C/N soybean; high C/N sugarcane). Each method compared N release patterns and N pools in context of microbial activity. We find that soil extractions and microdialysis provide differing perspectives of N availability over a 100-day litter decomposition experiment.

While both method detect similar patterns of total N release (lower and higher N availability in sugarcane- and soybean-litter amended soils, respectively), the detected N forms differed. Microdialysis detected mostly a higher proportion of organic N (amino acids) and less inorganic N than soil extractions. Furthermore, microdialysis measured consistent changes to the amino acid profile, indicating the potential to monitor sensitive processes at small spatial scales such as the rhizosphere.

3.4.1 Inorganic nitrogen dynamics

Previous work has highlighted the greater relative contribution of amino acids to microdialysis fluxes in comparison to extractions (Inselsbacher et al., 2014, Brackin et al., 2015, Brackin et al., 2016, Oyewole et al., 2016, Ganeteg et al., 2017) suggesting that in many soils, amino acids are a dominant pool of available N to plants and microbes. We show that the major disparity between microdialysis and soil extractions mostly lies in the estimation of the contribution and timing of inorganic N, particularly during periods of high microbial activity and N turnover. Although all sampling methods predicted an endpoint dominated by NO_3^-, N pathways to this endpoint varied considerably, with extractions being mostly dominated by NH_4^+ and NO_3^-, with some exceptions during periods of high microbial immobilisation (particularly in Cane Medium and High treatments).

We suggest that the higher prevalence of NH_4^+ in KCl extractions is mostly a methodological artefact promoting organic N mineralisation and release/exchange of adsorbed NH_4^+ (Rousk and Jones, 2010, Inselsbacher, 2014). In contrast, microdialysis minimises disturbance to soil environments, preventing significant mineralisation of dissolved organic N, and avoids releasing NH_4^+ bound to soil surfaces or from biological structures, resulting in smaller contributions of NH_4^+ to fluxes. This is supported by similarly small contributions of NH_4^+ to H_2O extractions, as both methods likely sample mostly from the dissolved pool – however the minor contributions of amino acids may highlight the potential of H_2O extractions to promote organic N mineralisation (Rousk and Jones, 2010). This firmly questions how available NH_4^+ truly is in soils, and whether the use of certain sampling methods may misrepresent bioavailable N forms under undisturbed conditions.

The magnitude of KCl-extractable NH_4^+ was influenced by the type and amount of litter present – for instance, extractable NH_4^+ was far more prominent at higher rates of soybean than with sugarcane. These differences may be in part driven by short-term microbial processes; for instance, NH_4^+ in sugarcane treatments may be more tightly held, or quickly transformed by microbes to balance internal metabolic and enzymatic demands under high C availability (Schimel and Weintraub, 2003, Geisseler et al., 2010). In contrast, the relative abundance of N in soybean treatments may promote microbial release acquired NH_4^+ as a waste product (Drury et al., 1991, Kowalchuk and Stephen, 2001, Geisseler et al., 2010). Released pools could be adsorbed to soil surfaces, but much may only be loosely-bound (Steffens and Sparks, 1997, Nieder et al., 2011), easily mobilised or exchanged in extractions – particularly given than H_2O extractions (which would not likely desorb bound NH_4^+) also observed high proportions in these treatments.

It is this adsorption that may have produced the lag in mineralisation observed in microdialysis fluxes, as early-stage releases of NH_4^+ (days 3 to 7) were taken out of solution and effectively out of the

sampling pool. As potential exchange sites fill over time, this could result in a greater dissolved pool at a later stage (days 11 to 17). Of course, this period was also a time of rapid growth in microbial biomass and activity, and so the lag may also represent significant consumption of NH_4^+ to address microbial metabolic requirements during this period, with a resulting delay in significant nitrification until day 17. Both explanations suggest very different outcomes for the plant-availability of NH_4^+, with rapid microbial consumption implying high availability but rapid turnover (and competition), but with adsorption implying relatively low immediate plant availability, but with potential for exchange (Steffens and Sparks, 1997, Nieder et al., 2011, Braun et al., 2018) (Figure 3-9).

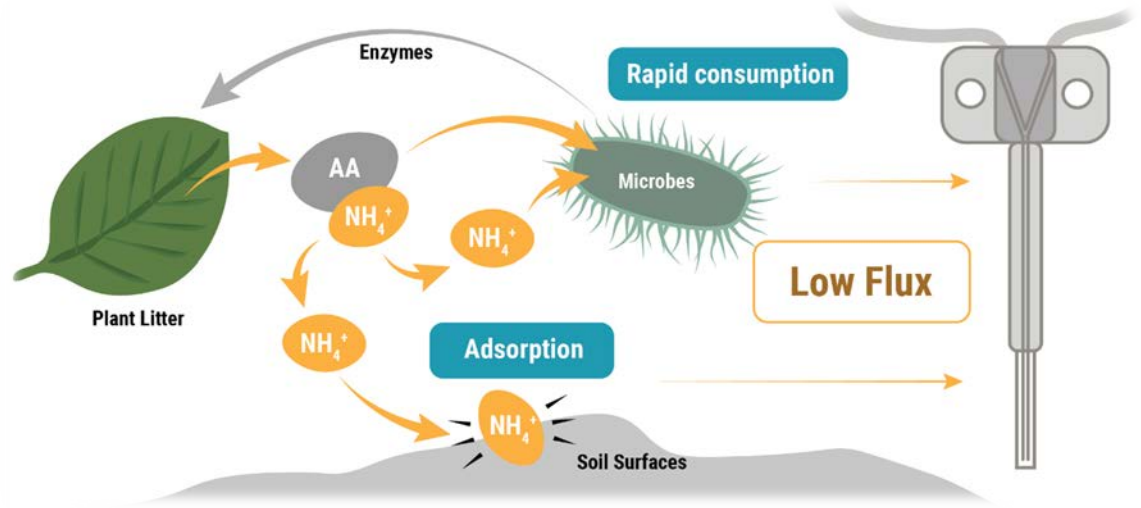

Figure 3-9. Two pathways which may result in low NH_4^+ diffusive fluxes as measured by microdialysis. Rapid consumption by microbes may result in immobilisation of NH_4^+, and abiotic adsorption to soil surfaces may reduce the pool of dissolved NH_4^+.

Both processes likely contribute simultaneously, but deciphering these contributions is difficult to clarify *in situ*. These will likely be the result of several factors: equilibria between NH_4^+ and other competing cations such as Ca^{2+} and K^+ (Chung and Zasoski, 1994, Evangelou and Lumbanraja, 2002), the spatial distribution and magnitude of exchange sites on soil particles such as clays or organic matter (Christensen, 2001, Skinner et al., 2001, Lehmann et al., 2007, Lehmann and Solomon, 2010, Braun et al., 2018), and the presence/activity of soil microbes (Kaiser et al., 2014, Kuzyakov and Blagodatskaya, 2015). Studies with ^{15}N-labelled organic and inorganic substrates have shown that the microbial biomass can rapidly acquire and retain the delivered N (St. Luce et al., 2014, Quan et al., 2018, Braun et al., 2018), but that abiotic fixation to mineral surfaces may also be significant (Hatton et al., 2012, Bimüller et al., 2013, Braun et al., 2018). *In situ* techniques such as microdialysis have great potential to be combined with labelling methods to provide a new perspective for such studies, with extractions (or similar *ex situ* methods) providing a measure of soluble and exchangeable

N, given context by microdialysis which integrates physical and biological factors affecting availability.

3.4.2 Amino acid dynamics

We also show that the contribution of amino acids to total N fluxes is mostly driven by microbial processes that shift inorganic N availability, given that the magnitude of these shifts was much larger for inorganic N forms during the experiment. For instance, amino acids will contribute least to total N fluxes during times of high mineralisation and nitrification (when NO_3^- dominates), and most during periods of high N immobilisation, as observed in sugarcane treatments during early incubation. The latter example is mostly the result of available inorganic N being rapidly used by microbes, presumably to balance stoichiometric demands for metabolic processes (Schimel and Weintraub, 2003, Geisseler et al., 2010), however it is interesting that amino acids appear mostly unaffected under higher C conditions. Such circumstances drive N limitation in microbial communities and are thought to increase demand for organic N forms by microbes to 'bypass' mineralisation (Geisseler et al., 2010, 2012, Pinggera et al., 2015). In sugarcane treatments, we observed decreases in fluxes – modestly at day 3 and strongly at day 7 – which may indicate some direct uptake of organic N was promoted, at least for a brief period during early stages of the experiment. Interestingly, there was no significant decrease observed in soil extractions during this time; in fact, KCl extractions of amino acids from Cane High treatments were significantly greater than controls on day 3.

This may be the result of masking through the dissolution of adsorbed amino acids, especially given that both extraction techniques sampled some amino acids differently to microdialysis. For instance, KCl extractions tended to measure greater proportions of positively-charged amino acids (particularly lysine and arginine), which would likely have been less available (Bartlett and Doner, 1988, Vieublé Gonod et al., 2006, Rothstein, 2010). Likewise, H_2O extractions saw greater proportions of uncharged (threonine, cysteine, proline) and negatively-charged (aspartic acid, glutamic acid) amino acids, which may have also been drawn from loosely-bound sources to mask any microbial depletion of free pools. As extractions of amino acids were significantly correlated with protease activity, this indicates that the fate of many amino acids after proteolysis could be adsorption, rather than dissolution in soil. As we expected microdialysis and H_2O extractions to sample from similar N pools (compounds dissolved in soil solution) it is interesting to observe such differences in amino acid sampling. H_2O extractions can potentially promote greater mineralisation during processing than salt extractions (Rousk and Jones, 2010), and so these differences may be the result of preferential loss or transformation of some amino acids, which would be of interest in future investigations.

3.4.3 Leucine fluxes are a consistent feature of short-term soybean decomposition

The stable measurement of leucine fluxes in soybean treatments also presents an interesting case study in how extractions and microdialysis differently quantify organic N cycling and availability. In previous work exploring N release from soybean and sugarcane litter (Buckley et al., 2016), we noted a consistent pattern of elevated leucine fluxes in soy-amended soils: between 38 – 57% of total amino acid fluxes after 17 days of decomposition. As leucine is enriched in soybean residues (Fontaine et al., 2001, Bøhn et al., 2014), we hypothesised that leucine may be a consistent outcome of soybean decomposition. In the present experiment we have replicated the phenomenon, observing a consistent pattern of greater leucine fluxes in soybean soils, although these much less stark, contributing 22 – 36% of total amino acid fluxes at day 17. However, we have also shown that this consistent pattern is not apparent in soil extractions, and this may be a further product of their transformational nature, promoting turnover of leucine to other N forms (Rousk and Jones, 2010, Inselsbacher, 2014), or releasing of adsorbed pools which could affect relative contributions (Ros et al., 2009).

As the half-life of leucine in soil is rapid – approximately three hours (Vieublé Gonod et al., 2006), such a consistent and relatively long-term shift in amino acid fluxes is notable, and suggests that decomposing litter can act as a substantial point source for specific N forms. Previous work has shown that leucine is a labile amino acid, given its neutral charge in most soils (including the current soil) reducing the potential for adsorption to soil surfaces (Vieublé Gonod et al., 2006, Rothstein, 2010). This will subsequently increase the likelihood of sampling with microdialysis (Inselsbacher et al., 2011), but may also increase the chances of interception by microbes, or via losses through physical leaching in soil water (Fischer et al., 2007, van Kessel et al., 2009). The influence of leguminous rotation crops on soil N has been well characterised, but mostly in regard to their role in increasing inorganic N availability through biological nitrogen fixation (Baggs et al., 2000, Garside and Bell, 2001, Crews and Peoples, 2004, 2005, Plaza-Bonilla et al., 2015, Büchi et al., 2015). Although our work supports the significant contribution soybean residues can make to inorganic N fluxes and concentrations (Crews and Peoples, 2004), we suggest there may be chemical characteristics of the litter itself (other than C/N or lignin/N) which may influence the temporal dynamics of N cycling (Kaiser et al., 2014). The potential to investigate the decomposition profiles of organic amendments using the microdialysis technique could provide further useful insights into how microbes truly interact with organic inputs, and how release rates could be better tailored match the uptake potential of plant roots *in situ* (Rovira et al., 2005, Rovira et al., 2008, Chin et al., 2018). There are also many research questions beyond soil N which could benefit from a sensitive, spatially-relevant tool, including monitoring of root exudates and microbial metabolites in the rhizosphere (van Dam and Bouwmeester, 2016, Oburger and Jones, 2018).

3.5 Conclusions

Our study identifies microdialysis as a sensitive *in situ* technique capable of measuring small but sustained changes in the forms of N released into soil during litter decomposition. The apparent differences between this technique and soil extractions may be in part due to the greater contact of microdialysis with the living soil environment, with minimal disturbance that avoids leakage of N from biological structures, and significant exchange with soil surfaces. Microdialysis also offers other unique advantages over extractions. For instance, microdialysis membranes provide a degree of sterile sampling through size-exclusion of soil microbes, minimising microbial conversion of organic N to inorganic after collection. Additionally, we show that repeated temporal measurements can be made without the need for destructive harvesting.

The dominance of organic N and NO_3^- in dialysates indicates that organic N initially released from litter is rapidly converted to NO_3^-, at least from the perspective of the dissolved soil N pool. A logical conclusion would then be that both N forms may be more relevant to plant nutrition than NH_4^+, and represents a small but significant shift in our understanding of plant-available N. However, the fate of NH_4^+ in soil is unclear, and warrants further investigation. We recognise that NH_4^+ fluxes are not always as low as in our study, and are affected by the ecosystem, soil characteristics, and N management (Shaw et al., 2014, Inselsbacher et al., 2014, Brackin et al., 2015). However, our findings that extractions over-estimate inorganic N aligns with previous work (Rousk and Jones, 2010, Inselsbacher, 2014). We may need to reconsider the assumptions made when deploying extractions, and the potentially contrasting findings when using *in situ* techniques like microdialysis.

3.6 Additional Figures and Tables

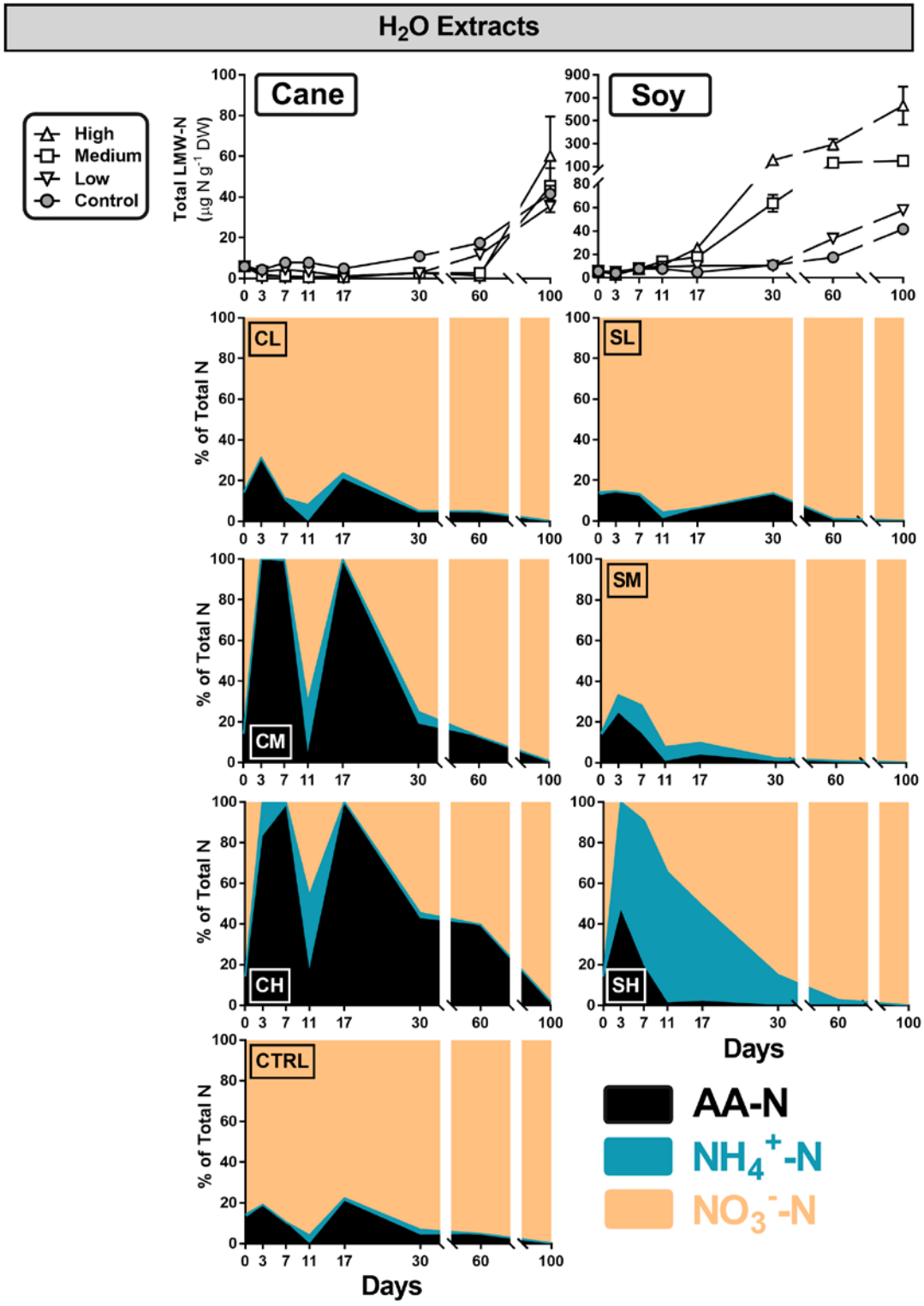

Figure 3-10. Total low molecular weight N (LMW-N) as sampled by H_2O extractions, from soil amended with three rates of sugarcane litter (cane) and soybean litter (soy). Also shown are proportions of total N as represented by amino acids (AA-N), ammonium (NH_4^+-N) and nitrate (NO_3^--N) for each litter treatment (CL – cane low; CM – cane medium; CH – cane high; SL – soy low; SM – soy medium; SH – soy high; CTRL – no-litter controls).

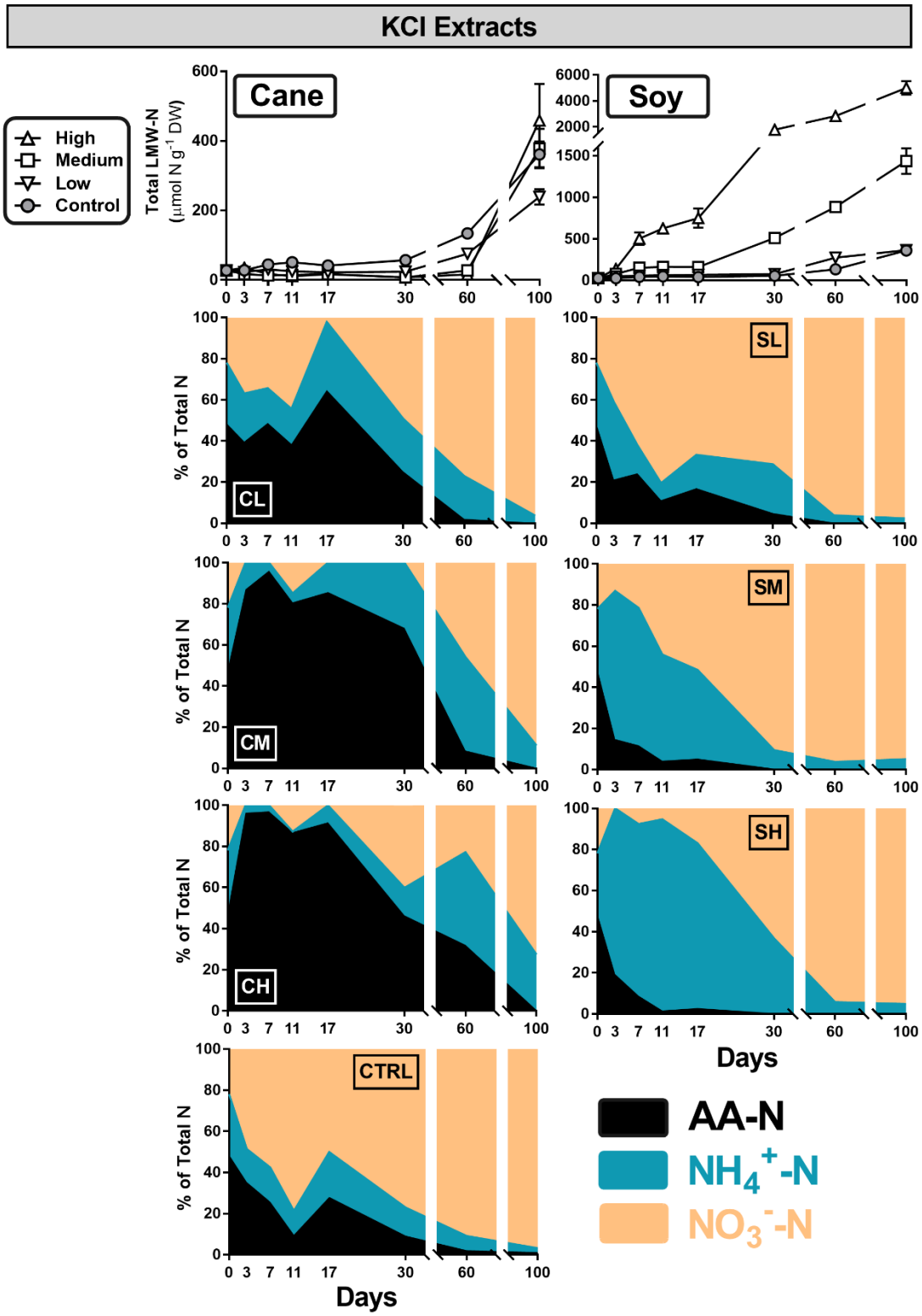

Figure 3-11. Total low molecular weight N (LMW-N) as sampled by KCl extractions, from soil amended with three rates of sugarcane litter (cane) and soybean litter (soy). Also shown are proportions of total N as represented by amino acids (AA-N), ammonium (NH_4^+-N) and nitrate (NO_3^--N) for each litter treatment (CL – cane low; CM – cane medium; CH – cane high; SL – soy low; SM – soy medium; SH – soy high; CTRL – no-litter controls).

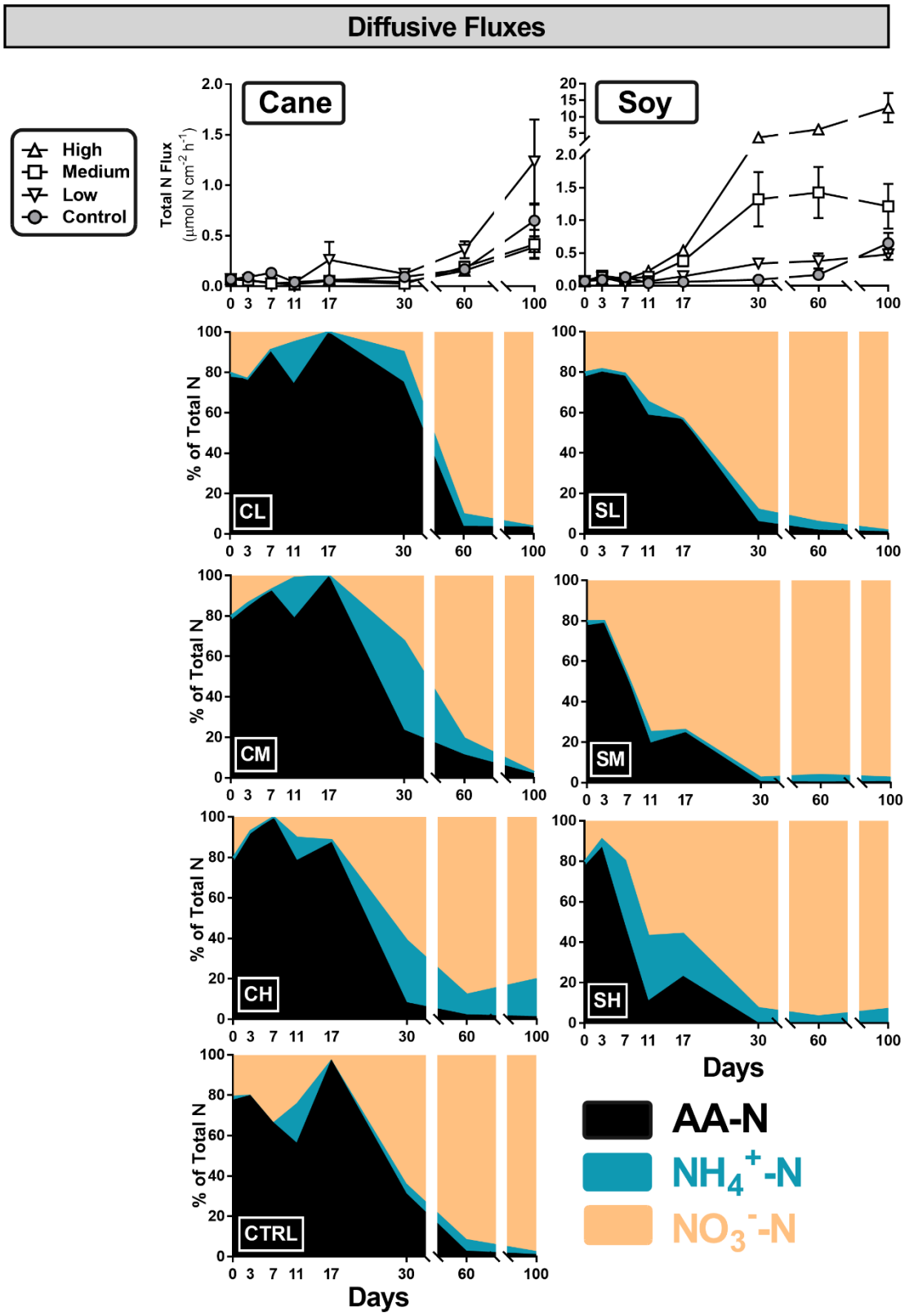

Figure 3-12. Total low molecular weight N fluxes (LMW-N) as sampled by microdialysis, from soil amended with three rates of sugarcane litter (cane) and soybean litter (soy). Also shown are proportions of total N as represented by amino acids (AA-N), ammonium (NH_4^+-N) and nitrate (NO_3^--N) for each litter treatment (CL – cane low; CM – cane medium; CH – cane high; SL – soy low; SM – soy medium; SH – soy high; CTRL – no-litter controls).

Table 3-5. Concentrations (μg N g^{-1} soil DW ± 1 SEM) of amino acids (AA-N), ammonium (NH_4^+) and nitrate (NO_3^-) and total nitrogen (Total N) as sampled by **H_2O extraction**, from soils amended with increasing rates of sugarcane litter (Cane) and soybean litter (Soy), or control soils with no litter. Samples were taken from destructive harvests at 8 timepoints over a 100-day incubation. Letters denote groupings of statistical significance (ANOVA, $p \leq 0.05$) between treatments in each N pool for a given timepoint, or for treatments across all time points (repeated measures ANOVA).

N Pool	Treatment	Day 0	Day 3	Day 7	Day 11	Day 17	Day 30	Day 60	Day 100
Total AA-N	**Control** [a]	**0.8** ± 0.17	**0.8** ± 1.0 [a]	**0.8** ± 0.17[a]	**0.02** ± 0.01 [a]	**0.64** ± 0.08 [a]	**0.3** ± 0.23 [a]	**0.83** ± 0.14 [ab]	**0.1** ± 0.02 [a]
	Cane Low [a]		**0.9** ± 0.2 [a]	**0.8** ± 0.14 [a]	**0.05** ± 0.02 [a]	**0.8** ± 0.16 [a]	**0.07** ± 0.01 [a]	**0.7** ± 0.16 [ab]	**0.2** ± 0.03 [a]
	Cane Medium [a]		**1.3** ± 0.16 [a]	**1.2** ± 0.2 [a]	**0.04** ± 0.01 [a]	**0.7** ± 0.2 [a]	**0.2** ± 0.03 [a]	**0.3** ± 0.03 [b]	**0.3** ± 0.1 [a]
	Cane High [a]		**1.3** ± 0.15 [a]	**1.1** ± 0.2 [a]	**1.0** ± 0.6 [a]	**1.2** ± 0.2 [a]	**0.3** ± 0.02 [a]	**0.7** ± 0.3 [ab]	**0.2** ± 0.06 [a]
	Soy Low [a]		**0.8** ± 0.1 [a]	**1.0** ± 0.2 [a]	**0.14** ± 0.06 [a]	**0.6** ± 0.2 [a]	**0.4** ± 0.2 [a]	**0.4** ± 0.03 [b]	**0.2** ± 0.07 [a]
	Soy Medium [a]		**0.8** ± 0.2 [a]	**1.1** ± 0.2 [a]	**0.13** ± 0.1 [a]	**0.7** ± 0.1 [a]	**0.4** ± 0.08 [a]	**1.2** ± 0.2 [a]	**0.12** ± 0.03 [a]
	Soy High [a]		**1.3** ± 0.2 [a]	**1.5** ± 0.1 [a]	**0.2** ± 0.1 [a]	**0.6** ± 0.2 [a]	**0.4** ± 0.02 [a]	**0.3** ± 0.02 [ab]	**0.2** ± 0.06 [a]
NH_4^+-N	**Control** [a]	**0.1** ± 0.03	**0.1** ± 0.005 [b]	**0.005** ± 0.0 [d]	**0.3** ± 0.05 [c]	**0.04** ± 0.01 [c]	**0.2** ± 0.03 [c]	**0** [b]	**0** [a]
	Cane Low [a]		**0.01** ± 0.005 [b]	**0.02** ± 0.01 [d]	**0.2** ± 0.02 [c]	**0.03** ± 0.01 [c]	**0.01** ± 0.01 [d]	**0** [b]	**0** [a]
	Cane Medium [a]		**0.001** ± 0.001 [b]	**0.02** ± 0.01 [d]	**0.2** ± 0.02 [c]	**0.01** ± 0.01 [c]	**0.1** ± 0.07 [c]	**0** [b]	**0** [a]
	Cane High [a]		**0.5** ± 0.3 [b]	**0.02** ± 0.01 [d]	**0.2** ± 0.02 [c]	**0.01** ± 0.01 [c]	**0.04** ± 0.02 [d]	**0** [b]	**0** [a]
	Soy Low [a]		**0.02** ± 0.01 [b]	**0.1** ± 0.01 [c]	**0.3** ± 0.06 [c]	**0.01** ± 0.01 [c]	**0.05** ± 0.05 [d]	**0** [b]	**0** [a]
	Soy Medium [a]		**0.3** ± 0.03 [b]	**1.0** ± 0.08 [b]	**0.9** ± 0.1 [b]	**1.0** ± 0.1 [b]	**0.9** ± 0.1 [b]	**0** [b]	**0** [a]
	Soy High [a]		**1.4** ± 0.14 [a]	**5.5** ± 0.23 [a]	**6.8** ± 0.4 [a]	**11.9** ± 0.9 [a]	**22.5** ± 2.5 [a]	**8.4** ± 3.2 [a]	**0** [a]
NO_3^--N	**Control** [a]	**5.0** ± 0.3	**3.5** ± 0.5 [a]	**7.0** ± 1.0 [a]	**7.5** ± 0.8 [ab]	**4.2** ± 1.0 [d]	**10.5** ± 2.3 [b]	**16.6** ± 1.1 [d]	**41.5** ± 4.0 [b]
	Cane Low [a]		**2.5** ± 0.7 [a]	**3.5** ± 1.1 [b]	**3.1** ± 0.9 [b]	**0.2** ± 0.1 [e]	**2.6** ± 0.6 [c]	**11.1** ± 0.8 [d]	**35.4** ± 3.0 [b]
	Cane Medium [a]		**0** [b]	**0** [c]	**0.5** ± 0.05 [c]	**0** [e]	**2.4** ± 0.9 [c]	**2.4** ± 0.5[e]	**45.1** ± 8.7 [b]
	Cane High [a]		**0** [b]	**0** [c]	**0.3** ± 0.06 [c]	**0** [e]	**2.4** ± 1.1 [c]	**0.6** ± 0.2 [f]	**60.1** ± 19.3 [b]
	Soy Low [a]		**4.5** ± 0.3 [a]	**7.2** ± 0.4 [a]	**9.1** ± 0.9 [a]	**9.6** ± 0.8 [c]	**10.0** ± 3.2 [bc]	**33.2** ± 1.4 [c]	**57.4** ± 3.5 [b]
	Soy Medium [a]		**2.0** ± 0.1 [a]	**5.7** ± 0,6 [a]	**13.1** ± 1.6 [a]	**16.1** ± 0.9 [a]	**62.4** ± 7.2 [a]	**131.6** ± 16.0 [b]	**149.9** ± 9.3 [b]
	Soy High [a]		**0** [b]	**0.7** ± 0.1 [c]	**3.8** ± 0.5 [b]	**12.9** ± 0.8 [b]	**133.9** ± 11.8 [a]	**284.0** ± 32.5 [a]	**631.1** ± 118 [a]
Total N	**Control** [b]	**5.9** ± 0.4	**4.3** ± 0.6 [b]	**7.8** ± 1.1 [a]	**7.8** ± 0.8 [b]	**4.8** ± 1.0 [d]	**11.0** ± 2.3 [cd]	**17.46** ± 1.1 [cd]	**41.6** ± 4.0 [c]
	Cane Low [b]		**3.4** ± 0.8 [bc]	**4.3** ± 1.1 [b]	**3.4** ± 0.9 [c]	**1.1** ± 0.2 [e]	**2.7** ± 0.6 [d]	**11.8** ± 0.7 [cd]	**35.6** ± 3.0 [c]
	Cane Medium [b]		**1.3** ± 0.16 [c]	**1.2** ± 0.21 [c]	**0.7** ± 0.1 [cd]	**0.7** ± 0.2 [e]	**2.7** ± 0.9 [d]	**2.7** ± 0.9 [d]	**45.5** ± 8.8 [c]
	Cane High [b]		**1.9** ± 0.3 [bc]	**1.1** ± 0.2 [c]	**0.7** ± 0.1 [cd]	**1.2** ± 0.2 [e]	**2.7** ± 1.1 [d]	**1.3** ± 0.5 [d]	**60.3** ± 19.3 [c]
	Soy Low [b]		**5.3** ± 0.3 [a]	**8.3** ± 0.5 [a]	**9.5** ± 0.9 [b]	**10.3** ± 0.8 [c]	**10.4** ± 3.2 [cd]	**33.6** ± 1.4 [cd]	**57.7** ± 3.5 [c]
	Soy Medium [b]		**3.1** ± 0.2 [b]	**7.8** ± 0.6 [a]	**14.1** ± 1.6 [a]	**17.8** ± 7.2 [b]	**63.6** ± 7.2 [b]	**133.0** ± 16.2 [b]	**150.0** ± 9.3 [b]
	Soy High [a]		**2.7** ± 0.3 [b]	**7.7** ± 0.2 [a]	**10.8** ± 0.6 [a]	**25.3** ± 0.7 [a]	**156.8** ± 12.5 [a]	**292.6** ± 48.4 [a]	**631.3** ± 166 [a]

Table 3-6. Concentrations (μg N g^{-1} soil DW ± 1 SEM) of amino acids (AA-N), ammonium (NH_4^+) and nitrate (NO_3^-) and total nitrogen (Total N) as sampled by **KCl extraction**, from soils amended with increasing rates of sugarcane litter (Cane) and soybean litter (Soy), or control soils with no litter. Samples were taken from destructive harvests at 8 timepoints over a 100-day incubation. Letters denote groupings of statistical significance (ANOVA, $p \leq 0.05$) between treatments in each pool for a given timepoint, or for treatments across all time points (repeated measures ANOVA).

N Pool	Treatment	Day 0	Day 3	Day 7	Day 11	Day 17	Day 30	Day 60	Day 100
Total AA-N	**Control** [a]	**1.8** ± 0.2	**1.3** ± 0.2 [b]	**1.5** ± 0.2 [b]	**0.6** ± 0.06 [b]	**1.3** ± 0.2 [b]	**0.5** ± 0.09 [b]	**0.4** ± 0.04 [b]	**0.5** ± 0.4 [a]
	Cane Low [a]		**1.1** ± 0.2 [b]	**1.9** ± 0.2 [b]	**1.0** ± 0.1 [ab]	**1.7** ± 0.2 [ab]	**0.7** ± 0.1 [b]	**0.2** ± 0.04 [c]	**0.07** ± 0.01 [a]
	Cane Medium [a]		**2.1** ± 0.3 [b]	**1.8** ± 0.2 [b]	**1.6** ± 0.1 [a]	**2.0** ± 0.2 [ab]	**0.7** ± 0.1 [b]	**14.6** ± 2.3 [b]	**0.1** ± 0.04 [a]
	Cane High [a]		**4.7** ± 0.7 [a]	**2.4** ± 0.4 [b]	**1.4** ± 0.2 [ab]	**2.1** ± 0.2 [ab]	**0.8** ± 0.2 [ab]	**18.0** ± 3.9 [ab]	**0.2** ± 0.03 [a]
	Soy Low [a]		**1.5** ± 0.3 [b]	**2.0** ± 0.2 [b]	**1.1** ± 0.2 [b]	**1.6** ± 0.2 [ab]	**0.2** ± 0.04 [b]	**0.1** ± 0.02 [c]	**0.1** ± 0.01 [a]
	Soy Medium [a]		**1.7** ± 0.3 [b]	**2.4** ± 0.1 [ab]	**1.0** ± 0.1 [b]	**1.2** ± 0.2 [b]	**0.3** ± 0.04 [b]	**3.1** ± 1.5 [a]	**0.2** ± 0.02 [a]
	Soy High [a]		**3.7** ± 0.4 [a]	**3.5** ± 0.6 [a]	**1.5** ± 1.9 [b]	**2.6** ± 0.4 [a]	**1.0** ± 0.1 [a]	**6.3** ± 2.2 [a]	**0.1** ± 0.01 [a]
NH_4^+-N	**Control** [c]	**1.2** ± 0.2	**0.6** ± 0.1 [d]	**1.0** ± 0.2 [b]	**0.8** ± 0.1 [c]	**1.1** ± 0.2 [cd]	**0.8** ± 0.1 [c]	**1.3** ± 0.3 [c]	**0.9** ± 0.2 [d]
	Cane Low [c]		**0.7** ± 0.2 [d]	**0.7** ± 0.1 [bc]	**0.5** ± 0.04 [d]	**1.4** ± 0.5 [cd]	**0.7** ± 0.1 [c]	**2.3** ± 0.4 [c]	**1.1** ± 0.2 [d]
	Cane Medium [c]		**0.3** ± 0.04 [e]	**0.06** ± 0.01 [c]	**0.09** ± 0.03 [e]	**0.4** ± 0.2 [cd]	**0.4** ± 0.2 [c]	**1.7** ± 0.2 [c]	**4.6** ± 0.6 [c]
	Cane High [c]		**0.2** ± 0.04 [e]	**0.06** ± 0.02 [c]	**0.003** ± 0.002 [f]	**0.2** ± 0.06 [d]	**0.3** ± 0.1 [d]	**1.1** ± 0.4 [c]	**9.8** ± 2.0 [b]
	Soy Low [c]		**2.7** ± 0.4 [c]	**1.1** ± 0.3 [b]	**0.7** ± 0.1 [c]	**1.5** ± 0.3 [c]	**0.7** ± 0.1 [c]	**1.4** ± 0.3 [c]	**1.1** ± 0.2 [d]
	Soy Medium [bc]		**8.0** ± 0.5 [b]	**14.8** ± 1.9 [a]	**11.7** ± 0.6 [b]	**11.3** ± 2.6 [b]	**6.4** ± 0.5 [b]	**3.9** ± 0.5 [b]	**9.9** ± 1.9 [b]
	Soy High [a]		**15.8** ± 1.1 [a]	**65.3** ± 9.9 [a]	**81.7** ± 3.8 [a]	**91.0** ± 15.6 [a]	**90.3** ± 6.0 [a]	**22.1** ± 2.1 [a]	**33.9** ± 3.9 [a]
NO_3^--N	**Control** [a]	**0.9** ± 0.2	**2.0** ± 0.3 [b]	**3.8** ± 0.7 [ab]	**5.7** ± 0.8 [a]	**3.4** ± 1.2 [b]	**6.7** ± 1.7 [bc]	**17.1** ± 0.6 [d]	**49.2** ± 5.4 [c]
	Cane Low [a]		**1.5** ± 0.5 [b]	**1.7** ± 0.6 [c]	**2.1** ± 0.6 [b]	**0.04** ± 0.04 [c]	**2.0** ± 0.6 [c]	**8.2** ± 1.1 [e]	**32.3** ± 3.1 [c]
	Cane Medium [a]		**0** [c]	**0** [d]	**0.3** ± 0.1 [c]	**0** [c]	**0** [d]	**1.9** ± 0.4 [f]	**48.3** ± 7.9 [c]
	Cane High [a]		**0** [c]	**0** [d]	**0.2** ± 0.07 [c]	**0.003** ± 0.003 [c]	**0.3** ± 0.1 [cd]	**0.6** ± 0.3 [g]	**54.3** ± 15.3 [c]
	Soy Low [a]		**2.9** ± 0.2 [a]	**5.0** ± 0.3 [a]	**7.2** ± 0.9 [a]	**6.3** ± 0.6 [a]	**9.0** ± 2.8 [b]	**38.1** ± 1.7 [c]	**49.9** ± 3.4 [c]
	Soy Medium [a]		**1.4** ± 0.1 [b]	**4.3** ± 0.5 [ab]	**10.1** ± 0.9 [a]	**10.2** ± 1.2 [a]	**65.1** ± 3.1 [a]	**119.2** ± 5.0 [b]	**191.0** ± 5.0 [b]
	Soy High [a]		**0** [c]	**1.6** ± 0.2 [b]	**4.6** ± 0.5 [a]	**11.6** ± 1.2 [a]	**156.9** ± 7.4 [a]	**375.2** ± 23.6 [a]	**665.9** ± 70.1 [a]
Total N	**Control** [b]	**3.8** ± 0.2	**4.0** ± 0.5 [c]	**6.3** ± 0.8 [c]	**7.2** ± 0.8 [c]	**5.8** ± 1.0 [c]	**8.0** ± 1.8 [c]	**18.8** ± 0.7 [d]	**50.6** ± 5.2 [c]
	Cane Low [b]		**3.2** ± 0.7 [c]	**4.3** ± 0.6 [cd]	**3.5** ± 0.9 [d]	**3.1** ± 0.6 [de]	**3.4** ± 0.6 [de]	**10.6** ± 1.4 [e]	**33.5** ± 3.1 [c]
	Cane Medium [b]		**2.4** ± 0.3 [c]	**1.9** ± 0.2 [d]	**2.0** ± 0.1 [de]	**2.4** ± 0.3 [e]	**1.1** ± 0.2 [e]	**18.2** ± 2.4 [f]	**53.0** ± 7.9 [c]
	Cane High [b]		**4.9** ± 0.7 [b]	**2.5** ± 0.4 [d]	**1.6** ± 0.2 [e]	**2.3** ± 0.2 [e]	**1.4** ± 0.3 [e]	**19.7** ± 4.0 [g]	**64.2** ± 14.8 [c]
	Soy Low [b]		**7.1** ± 0.5 [b]	**8.2** ± 0.4 [c]	**9.0** ± 1.2 [c]	**9.6** ± 0.7 [c]	**9.9** ± 2.8 [c]	**38.6** ± 1.9 [c]	**51.1** ± 3.4 [c]
	Soy Medium [ab]		**11.0** ± 0.7 [ab]	**21.5** ± 2.2 [b]	**22.8** ± 1.3 [b]	**22.7** ± 3.5 [b]	**71.7** ± 2.9 [b]	**126.2** ± 6.1 [b]	**201.1** ± 21.6 [b]
	Soy High [a]		**19.6** ± 0.9 [a]	**70.5** ± 10.5 [a]	**87.8** ± 3.7 [a]	**105.2** ± 16.2 [a]	**248.2** ± 7.6 [a]	**403.6** ± 23.8 [a]	**700.0** ± 72.5 [a]

Table 3-7. Diffusive fluxes (nmols N cm^{-2} h^{-1} ± 1 SEM) of amino acids (AA-N), ammonium (NH_4^+) and nitrate (NO_3^-) and total nitrogen (Total N) as sampled by **microdialysis**, from soils amended with increasing rates of sugarcane litter (Cane) and soybean litter (Soy), or control soils with no litter. Samples were taken from non-destructive harvests at 8 timepoints over a 100-day incubation. Letters denote groupings of statistical significance (ANOVA, $p \leq 0.05$) between treatments in each pool for a given timepoint, or for treatments across all time points (repeated measures ANOVA).

N Pool	Treatment	Day 0	Day 3	Day 7	Day 11	Day 17	Day 30	Day 60	Day 100
Total AA-N	**Control** [a]	**54.7** ± 8.6	**73.9** ± 8.8 [ab]	**64.3** ± 8.7 [a]	**17.33** ± 2.0 [a]	**59.8** ± 11.5 [b]	**23.9** ± 4.1 [a]	**3.9** ± 1.0 [a]	**6.2** ± 1.4 [a]
	Cane Low [a]		**50.0** ± 16.3 [ab]	**24.1** ± 2.6 [b]	**14.9** ± 1.6 [a]	**54.4** ± 8.9 [b]	**20.6** ± 2.9 [a]	**5.0** ± 1.6 [a]	**5.7** ± 1.5 [a]
	Cane Medium [a]		**46.5** ± 6.6 [b]	**31.7** ± 3.5 [b]	**14.1** ± 0.9 [a]	**69.9** ± 9.1 [b]	**7.6** ± 1.5 [a]	**19.5** ± 9.7 [a]	**6.1** ± 1.0 [a]
	Cane High [a]		**56.7** ± 11.3 [ab]	**30.7** ± 4.5b [b]	**21.4** ± 2.9 [a]	**88.4** ± 11.9 [b]	**6.2** ± 2.0 [a]	**5.1** ± 1.1 [a]	**7.1** ± 1.3 [a]
	Soy Low [a]		**107.8** ± 28.0 [ab]	**31.0** ± 3.1b [b]	**22.8** ± 2.3 [a]	**66.7** ± 14.7 [b]	**19.4** ± 11.7 [a]	**5.9** ± 1.2 [a]	**9.6** ± 7.3 [a]
	Soy Medium [a]		**121.5** ± 18.1 [a]	**37.7** ± 4.1b [a]	**17.94** ± 1.8 [a]	**75.9** ± 13.7 [b]	**8.3** ± 2.0 [a]	**5.6** ± 1.3 [a]	**5.8** ± 2.2 [a]
	Soy High [a]		**95.4** ± 11.5 [ab]	**36.8** ± 4.1b [a]	**19.1** ± 2.7 [a]	**123.6** ± 25.1[a]	**8.6** ± 1.5 [a]	**20.2** ± 9.6 [a]	**21.2** ± 8.5 [a]
NH_4^+-N	**Control** [b]	**1.7** ± 1.1	**0.1** ± 0.1 [a]	**0** [b]	**6.5** ± 0.9 [b]	**0** [b]	**3.0** ± 0.8 [c]	**8.3** ± 1.9 [b]	**7.5** ± 1.3 [b]
	Cane Low [b]		**0** [a]	**0.2** ± 0.2 [b]	**3.9** ± 0.8 [b]	**0** [b]	**3.8** ± 0.8 [c]	**6.6** ± 1.5 [b]	**0** [c]
	Cane Medium [b]		**1.2** ± 0.8 [a]	**0** [b]	**3.5** ± 0.4 [b]	**0** [b]	**13.9** ± 1.5 [b]	**10.1** ± 1.6 [b]	**5.6** ± 5.3 [bc]
	Cane High [b]		**0.6** ± 0.6 [a]	**0** [b]	**3.7** ± 1.0 [b]	**0.8** ± 0.4 [b]	**20.1** ± 2.2 [b]	**34.8** ± 12.3 [b]	**67.4** ± 19.4 [b]
	Soy Low [b]		**0.4** ± 0.4 [a]	**2.3** ± 2.3 [b]	**4.3** ± 2.5 [b]	**0** [b]	**16.4** ± 2.6 [b]	**10.3** ± 1.6 [b]	**1.3** ± 0.9 [c]
	Soy Medium [ab]		**1.5** ± 0.7 [a]	**2.1** ± 1.3 [b]	**6.0** ± 1.0 [b]	**5.7** ± 2.9 [b]	**17.9** ± 3.4 [b]	**20.4** ± 5.5 [b]	**29.4** ± 12.9 [b]
	Soy High [a]		**3.8** ± 1.8 [a]	**53.1** ± 10.7 [a]	**74.3** ± 18.0 [a]	**110.3** ± 12.7 [a]	**252.5** ± 36.3 [a]	**176.2** ± 41.5 [a]	**455.6** ± 174[a]
NO_3^--N	**Control** [c]	**15.2** ± 4.6	**18.3** ± 4.4 [a]	**69.3** ± 49.3 [a]	**18.3** ± 11.3 [b]	**1.8** ± 1.5 [c]	**67.0** ± 22.0 [c]	**156.5** ± 22.7 [c]	**637.8** ± 158 [b]
	Cane Low [c]		**10.4** ± 4.5 [a]	**5.1** ± 4.6 [b]	**1.6** ± 1.6 [b]	**0** [c]	**3.3** ± 1.7 [d]	**181.3** ± 55.4 [c]	**410.5** ± 144 [b]
	Cane Medium [c]		**9.6** ± 7.0 [a]	**5.1** ± 5.1 [b]	**0.2** ± 0.2 [b]	**0** [c]	**20.6** ± 9.4 [c]	**125.9** ± 30.7 [c]	**378.2** ± 103 [b]
	Cane High [c]		**6.8** ± 5.9 [a]	**0** [b]	**13.4** ± 13.4 [b]	**0** [c]	**97.5** ± 41.9 [c]	**323.3** ± 72.7 [c]	**1150.8** ± 418 [b]
	Soy Low [c]		**21.3** ± 8.1 [a]	**14.3** ± 8.0 [ab]	**41.9** ± 20.4 [b]	**79.5** ± 38.0 [b]	**304.3** ± 52.4 [b]	**363.3** ± 115 [c]	**468.9** ± 76.6 [b]
	Soy Medium [bc]		**29.1** ± 10.9 [a]	**71.3** ± 26.4 [a]	**114.3** ± 28.6 [a]	**297.9** ± 76.5 [ab]	**1298.6** ± 411 [ab]	**1401.9** ± 394 [b]	**1182.3** ± 331 [b]
	Soy High [a]		**9.4** ± 5.5 [a]	**32.6** ± 19.4 [ab]	**135.2** ± 33.9 [a]	**308.9** ± 43.4 [a]	**3487.4** ± 668 [a]	**6000.5** ± 1382 [a]	**12315.9** ± 4494 [a]
Total N	**Control** [b]	**71** ± 11.2	**92. 3** ± 9.9 [ab]	**133.5** ± 48.8 [a]	**42.2** ± 12.1 [c]	**61.6** ± 12.1 [b]	**93.9** ± 24.9 [c]	**168.7** ± 22.9 [c]	**651.4** ± 158 [b]
	Cane Low [b]		**60.4** ± 15.0 [b]	**29.3** ± 5.4 [b]	**20.4** ± 22 [c]	**54.4** ± 8.9 [b]	**27.8** ± 3.8 [c]	**192.9** ± 54 [c]	**416.2** ± 143 [b]
	Cane Medium [b]		**58.5** ± 8.3 [b]	**36.7** ± 6.3 [b]	**17.8** ± 1.1 [c]	**69.9** ± 9.1 [b]	**42.1** ± 8.9 [c]	**155.6** ± 33.6 [c]	**389.9** ± 107 [b]
	Cane High [b]		**64.1** ± 13.1 [b]	**30.7** ± 4.8 [b]	**38.6** ± 13.7 [c]	**262.0** ± 179 [b]	**123.7** ± 43.7 [c]	**363.3** ± 83.6 [c]	**1235.6** ± 415 [b]
	Soy Low [b]		**129.5** ± 31.1 [ab]	**46.5** ± 10.3 [b]	**69.0** ± 23.3 [bc]	**146.3** ± 42.8 [b]	**340.1** ± 48.5 [c]	**379.5** ± 116 [c]	**479.8** ± 81.6 [b]
	Soy Medium [ab]		**152.1** ± 16.1 [a]	**109.1** ± 28.2 [a]	**138.2** ± 31.0 [ab]	**379.5** ± 81.6 [a]	**1324.8** ± 415 [b]	**1427.9** ± 393 [b]	**1217.5** ± 343 [b]
	Soy High [a]		**108.6** ± 9.0 [ab]	**97.6** ± 24.8 [a]	**228.6** ± 51.5 [a]	**542.8** ± 56.4 [a]	**3748.5** ± 700 [a]	**6197.0** ± 1421 [a]	**12792.6** ± 4474 [a]

4 Quantifying inorganic N availability in sugarcane soils

4.1 Introduction

Nitrogen (N) availability is a critical component of soils and represents the fraction of total soil N that is bioavailable to both soil microbes and plant roots. However, it is a difficult measure to derive and interpret, in part due to the diverse, heterogeneous nature of soils, which can vary widely in physical, chemical and biological complexity. Further, artefacts arise from the extraction methods used to quantify soil N availability, and these influence the estimates of available N (Jones and Willett, 2006, Ros et al., 2009, Inselsbacher, 2014). It is therefore of interest to consider alternatives, and the *in situ* technique microdialysis is explored here as a method to quantify bioavailable soil N.

This chapter expands on findings from Chapter 2, which showed that microdialysis offers a differing perspective of N availability compared to conventional soil extractions – particularly, the small contribution that NH_4^+ makes to total N fluxes, which contrasts to its greater prevalence in soil extracts. This finding mirrors other microdialysis studies exploring soil N fluxes (Inselsbacher and Näsholm, 2012a, Inselsbacher et al., 2014). Microdialysis samples solutes freely mobile in soil solution, and we hypothesise that as a cation, NH_4^+ is adsorbed to soil surfaces and thus is mostly excluded from sampling with microdialysis. In contrast, extractions can mobilise loosely bound NH_4^+ (H_2O extraction) and exchangeable NH_4^+ (salt extractions), which increase the presence of NH_4^+ in the total pool of extracted N. However, the magnitude of NH_4^+ released from soil exchange sites is likely to be influenced by soil type, climate and N management. For instance, recently fertilised soils can have increased NH_4^+ fluxes compared to unfertilised soils (Inselsbacher et al., 2014, Brackin et al., 2015). Likewise, Shaw et al. (2014) found that NH_4^+ fluxes were dominant in four out of eight British grassland soils along an altitudinal gradient. From this we may conclude that prevailing soil parameters can influence NH_4^+ fluxes, but the parameters remain unclear.

In this study, we measured inorganic N (NH_4^+ and NO_3^-) availability in 24 sugarcane soils from the main growing regions in tropical and subtropical Queensland, Australia. The soils represent a diversity of soil types, with wide-ranging soil texture (clay content 4.6 to 54 %) and chemical properties (e.g. organic C content 0.68 to 3.3 %; total N content 0.05 to 0.24 %). The soils provide an opportunity to compare the contribution of NH_4^+ and NO_3^- to microdialysis fluxes and soil extractions, and permit an examination of the soil parameters that affect diffusive N fluxes and concentrations.

Fluxes determined by microdialysis may integrate many factors that affect the diffusion of nitrogen through soil, including molecular interactions with the soil environment (Tinker and Nye, 2000, Miró

and Frenzel, 2005). Physical soil parameters such as soil texture and bulk density, and chemical parameters such as total N, total organic carbon (OC) and electro-conductivity (EC) are routinely investigated in soil studies, but the influence of these factors on N fluxes have yet to be explored in depth. Solute charge affects N recoveries with microdialysis (Inselsbacher et al., 2011, Chapter 5), with positively-charged ions (e.g. NH_4^+, lysine) more likely to adsorb to the negatively-charged soil surfaces (Bartlett and Doner, 1988, Vieublé Gonod et al., 2006, Rothstein, 2010, Nieder et al., 2011), and so these interactions may increase with greater clay or soil OC, decreasing availability. In contrast, ions with a negative charge (e.g. glutamate, NO_3^-) can be repelled from anionic soil surfaces to be more mobile in soil solution (Tinker and Nye, 2000), with coarser soil texture increasing the potential for greater movement through the soil profile (Di and Cameron, 2002, Cameron et al., 2013). Anion exchange capacity of soils may also influence NO_3^- adsorption, particularly at low pH where protonation of surface-based hydroxyl groups can increase the prevalence of positively-charged soil sites (Havlin, 2017).

Biological influences can also alter N fluxes, given that in most natural soil systems depolymerisation and mineralisation of organic N form the primary bottleneck to N availability (Schimel and Bennett, 2004). Fluxes could be further modified by microbial uptake of the products of mineralisation (Geisseler et al., 2010). As microbes act as sinks and sources for N compounds (Geisseler et al., 2010), we hypothesise that a relationship between higher rates of mineralisation and fluxes exists. Nitrogen mineralisation assays often combine standardised laboratory incubations and extraction techniques to estimate available N mineralised over a specific timeframe (Stanford and Smith, 1972). We use these methods to estimate N mineralisation rates over a 14-day incubation and compare with the N fluxes collected over the course of one day.

In addition to the quantification of N in extracts and fluxes, microdialysis sampling may be useful to characterise N movement in contrasting soils. For instance, depletion of a solute around probes is a common feature of microdialysis sampling (Inselsbacher et al., 2011). As solutes diffuse across the microdialysis membrane, concentrations decrease in zones immediately around the probe. Subsequently, solutes diffuse from regions further away from the probe at rates dependent on their effective diffusion coefficient in that soil environment (Tinker and Nye, 2000). Generally, replenishment rates rarely equal initial depletion rates, and so with time fluxes will decrease non-linearly (Figure 4-1). As depletion curves are dependent on the integrated resistive properties of soil environments, combined with diffusive properties of a solute (Miró and Frenzel, 2005), these curves might provide useful information about solute movement through soils, and their dependence on particular soil properties. As these depletion conditions likely represent similar circumstances encountered by plant roots during nutrient uptake (Tinker and Nye, 2000, Hinsinger, 2001, York et

al., 2016), we may be able to infer understanding regarding plant-availability of a solute in context with the physical soil environment. In this study, we particularly focus on the *plateau* – the steady-state flux that follows the initial depletion, as predicted by non-linear regression (Figure 4-1). We hypothesise that the plateau represents a measure of solute transport – likely most affected by soil properties which affect path tortuosity, such as soil texture.

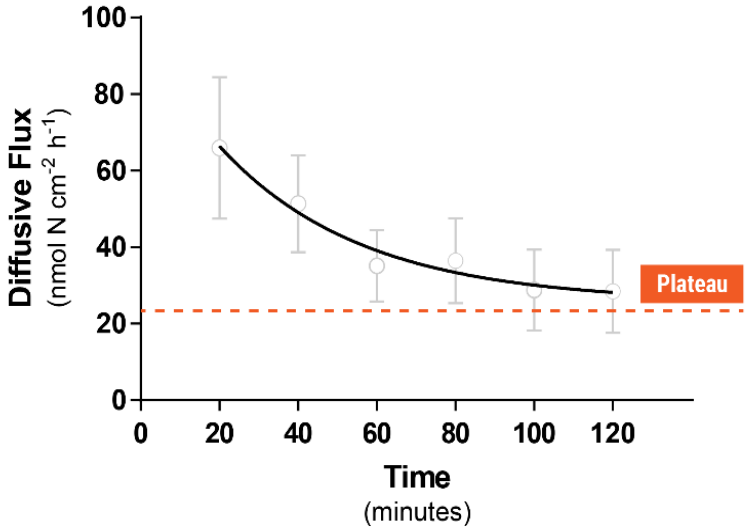

Figure 4-1. An example of a depletion curve for a solute sampled at 20 minutes intervals over 120 minutes. The plateau (indicated by the orange dotted line) represents a steady-state flux estimated by the non-linear regression.

Fluxes as measured by microdialysis are therefore an integrated measurement of solute movement influenced by solute pool size, solute movement through the soil environment, and microbial sink and source strength. This study compares soils with contrasting properties to investigate the influence of physical, chemical and biological parameters on NH_4^+ and NO_3^- and fluxes and depletion curves.

We focus on inorganic N in an effort to simplify the study, however we recognise that organic N (such as amino acids) is an important part of any consideration of bioavailable N, and represents a diverse pool of compounds with wide-ranging electrical charge and molecular characteristics which will influence their availability in soil solution (Chapter 6, Vieublé Gonod et al., 2006, Rothstein, 2010).

4.2 Methods

4.2.1 Soil collection and storage

Twenty-four sugarcane soils were collected from a diversity of sugarcane agricultural sites across Queensland, Australia, as part of Sugar Research Australia project "Decision support for informed nitrogen management: soil N mineralisation" (SRA 2015/069). Collaborators at the Department of Science and Environment (DES, formerly DSITI) were responsible for soil characterisation (4.2.2) and mineralisation assays (4.2.3). Soils were collected from the top 20 cm of soil from plots with a management history of zero-N fertiliser application. Soils were stored in resealable plastic bags at 4 °C for up to three months before handling for microdialysis and extractions.

4.2.2 Soil characterisation

Soils properties (Table 4-1) were characterised as per Rayment and Lyons (2011) including particle size (sand, silt, clay), total soil organic C (Total OC) (by combustion), total N (by combustion), pH and electro-conductivity (1:5 with water) and exchangeable cations (Ca, Mg, K, Na)

4.2.3 Mineralisation assays

Incubations were performed similarly to Stanford and Smith (1972). Briefly, sampled soils were air-dried at 40 °C, before sieving (2 mm). 80 g of air-dried soil was weighed into plastic jars, with water added to reach field capacity. Jars were placed in a dark incubator at 30 °C for 14 days, at which time samples were harvested. Sub-samples were extracted with 2 M KCl, with NH_4^+ and NO_3^- analysed using an automated colourimetric assay, as per Rayment and Lyons (2011), methods 7C1 and 7C2. Potentially mineralisable N was calculated as the difference between total inorganic N at day 14 and day 0 (sampled prior to incubation).

4.2.4 Soil preparation for microdialysis and extractions

Twenty mL soil microcosms were constructed from modified 50 mL centrifuge tubes (Inselsbacher et al., 2009), with the top half of the tubes sawn off to permit microdialysis of smaller soil volumes. Microcosms were filled with soils, with moisture levels at field capacities. Soils were tapped down to compact and watered to reach 70% water-holding capacity (WHC) for each soil. This resulted in a variable bulk-density across soil types, which ranged from 0.75 – 1.49 g^{-1} soil DW cm^{-3}. Soils were incubated at room temperature (approximately 24 °C) for two days prior to sampling with microdialysis and extractions – soils were re-watered to maintain 70% WHC during incubation.

Table 4-1. List of properties for soils measured in this chapter, sampled from sugarcane-growing regions in Queensland, Australia. Properties are presented as means of three replicates ± standard error.

						Texture			
ID	**Growing Region**	**EC (µS)**	**pH**	**Total C (%)**	**Total N (%)**	**Coarse Sand (%)**	**Fine Sand (%)**	**Silt (%)**	**Clay (%)**
1	Mackay	79.3 ± 0.7	5.8 ± 0.03	1.1 ± 0.01	0.09 ± 0	17.0 ± 0.4	43.7 ± 0.7	21.5 ± 0.07	19.9 ± 0.07
2	Mackay	67.2 ± 3.7	5.9 ± 0.3	1.2 ± 0	0.06 ± 0	27.5 ± 0.4	37.7 ± 0.7	21.3 ± 0.2	14.7 ± 0.11
3	Mackay	64.1 ± 0.1	5.9 ± 0.01	1.3 ± 0.02	0.09 ± 0.003	15.2 ± 0.3	46.1 ± 0.1	23.3 ± 0	16.4 ± 0
4	Mackay	130 ± 5.1	5.2 ± 0.03	1.2 ± 0.002	0.07 ± 0.003	14.8 ± 0.4	40.7 ± 0.3	23.0 ± 0.03	21.4 ± 0.03
5	Mackay	45.6 ± 0.5	6.1 ± 0.01	1.6 ± 0	0.1 ± 0.003	26.4 ± 1.0	41.0 ± 1.0	20.7 ± 0.5	14.3 ± 1.17
6	Ingham	69 ± *n.a.*†	6.2 ± *n.a.*†	2.3 ± *n.a.*†	0.13 ± *n.a.*†	16.2 ± 0.1	57.4 ± 0.4	12.9 ± 0	14.5 ± 0
7	Silkwood	38.9 ± 1.6	5.2 ± 0.03	5.3 ± 0	0.27 ± 0	9.0 ± 0.1	30.0 ± 1.0	21.6 ± 0.5	37.1 ± 0.3
8	Tully	35.9 ± 0.8	5.1 ± 0.03	1.2 ± 0.07	0.11 ± 0.005	6.3 ± 0.1	27.5 ± 0.6	33.0 ± 0	35.4 ± 0
9	Tully	34.0 ± 0.5	5.2 ± 0.03	1.4 ± 0.01	0.12 ± 0	1.2 ± 0.05	22.7 ± 1.3	42.8 ± 0.09	40.2 ± 0.1
10	Ingham	107.1 ± 0.3	5.3 ± 0.03	1.2 ± 0.004	0.1 ± 0	6.9 ± 0.2	37.3 ± 1.8	33.2 ± 0.03	26.5 ± 0.03
11	Tully	48.5 ± 1.2	5.0 ± 0.01	3.1 ± 0.004	0.21 ± 0	2.9 ± 0	5.1 ± 0	35.7 ± 0	54.1 ± 0
12	Tully	44.9 ± 1.4	5.1 ± 0.04	3.4 ± 0.01	0.24 ± 0	3.8 ± 0.03	5.2 ± 0.8	36.7 ± 0.03	52.5 ± 0.03
13	Bundaberg	157.7 ± 0.4	6.7 ± 0.03	0.7 ± 0.01	0.05 ± 0.003	41.3 ± 0.6	46.7 ± 0.6	9.1 ± 0	5 ± 0
14	Mackay	49.3 ± 0.5	6.6 ± 0.03	2.3 ± 0.002	0.14 ± 0.003	6.8 ± 0.06	20.3 ± 0.6	28.2 ± 0.06	47.6 ± 0
15	Ingham	87.3 ± 3.7	5.7 ± 0.02	1.1 ± 0.003	0.1 ± 0	7.4 ± 0.03	30.4 ± 0.3	33.1 ± 0.09	28.1 ± 0.07
16	Ingham	71.5 ± 2.8	5.9 ± 0.04	2.1 ± 0.01	0.12 ± 0	12.4 ± 0.3	59.6 ± 0.3	12.9 ± 0	16.4 ± 0
17	Bundaberg	98.3 ± 2.3	6.6 ± 0.05	0.8 ± 0.005	0.06 ± 0	53.5 ± 0.5	36.3 ± 0.4	7.2 ± 0.11	5 ± 0
18	Bundaberg	78.1 ± 2.4	6.5 ± 0.09	0.8 ± 0	0.05 ± 0.003	54.2 ± 1.1	36.2 ± 0.7	6.3 ± 0.1	4.6 ± 0
19	Tully	28.5 ± 0.9	5.5 ± 0.1	1.7 ± 0.003	0.11 ± 0	20.5 ± 0.3	26.5 ± 0.7	19.1 ± 0	35.2 ± 0.03
20	Tully	36.1 ± 3.4	5.4 ± 0.1	2.9 ± 0	0.2 ± 0	4.5 ± *n.a.*†	11 ± *n.a.*†	38 ± *n.a.*†	46.9 ± *n.a.*†
21	Tully	33.2 ± 0.5	5.7 ± 0.03	2.6 ± 0.005	0.18 ± 0	3.2 ± 0.1	16.5 ± 0.2	35.0 ± 0.03	51.0 ± 0.03
22	Burdekin	124.6 ± 2.1	7.4 ± 0.05	1.2 ± 0	0.1 ± 0	2.9 ± 0.1	25.5 ± 0.4	24.6 ± 0.07	48.6 ± 0.06
23	Burdekin	112.4 ± 2.1	7.7 ± 0.1	1.1 ± 0	0.09 ± 0	2.5 ± 0.03	30.3 ± 0.3	24.9 ± 0.1	47.3 ± 0.01
24	Tully	31.4 ± 1.7	5.5 ± 0.05	1.7 ± 0.01	0.11 ± 0	18.9 ± 0.2	21.1 ± 1.3	19.9 ± 0.09	46 ± 0.1

† Standard error unavailable as only one replicate available.

4.2.5 Soil Sampling

Five g of unsieved soil was shaken with 10 mL of 1M KCl, or distilled H_2O for one hour on an orbital shaker. Extracts were then centrifuged at 4500 rpm for three minutes, with supernatants collected and frozen at -20 °C until analysis.

Microdialysis sampling was performed using a system consisting of a two syringe pumps (CMA 4004, CMA Microdialysis AB, Kista, Sweden), each equipped with four micro-syringes (2.5 mL, SGE Analytical Sciences, Ringwood, Australia) connected to four microdialysis probes (CMA 20). These were then connected to collection tubes (see below). The probes each featured a polyarylethersulphone (PAES) membrane, 10 mm long, 0.5 mm diameter, and with a molecular weight cut-off of 20 kDa. In each soil tested, holes were made using a small introducing needle, and probes inserted into the holes.

For comparisons of fluxes with mineralisation assays, probes were perfused with ultra-pure water (Milli-Q, Millipore Corporation) at a flow rate of 1 µL min^{-1} for 24 hours. Probes were connected to 1.5 mL centrifuge tubes, kept on ice at all times. After sampling, dialysates were frozen at -20 °C until analysis.

For calculations of N depletion curves, probes were connected to a refrigerated fraction collector (CMA 470), kept at 6°C, and fitted with 300 µL collection vials. The fraction collector was programmed to shift vials at 20-minute intervals during sampling. Probes were then perfused with ultra-pure water (Milli-Q, Millipore Corporation) at a flow rate of 1 µL min^{-1} for two hours. After sampling, dialysates were frozen at -20 °C until analysis.

4.2.6 Nitrogen measurements

NO_3^- concentrations of microdialysis samples and extractions (KCl and H_2O) were determined via the reduction of nitrate to nitrite with vanadium (III) chloride (VCl_3), followed by the Griess reaction, described by Miranda et al. (2001). NH_4^+ concentrations were measured as per Kandeler and Gerber (1988).

Microdialysis fluxes (*D*; in nmol N cm^{-2} h^{-1}) were calculated as follows:

$$D = C_{dial}/A_m \times t \qquad (1)$$

where C_{dial} is the concentration of a solute in the dialysate, A_m is the area of the membrane, and *t* is the sampling time.

4.2.7 Statistical analysis

Pearson moment correlations were performed to compare measurements of N using microdialysis and extractions, and between soil parameters (GraphPad Prism 6, Graphpad Software, Inc.). To determine significant differences between proportional data for N measured by each sampling method, a one-way ANOVA was performed, followed by a Tukey's HSD post-hoc test to determine significant differences between groups (GraphPad Prism 6, Graphpad Software, Inc.). Flux plateaus were calculated using non-linear regression (one-phase decay), based on N fluxes collected over two hours (GraphPad Prism 6, Graphpad Software, Inc.). Principal components analysis (PCA) was performed to determine general relationships between relationships between soil parameters and N measurements (Statistica 13, Tibco Software Inc.), with analyses based on correlations.

Before analyses, all data was tested using a D'Agostino & Pearson normality omnibus test, and was $\log_{10}$-transformed to meet requirements for normality (GraphPad Prism 6, Graphpad Software, Inc.).

4.3 Results

4.3.1 Contribution of nitrogen pools as sampled by extraction and microdialysis methods

Mean contributions of NH_4^+ and NO_3^- to total N (in extract or flux) differed between extraction and microdialysis methods (Figure 4-2). The contribution of NH_4^+ ranked KCl>H_2O>microdialysis with 60.5 ± 6.8, 44.9 ± 7.4 % and 14.3 ± 5.5 %, and the remainder contributed by NO_3^-. Proportions of NH_4^+ in fluxes were significantly less than KCl and H_2O extractions ($F_{2,68} = 12.3$, $p < 0.001$).

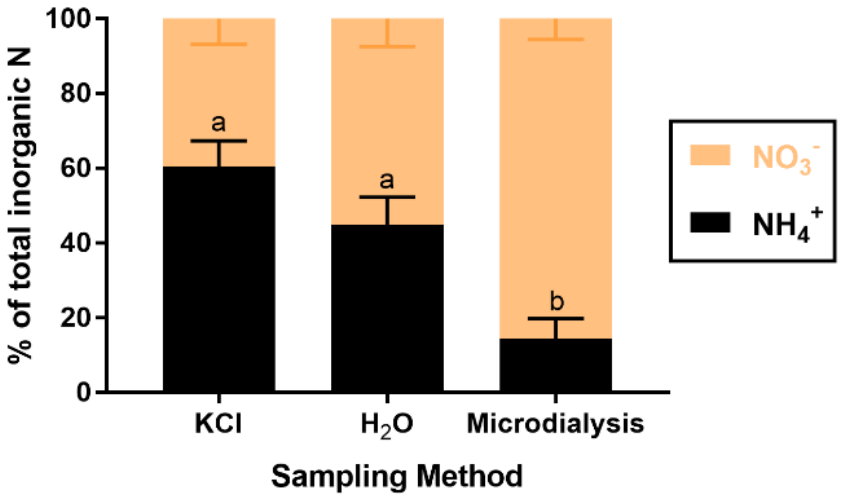

Figure 4-2. Proportions of total inorganic N as NH_4^+ (in black) and NO_3^- (orange) as estimated by extractions (KCl, H_2O) and microdialysis. Error bars represent ± 1 SEM; letters denote statistical differences between sampling methods ($p < 0.05$).

4.3.2 Relationships between extraction and microdialysis methods

H_2O extractions shared a significant positive correlation with microdialysis fluxes for both NO_3^- (r = 0.8, $p < 0.001$) and NH_4+ (r = 0.7, p = 0.001) (Figure 4-3, top graphs). KCl extractions also shared a significant positive correlation with fluxes for NO_3^- (r = 0.66; p = 0.005) but was not significantly correlated for NH_4^+ (r = 0.25, p = 0.23). As H_2O extractions shared a relatively close relationship with microdialysis fluxes, we used measures of total N ($NH_4^+ + NO_3^-$) in a linear regression (Figure 4-3, bottom graph) to generate an equation predicting total N fluxes (in µmols N cm^{-2} day^{-1}) using H_2O extractions (mg N kg^{-1} soil DW), as follows:

$$y = 1.398x + 2.912 \qquad (r^2 = 0.64, p < 0.001) \qquad (2)$$

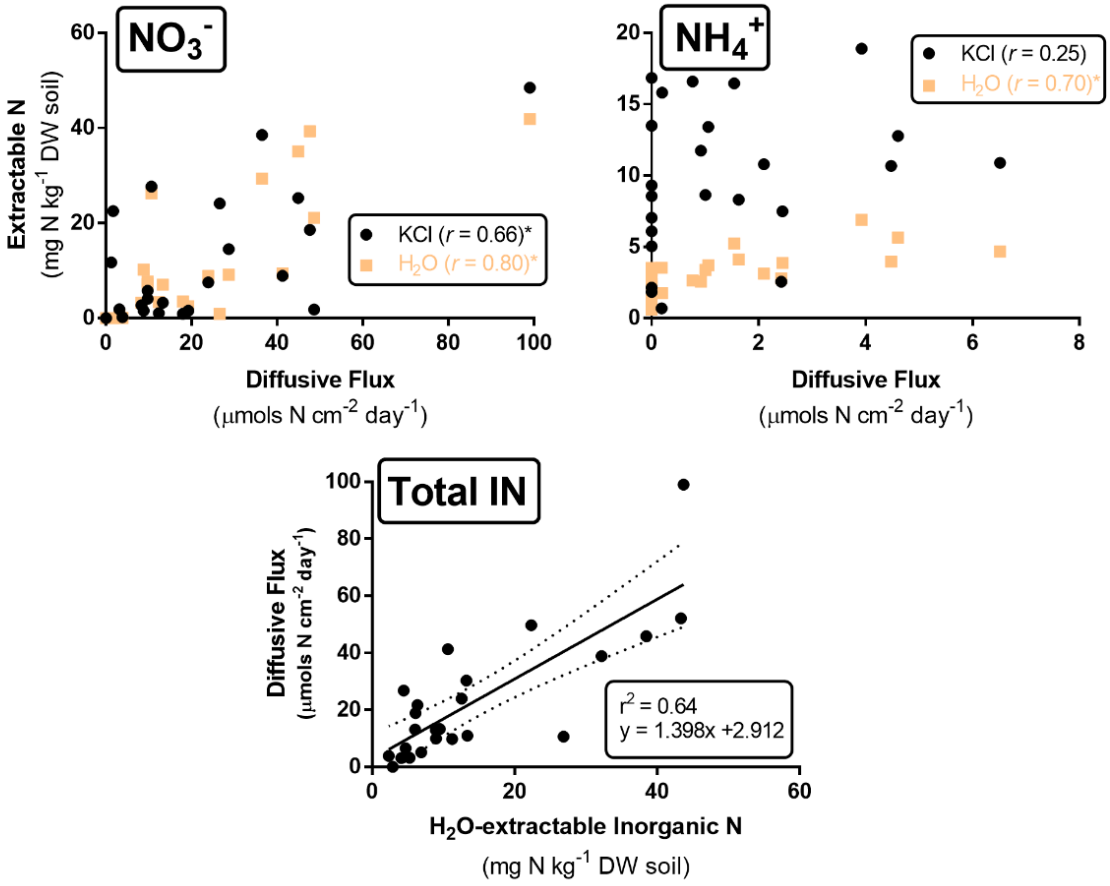

Figure 4-3. Top graphs: Correlations of NO_3^- and NH_4^+ fluxes (µmols N cm^{-2} day^{-1}) versus extractable-N (KCl, in black; H_2O, in orange). **Bottom graph:** Linear regression of total N fluxes and H_2O-extractable inorganic N; dotted lines represent 95% confidence intervals

4.3.3 Nitrogen relationships with physical, chemical and biological soil parameters

We compared N fluxes, N plateaus and N concentrations (from extractions) with physical and chemical soil parameters to determine relationships between soil environments and N availability (Table 4-2). Flux plateaus for NO_3^- were negatively correlated with Total N and clay content, but were positively correlated with EC. Plateaus for combined N (NO_3^- + NH_4^+) were also had a negative correlation with clay content but were positively correlated with EC. In contrast, plateaus for NH_4^+ were negatively correlated with pH.

N fluxes had little correlation with soil parameters, except for NO_3^-, which was positively correlated with pH, and combined N fluxes, which were positively correlated with EC and pH (Table 4-2).

Table 4-2. Pearson moment correlations between estimated flux plateaus (from non-linear regressions of total N depletion), N Flux, and KCl extractable-N, versus soil parameters – Total N (%), Clay content (%), electro-conductivity (EC). Numbers represent Pearson r values, asterisks represent significant relationship (* - $p < 0.05$; ** - $p < 0.01$). Dashes represent correlations which were not significant ($p \geq 0.05$)

	Total N	**% Clay**	**EC**	**pH**
N Plateau				
NO_3^-	**-0.50***	**-0.53***	**0.46***	-
NH_4^+	-	-	-	**-0.43***
Combined N	-	**-0.51***	**0.43***	-
N Flux				
NO_3^-	-	-	-	**0.43***
NH_4^+	-	-	-	-
Combined N	-	-	**0.44***	**0.48***
KCl Extractable-N				
NO_3^-	-	**-0.61****	**0.46***	**0.42***
NH_4^+	**0.45***	**0.55****	-	**-0.59****
Combined N	-	**-0.46***	-	-
H_2O Extractable-N				
NO_3^-	-	-	**0.46***	-
NH_4^+	-	-	-	**-0.41***
Combined N	-	-	**0.47***	-

NO_3^- concentrations in KCl extractions were positively correlated with EC and pH, but were negatively correlated with clay content. In contrast, NH_4^+ concentrations in KCl extractions were positively correlated with Total N and clay content, but were negatively correlated with pH. Combined N concentrations in KCl extractions shared only a negative correlation with clay content.

Nitrogen concentrations in H_2O extractions did not significantly correlate with soil properties, with the exception of NO_3^- and combined N, where both measures were positively correlated with EC, and NH_4^+, which negatively correlated with pH.

Bulk density did not correlate with N measurements, except for NH_4^+ in KCl extractions, where there was a negative relationship (Pearson r = -0.44, p = 0.03).

When combining these variables into a principle components analysis, we observed that observed that for measurements of NO_3^- (Figure 4-4, NO_3^-), extractions and microdialysis measurements (absolute fluxes and plateaus) were similarly affected by Factor 1 (Eigenvalue = 5.24 explained variance = 47.7 %), likely related to soil texture and chemical properties. However, fluxes and H_2O extractions were most affected by a second factor (Eigenvalue = 1.94, explained variance 17.7%) which may be related to the magnitude of the soluble N pool. Both factors combined explained 65.34% of the variation in the combined dataset.

Absolute NH_4^+ fluxes and plateaus, as well as H_2O-extractable NH_4^+ was mostly unaffected by factor 1 (Eigenvalue = 4.65, explained variance 42.3%), but was greatly influenced by factor 2 (Eigenvalue = 2.57, explained variance 23.44%) (Figure 4-4, NH_4^+). In contrast, KCl-extractable NH_4^+ was mostly influenced by factor 1, and was closely related along the same axis as variables such mineralisation, Total OC, Total N and clay content. Both factors combined explained 65.73% of the variation in the combined dataset.

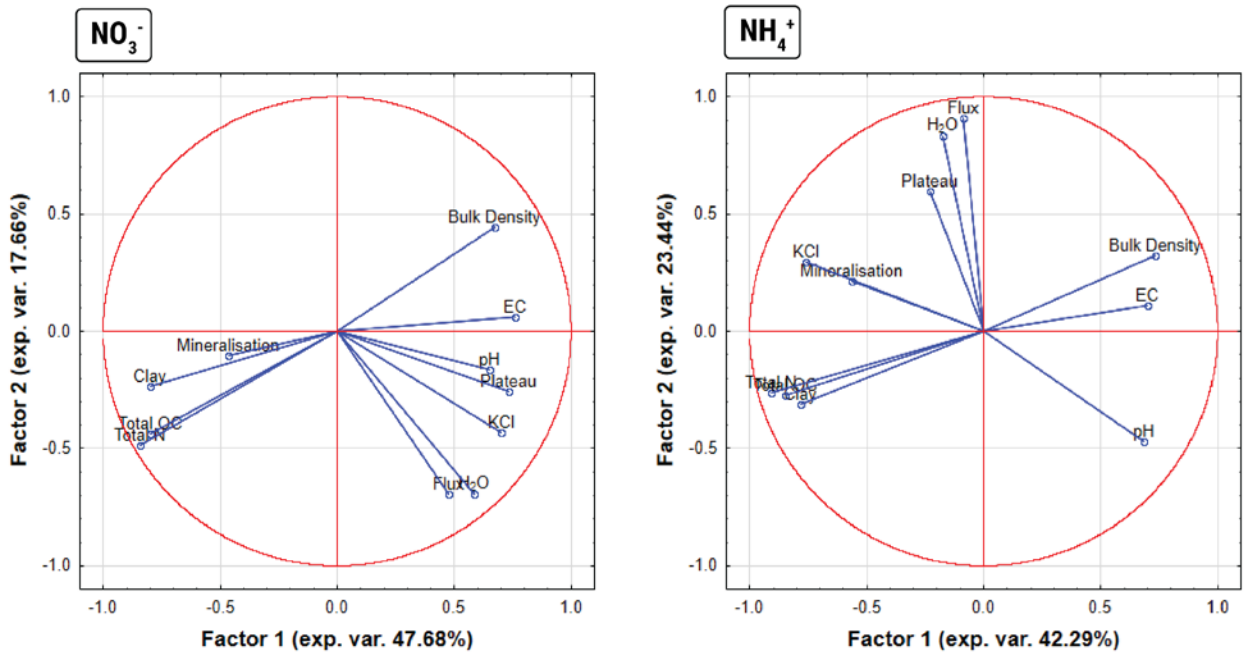

Figure 4-4. Principal components analysis of soil physical (clay content, bulk density) and chemical (total N, total OC, EC, pH, mineralisation) properties, and measurements of NO_3^- and NH_4^+ using extractions (H_2O, KCl) and microdialysis (flux, plateau).

4.3.4 Relationship between fluxes and potentially-mineralisable nitrogen

To determine if a relationship exists between fluxes sampled over the course of a day, with measures of mineralised N released over a 14-day incubation. Neither absolute fluxes or plateaus had a significant correlation with potentially-mineralisable N (PMN) (N fluxes: Pearson r = -0.03, p = 0.9; plateaus: Pearson r = -0.06, p = 0.76).

4.3.5 *Relationships between soil parameters*

Total N, Total OC and clay content shared highly significant positive correlations with each other, and significantly negative correlations with bulk density and EC (

Table **4-3**). Soil cations (Ca, Na, Mg, K) shared no relationship with EC (p > 0.05).

To investigate whether the relationship between soil texture and bulk density could be explained by starting moisture conditions (field capacity) at time of microcosm-filling, we found that clay content had a significantly positive correlation with field capacities of each soil (Pearson r = 0.59, p = 0.002).

Table 4-3. Pearson moment correlations between soil parameters. Numbers represent Pearson r values, asterisks represent significant relationship (* - $p < 0.05$; ** - $p < 0.01$, *** - $p < 0.001$). Dashes represent non-significant relationships ($p \geq 0.05$).

	Total OC	**% Clay**	**EC**	**pH**	**Bulk Density**
Total N	**0.95*** **	**0.75*** **	**-0.62****	**-0.47***	**-0.66*** **
Total OC		**0.62****	**-0.65****	**-0.47***	**-0.60****
% Clay			**-0.47***	**-**	**-0.68*** **
EC				**0.52***	-
pH					-

4.4 Discussion

4.4.1 KCl extractions release adsorbed pools of ammonium

Although soil scientists and ecologists have relied extensively on extractions to improve the understanding of N cycling and availability in soils (Ros et al., 2009), it is recognised that these methods are disruptive and introduce artefacts which can bias estimates of available N (Jones and Willett, 2006, Rousk and Jones, 2010, Inselsbacher, 2014). In 24 sugarcane soils that had not been N fertilised for at least 12 months, extractions (particularly using KCl) mobilise adsorbed NH_4^+ fractions

from soils with greater total N contents and increase the presence of NH_4^+ in the extracts. In contrast, NH_4^+ contributes significantly less to total N fluxes when sampled by microdialysis, regardless of total N status. Additionally, NH_4^+ flux plateaus shared no obvious relationship with the majority soil parameters tested here, and so the smaller contribution of NH_4^+ to fluxes could be attributed to the minimal disturbance of adsorbed NH_4^+ on soil surfaces.

That KCl extractions sample from adsorbed N pools is not a new finding, as it is generally accepted that salt extractions provide a measure of *exchangeable* N – the pool of adsorbed soil N which can be displaced by exchange with potassium ions at charged soil sites (Ros et al., 2009). However, application of KCl extractions are routinely used to inform N management decisions in crop systems, to estimate potentially-available N; included as part of industry-standard technical manuals (e.g. Rayment and Lyons (2011). Cost and ease of deployment are significant factors in such settings, but the use of salt extractions will inevitably provide a skewed view of available N, with a contribution from previously unavailable sources. Historically, this may have been viewed as an indication of a healthy soil nutrient status, with inorganic N viewed as the only forms available for plant uptake in recent decades despite evidence that plants also acquire organic forms of N (Paungfoo-Lonhienne et al., 2012). Now, with our understanding of the greater diversity of N forms that plants are capable using (Näsholm et al., 2009, Schmidt et al., 2013, Selosse et al., 2017), such views are being confronted. The much smaller contribution of NH_4^+ fluxes may suggest that NH_4^+ is, under *in situ* conditions, mostly unavailable; in contrast to both NO_3^- and organic N (such as amino acids) which may constitute a larger proportion of available N (Chapter 3, Chapter 6, Inselsbacher and Näsholm, 2012a, Oyewole et al., 2014, Oyewole et al., 2016).

We show that major differences between KCl extractions and microdialysis will lie in the disturbance of adsorbed NH_4^+ pools, which are dependent on soil factors that enhance N adsorption, such as increased clay content (Figure 4-5). As microdialysis minimises disturbances and exchange within these pools, NH_4^+ fluxes remain minimal. With this in mind, we suggest that both microdialysis and KCl extractions are complimentary methods, which when combined can provide useful information about the locations and abundance of N pools – whether they are labile in soil solution, or adsorbed to soil surfaces.

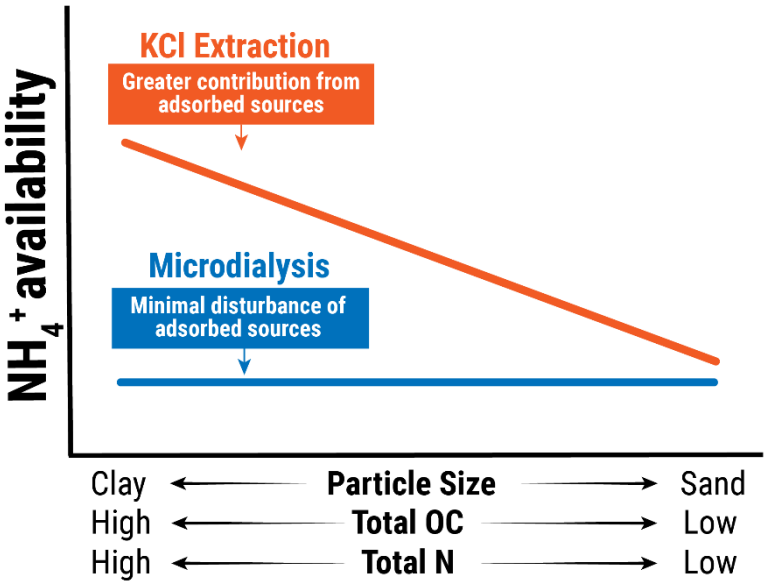

Figure 4-5. Conceptualisation of NH_4^+ availability in sugarcane soils, as estimated by extraction (in orange) and microdialysis (in blue), based on soil parameters tested in this study – particle size, total organic C (total OC) and total N.

These findings may not be true under conditions which artificially increase NH_4^+ concentrations – for instance, recently fertilised soils (Inselsbacher et al., 2014, Brackin et al., 2015), and this may be in part due to saturation of soil exchange sites, driving a greater mobility of NH_4^+ in solution (Gunda and Egbert, 1996, Nieder et al., 2011). Yet, these conditions may be transient as N is acquired by plants, immobilised by soil microbes, or lost through leaching or gaseous N emissions (Robinson et al., 2011), and so under conditions with limited inputs, Figure 4-5 may represent NH_4^+ availability as sampled by each method.

4.4.2 Nitrate mobility increases in sandier soils

In contrast to NH_4^+, the proportion of NO_3^- in KCl extracts and in fluxes showed similar relationships with the soil environment, with increased availability with larger soil particle size. This demonstrates the increased mobility of NO_3^- in soil environments with large pore spaces – particularly in moist soil conditions, where interactions with soil surfaces are minimised (Tinker and Nye, 2000). A wealth of research has highlighted the greater NO_3^- mobility in highly porous soils, particularly soils under agricultural management (Di and Cameron, 2002), and so this finding supports the potential for flux plateaus to be used as a measure of ion mobility in context with the physical soil environment.

NO_3^- was also weakly correlated with greater EC for both flux plateaus and N concentrations measured by KCl and H_2O extractions. Although EC could indicate the presence of concomitant ions which can facilitate faster diffusion of NO_3^- (Tinker and Nye, 2000), EC often increases under

historical fertiliser application (Zhao et al., 2014), suggesting that greater availability of NO_3^- observed here could be linked to previous fertiliser inputs at some soil sites. EC is a complex measure of both conductive properties of dissolved ions and water availability (Friedman, 2005), and is often used as a predictor of NO_3^- itself (Eigenberg et al., 2002, Hu et al., 2005). Given that other cations (Ca, Na, K and Mg) shared no correlation with EC, NO_3^- concentrations in soil solutions may have influenced EC measurements here, and consequently contributed to our observed correlations. pH also influenced NO_3^- fluxes and KCl-extractable NO_3^-, but not plateaus, suggesting that pH-dependent effects (such as anion exchange capacity, Havlin, 2017) may affect the NO_3^- pool size, but not necessarily its movement.

It is important to note that – given the minimal contribution of NH_4^+ to N fluxes in these soils – changes to NO_3^- fluxes will have disproportionate influence on total inorganic N fluxes. Our microdialysis findings suggest that soils with higher clay content, although containing greater total N, may have less N mobility, at least when disregarding fresh organic or inorganic inputs.

4.4.3 Fluxes are similar to water extractions

We found that N concentrations sampled by H_2O extractions shared a closer relative relationship with fluxes than KCl extractions. This finding is somewhat expected given that both likely sample solutes which are freely available and water soluble (Ros et al., 2009). Using this relationship, we have calculated a linear equation (Eqn. 2) that may assist with approximate conversion between soluble total inorganic N concentrations and fluxes. Given that fluxes are reported in similar units as uptake rates for plant roots, such information may be useful for N management decisions which maximise crop uptake of fertiliser-N in agricultural systems (Brackin et al., 2015).

However, we advise some caution in its application, given that NH_4^+ representation was still significantly greater in H_2O extractions than in fluxes. There is likely some degree of release from loosely bound N pools on soil surfaces; however, biological influences such as organic N mineralisation during extraction may have played a more important role in increasing NH_4^+ concentrations here. Inselsbacher (2014) has shown the potential of KCl and H_2O extractions to mineralise organic N; additionally, Rousk and Jones (2010) demonstrated significantly greater mineralisation of ^{14}C-labelled glucose and amino acids in H_2O extractions (*c.f.* K_2SO_4). Mineralisation during extraction may therefore be a more significant artefact in H_2O extraction than in KCl extraction, and may reduce their effectiveness to relate to microdialysis fluxes.

4.4.4 Inorganic nitrogen fluxes are unrelated to nitrogen mineralisation assays

We found no relationship between N fluxes and potentially mineralisable N, which initially suggests that biological mineralisation of organic N has minimal influence on N fluxes measured by

microdialysis. Mineralisation is predominantly mediated by soil microbes (Schimel and Bennett, 2004, Geisseler et al., 2010), which given their diversity and short generation times (Nemergut et al., 2013) can quickly respond to nutrient gradients on short time scales (hours to days), and so processes and measurements at the scale of a single day may not necessarily be representative of longer-term measures.

As standard mineralisation assays also rely on KCl extractions to measure mineralised N between two or more time points (Rayment and Lyons, 2011), adsorbed NH_4^+ (which may accumulate during incubation) may be sampled by extraction, but not apparent when measured with microdialysis fluxes. However, this may highlight the importance of deploying both sampling methods when investigating the contribution of mineralised N to bio-available pools, particularly if much of the mineralised N will be rapidly adsorbed or immobilised. Microdialysis will therefore provide a plant-relevant view of N availability over time, whereas KCl extractions will generate a broader picture of total mineralised N, and their locations within the soil environment.

4.5 Conclusions

We show that there are distinct differences in N pools measured by KCl extractions and microdialysis, and suggest that both methods provide complimentary measures of soil N, where KCl extractions will represent a total view of N within a system, while microdialysis fluxes represent the bio-available perspective – in context with prevailing soil conditions. By combining these methods, we have shown that in unfertilised sugarcane soils, NH_4^+ remains largely bound to soil surfaces, and its bioavailability may be lower than estimated by H_2O extractions. We also show that inorganic N fluxes are not directly linked to mineralisation rates, which may be in part related to the degree of recently mineralised NH_4^+ that is quickly adsorbed, and thus excluded from microdialysis sampling.

We find that flux plateaus calculated *via* depletion curves have potential for exploring transport dynamics of solutes, overcoming initial depletion of N pools around the microdialysis probe to observe rates of diffusive supply from surrounding soil regions. It is interesting that NH_4^+ plateaus showed little relationship with greater clay or C content, already known to increase the likelihood of fixation (Nieder et al., 2011). However, this study was limited by the degree of native inorganic N observable in these soils, and given the prospect that native NH_4^+ was mostly adsorbed, precise *in situ* calculations of plateaus use may be limited to solutes which are more mobile in solution. Alternatively, NH_4^+ plateaus are more closely linked with other soil properties not tested here, such as microbial activity or de-fixation processes. In any event, plateaus may provide a useful measure for quantifying solute mobility *in situ*, and future work using standard concentrations of solutes may provide new insights into the roles of their mobility on bioavailability.

5 Microdialysis as an *in situ* technique for sampling soil enzymes

5.1 Introduction

The decomposition of necromass from plants, animals and microbes is a critical ecological process that releases bio-available forms of nutrients from otherwise inaccessible structures. One of the primary agents of decomposition in soil are extracellular enzymes (exoenzymes), which catalyse the breakdown of specific molecular bonds within complex polymeric molecules to generate smaller bio-available forms. Exoenzymes are secreted by both plants and saprobiont microbes such as fungi and prokaryotes to digest necromass, and are a strategy to mobilise nutrients for uptake. Ectomycorrhizal fungi and ectomycorrhizas are recognised for their ability to mobilise and transfer nutrients from soil organic matter to woody plants (e.g. Chalot and Brun, 1998).

Given the diverse and complex nature of organic materials in soil, both recent necromass and processed soil organic matter (Schmidt et al., 2011), a broad suite of enzymes with varied catalytic activities must be deployed to forage what nutrients are secured there – primarily C, N and P. This may involve a succession of bacteria and fungi, each with an arsenal of enzymes which depolymerise not only labile, but also more recalcitrant molecules resistant to most enzymatic attack (Berg and McClaugherty, 2014). It is clear then that the regulation of nutrient bio-availability is dependent on exoenzyme activity (EEA), and the ability and responses of soil microbes and plants that produce them (Schimel and Bennett, 2004).

Numerous methods have been developed to study EEA, with most following a similar approach (Figure 5-1): incubation of a diluted soil subsample with a substrate which investigates an enzyme of interest, for instance a model protein such as casein to quantify protease activity, and measuring the breakdown product with colourimetric or fluorometric assays. These assays provide a measure of 'potential' EEA, i.e. activity levels that could be possible under optimum conditions of pH, temperature, substrate availability and soil homogenisation (Wallenstein and Weintraub, 2008, Burns et al., 2013). However, quantifying EEA at natural, or 'actual' levels is ecologically relevant, and likely to be more aligned with true rates of nutrient cycling and bioavailability (Caldwell, 2005). To address this concern, some studies have modified conventional assays by using buffers and incubation temperatures that reflect soil conditions, and monitoring EEA without artificial addition of substrates (Weintraub and Schimel, 2005, Reiskind et al., 2011). However, it can be difficult to compare soils with variable substrate availability, which is typically limiting and spatially heterogeneous (Vranova

et al., 2013, Kuzyakov and Blagodatskaya, 2015). Hence, standardisation of assays using non-limiting substrate concentrations are useful to compare between soils – so called 'V_{max}', based on Michaelis-Menten kinetics, where enzyme reaction rates are no longer dependent on substrate concentration (Dick, 2011) (Figure 5-1).

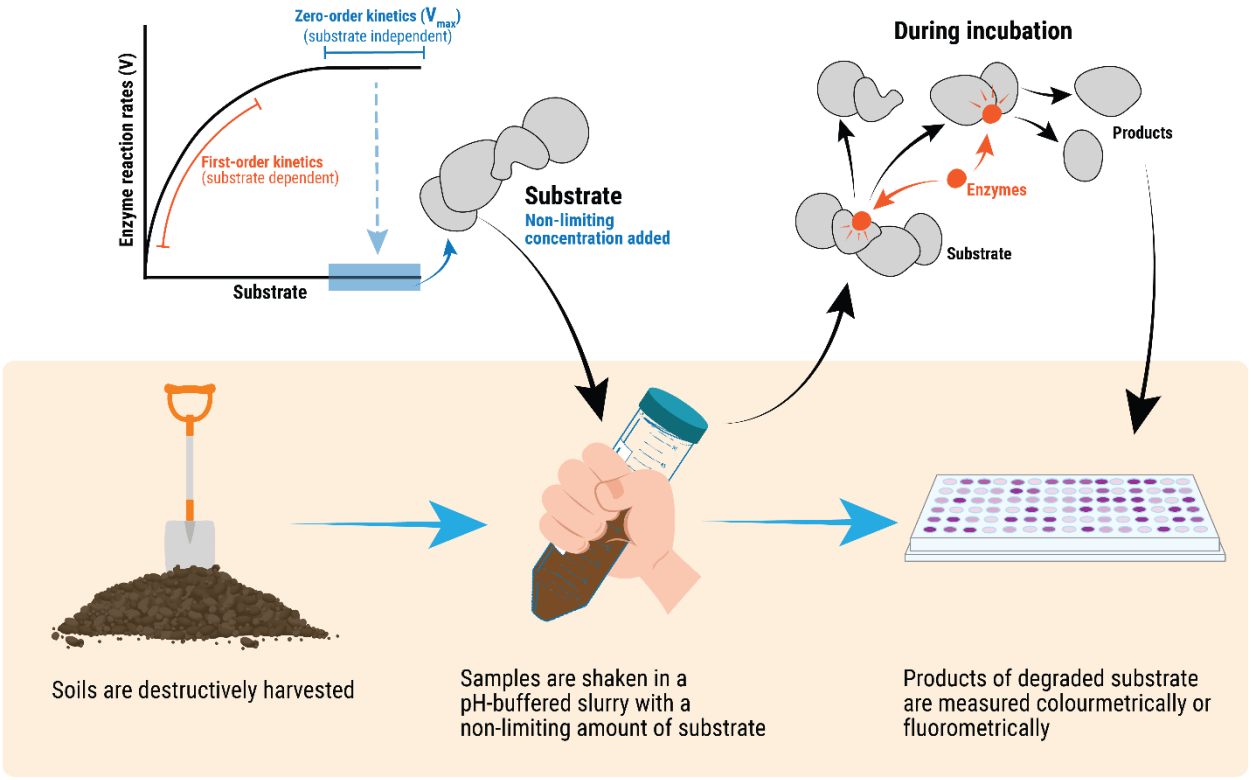

Figure 5-1. An outline of how conventional enzyme assays are performed. Soils are destructively harvested, with subsamples shaken in a slurry with a non-limiting amount of substrate. Products of enzymatic degradation are then measured colourmetrically or fluorometrically.

Conventional EEA assays also fail to differentiate between spatially distinct enzyme pools – those which are free in soil solution, and those which are bound to clay minerals and organic matter are stabilised (Nannipieri, 2006, Bonmatí et al., 2009). Such stabilised enzymes are thought to contribute most of the activity observed in conventional assays (Kandeler, 1990, Wallenstein and Weintraub, 2008) – however, conformational changes, blockage of active sites, and spatial isolation from substrates means that adsorption likely reduces the activity of stabilised enzymes *in situ* (Allison and Jastrow, 2006, Nannipieri, 2006). Nevertheless, prolonged protection from denaturation or proteolysis (Nannipieri et al., 1988, Burns et al., 2013) means that stabilised enzymes are likely to dominate the total enzyme pool. As the sampling and shaking of soils in conventional soil enzyme assays disrupts the natural aggregation of soils, and removes diffusional barriers between enzymes and substrates, the contribution of this fraction of soil-residing enzymes to measured enzyme activity is likely to be substantial (Nannipieri et al., 2012).

In situ approaches to quantifying EEA may provide a more accurate picture of the relationships held between spatial and temporal soil processes and enzyme activity, but only a few methods exist. Zymography is a quantitative method to assess the distribution of enzymes within an intact 2D soil profile (Dong et al., 2007, Spohn et al., 2013, Spohn and Kuzyakov, 2014, Hofmann et al., 2016). The most recent iteration of the technique involves incubating an exposed soil face with a thin polyamide membrane sheets soaked in a substrate of interest (Spohn and Kuzyakov, 2014, Hofmann et al., 2016). The membrane and soil face are separated by a thin agarose gel (0.1 cm). After incubation, membranes are imaged colourmetrically or fluorometrically to measure the spatial depletion of substrate remaining on the membrane. Zymography is non-destructive, and also enables temporal resolution of enzyme distributions (Spohn and Kuzyakov, 2014) but requires delivery of non-limiting substrate to exposed soil faces, and therefore may represent 'potential' *in situ* activity rather than actual activity (Wallenstein and Weintraub, 2008).

Genomic and transcriptomic approaches explore the potential for enzyme expression and actual enzyme expression within a microbial community, but with limitations surrounding lack of knowledge of enzyme-encoding gene diversity, and low concentrations of extractable target mRNA (Wallenstein and Weintraub, 2008, Carvalhais et al., 2012, Garoutte et al., 2016). Additionally, post-translational modifications, re-assimilation and short life-spans of free enzymes may mean transcriptional information does not necessarily correlate with enzyme activity (Nannipieri et al., 2012, Rocca et al., 2014). However, ongoing advances are being made in understanding and linking transcription products with the metaexoproteome in complex matrices (Alessi et al., 2017). Proteomic approaches can directly identify a diversity of soil proteins including enzymes (Schulze et al., 2004, Alessi et al., 2017), but detection can be hampered by high turnover of proteins, along with low concentrations and heterogeneity in soil (Giagnoni et al., 2011, Nannipieri et al., 2012, Keiblinger et al., 2012). Dissolved phenolic compounds present in soils can also interfere with common protein quantification methods (Roberts and Jones, 2008, Redmile-Gordon et al., 2013). Clearly, there is a need for alternative *in situ* methods to distinguish between soluble and bound exoenzymes and allow their *ex situ* characterisation.

Here we investigate the potential of the microdialysis technique to sample enzymes *in situ* – described in greater detail in Chapter 2. Originally developed for biomedical research, the method has seen increased use in environmental and soil research, sampling solutes from soils in laboratory and field settings (Miró and Frenzel, 2005, Sulyok et al., 2005, Inselsbacher et al., 2011, Oyewole et al., 2014, 2016, 2017, Brackin et al., 2015, Jämtgård et al., 2018). The method involves the insertion of small probes into soil, each with a semi-permeable membrane. Perfusate (usually water) is pumped behind the membrane, and solutes in the soil solution diffuse down a concentration gradient, pass over the

membrane, and are collected for later analysis. The small probe size the passive nature of sampling (using diffusion alone) provides a measure of solute fluxes under near undisturbed conditions, and will likely sample mobile solutes free in soil solution (Miró et al., 2005, Inselsbacher et al., 2011).

Microdialysis could therefore provide a halfway point between conventional EEA assays (which rely on optimal incubation conditions) and modified assays exploring 'actual' EEA (under prevailing soil conditions) (Figure 5-2). Enzymes are sampled non-destructively, and size discrimination imparted by the membrane may allow for enzymes to be separated from their microbial producers. Substrate incubation could then be performed directly with enzymes only – allowing quantification of enzyme activity relevant to *in situ* enzyme concentrations but using non-limiting substrates to calculate EEA at V_{max}. This may allow for an *in situ* measure of EEA that is also comparable between soils with variable native substrate availability. Furthermore, the innate bias of microdialysis towards free solutes in soil solution provides an avenue to explore the contribution of free and stabilised enzyme pools to total enzyme activity.

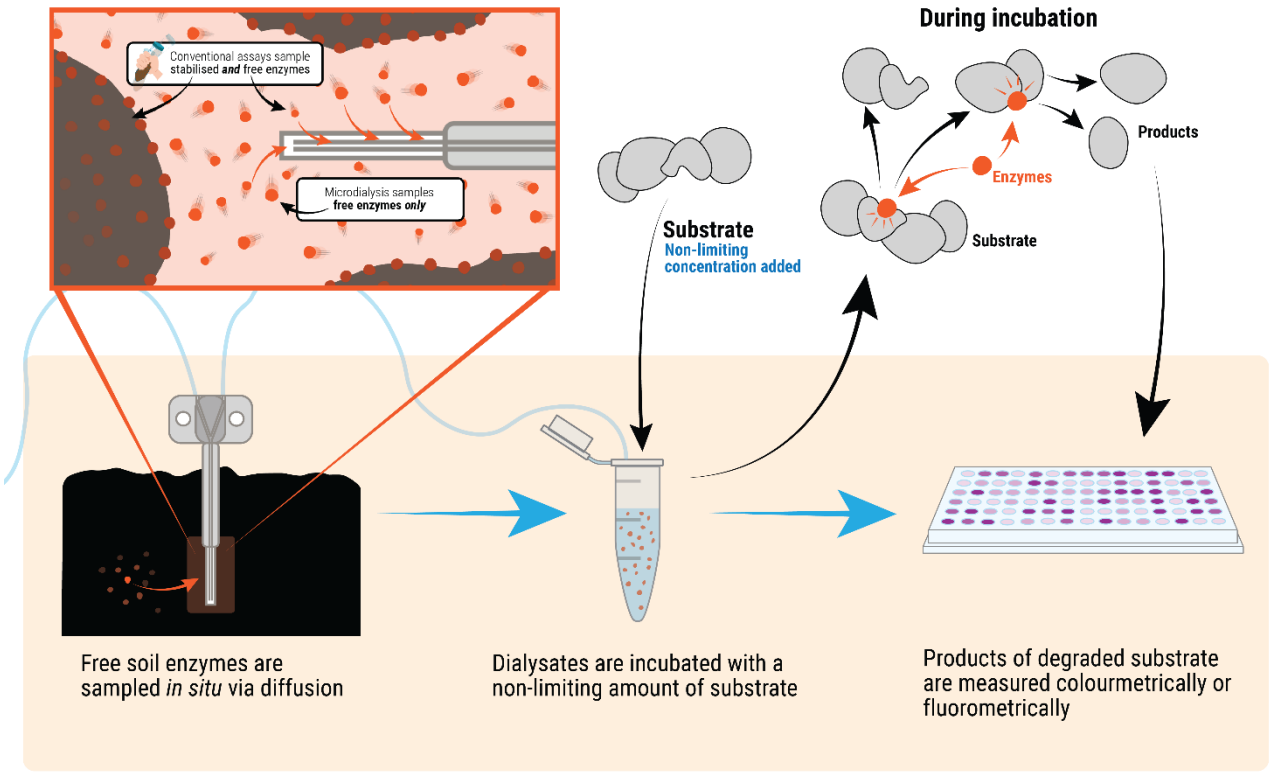

Figure 5-2. An outline of *in situ* enzyme sampling using microdialysis. Microdialysis probes are positioned into intact soil *in situ*, with free enzymes sampled via diffusion. Dialysates are incubated similarly to conventional assays, with non-limiting concentrations of substrate, before products of enzymatic degradation are measured colourmetrically or fluorometrically.

Problems observed with sampling proteins (such as enzymes) using microdialysis in biomedical studies include the slow diffusion of proteins and resulting in low protein concentrations in diffusate (Kjellström et al., 1999, Schutte et al., 2004, Rosenbloom et al., 2005). Such studies have sampled from protein solutions where barriers to diffusion are minimal (Bungay et al., 1990), but soil represents a far more complex environment. Protein diffusion in soil decreases with interactions in the soil matrix and may be impacted by low soil moisture content and interception by microbes or proteolytic enzymes (Tinker and Nye, 2000, Geisseler et al., 2010).

We therefore explore the effects of soil environments on enzyme diffusion by comparing microdialysis recoveries of a standard protease solution from simple to complex environments, from the standard enzyme solution and from soil spiked with the standard solution. We also examine the effect of decreasing soil moisture on protein recoveries. As the addition of fresh plant litter generally increases EEA (as observed in Chapter 3), we use the expected increase to study the sensitivity of microdialysis for detecting changes in *in situ* protease activity in lab-incubated soils amended with soybean litter, and compare the findings to a conventional protease activity assay. Lastly, we use the same soil-litter set-up to explore the contribution of free and stabilised enzymes to total hydrolytic activity, using microdialysis sample enzymes *in situ* and from soil extractions to estimate 'free' and 'free + stabilised' enzyme fractions.

5.2 Methods

5.2.1 Microdialysis system setup

Four infusion pumps (CMA 4004; CMA Microdialysis AB, Solna, Sweden) were equipped with a total of 16 micro-syringes (2.5 mL; SGE Analytical Science / Trajan Scientific, Ringwood, Australia). Each syringe was connected to a microdialysis probe, through-connected to a 1.5 mL collection tube, kept on ice during sampling. CMA 20 microdialysis probes (CMA Microdialysis AB, Solna, Sweden) were used for all sampling, and feature a membrane of 10 mm × 0.5 mm (surface area 0.159 cm^2), with a 100 kDa molecular weight cut-off (MWCO). Where possible, negative hydrostatic pressure was encouraged by positioning the probes at a height above the collection tubes, to prevent excessive perfusate loss (Rosenbloom et al., 2005, Chu et al., 2014).

5.2.2 Soil collection

Two soils were collected as part of a Sugar Research Project: "Decision support for informed nitrogen management: soil N mineralisation" (SRA 2015/069) described in Chapter 4. These soils were chosen for their differing clay content (Table 5-1).

Table 5-1. Soil information for two soils used in this enzyme study.

Soil ID	Clay content	pH	EC (µS)	Total C	Total N
"Sandy"	4.6 %	6.5	78.2	0.76 %	0.05 %
"Clay"	48.6 %	7.4	124.6	1.19 %	0.1 %

5.2.3 Preparation of protease standard solution

Protease Type XIV from *Streptomyces griseus* (product number P5147, Sigma-Aldrich) was used for the formulation of a standard protease solution and consists of three caseinolytic enzymes: *S. griseus* Protease A (18,093 kDa), *S. griseus* Protease B (18,629 kDa) and *S. griseus* Trypsin (22,918 kDa) (Sigma-Aldrich, 2018), although previous work has reported active components between 16 – 27 kDa (Sweeney and Walker, 1993).

Protease Type XIV was dissolved in a 0.01 M sodium acetate buffer (pH 7.5), with 0.005 calcium chloride added as a precaution to protect enzymes from autolysis (Sweeney and Walker, 1993). A 20 mg mL^{-1} stock was prepared, from which a 1 mg mL^{-1} solution was made.

5.2.4 Recovery of protease standard from solution

Sixteen microdialysis probes were inserted into 1.5 mL tubes filled with either 1 mg mL^{-1} protease solution, or Na-Acetate buffer (each with eight replicates) and perfused with Na-Acetate buffer at a flow rate of 1 µL min^{-1} for 5 hours. Protease solution was kept at room temperature (24°C) during sampling, and dialysate collection tubes (1.5 mL centrifuge tubes) were kept on ice at all times. Immediately following sampling, dialysate and protease standard was analysed for total protein concentration, and potential protease activity.

As autolysis was found to occur in protease samples even after freezing at -20 °C, the experiment was repeated to collect samples for SDS-PAGE analysis, with samples used fresh (but kept on ice).

5.2.5 Recovery of protease standard from soil solution

5 g of air-dried clay soil was weighed into 10 mL sterile sample tubes and wetted with 3.2 mL, 1.6 mL and 1.12 mL of Na-Acetate buffer + protease standard, wetting the soil to 200%, 100% and 70% WHC; for each, the protease concentration was adjusted to deliver the same dose of protease (3.2 mg) to each soil replicate. Microdialysis probes were inserted into soils (one probe per tube, eight per treatment) and sampled at 1 µL min^{-1} for 5 hours. Soils were kept at room temperature (approximately 24°C) during sampling, and dialysate collection tubes were kept on ice at all times. Following sampling, dialysates were analysed for total protein concentration and potential protease activity, with

remaining dialysate frozen at -20°C for SDS-PAGE. Additionally, soil solutions were collected by centrifugation at 4500 RPM for three minutes, with supernatants collected for analyses as above.

As autolysis was found to occur in samples even after freezing at -20 °C, the experiment was repeated to collect samples for SDS-PAGE analysis, with samples used fresh (but kept on ice).

5.2.6 Estimation of native enzyme activity in soil amended with soybean litter

To investigate the native enzymatic response of microbes to the input of fresh litter, we sampled enzymes *in situ* using the microdialysis technique. 20 g of fresh 'sandy' soil (16.31 g dry weight) and 'clay' soil (17.12 g dry weight) was mixed with 0.2 g of soybean litter (dried and ground; C/N: 12.6) and inserted into microcosms consisting of a modified centrifuge tube (Inselsbacher et al., 2009). Control soils (not mixed with soybean litter) were also included; for each treatment, n = 4. Soils were incubated on a lab bench for two days at approximately 24 °C, and at 70 % WHC. On the morning of sampling, soils were watered to 100% WHC. Microdialysis probes were then inserted into microcosm (one probe per microcosm) and perfused with ultrapure water at a flow rate of 1 µL min^{-1} for 5 hours. Soils were kept at room temperature (approximately 24 °C) during sampling, and dialysate collection tubes were kept on ice at all times. Following sampling, dialysates were analysed for potential protease activity.

Additionally, dedicated microcosms (n = 4 for each treatment) were destructively harvested using a conventional potential protease assay, described in 5.2.10.

5.2.7 Effect of non-sterile setup on dialysate collection

To investigate the potential effect of a non-sterile microdialysis setup on subsequent measurements of protease activity, we sampled enzymes from litter soil treatments as above (incubated for three days prior), with the addition of 0.1M sodium azide (10% final concentration, or milliQ water as control) in collection tubes, with protease activity tested as above.

5.2.8 Estimating free and stabilised enzyme pools

Clay soil was hand-mixed with soybean litter at the same ratios in 5.2.6, with 5 g of mixed soils added to 10 mL sterile sample tubes. Soils were water to 70% WHC and incubated overnight at room temperature (approximately 24 °C). The following morning, soils were watered to 100% WHC. To estimate free enzyme pool, microdialysis probes were inserted into soils and perfused with ultrapure water at a flow rate of 1 µL min^{-1} for two hours. Soils were kept at room temperature (approximately 24 °C) during sampling, and dialysate collection tubes were kept on ice at all times. To estimate stabilised + free enzyme pool, soils were then extracted with 25 mL of 0.1 M trishydroxymethyl-methane (THAM) buffer (pH 8.1) for one hour. Soil extracts were then resampled using microdialysis

as described above. All samples were analysed for total enzyme activity using a modified fluorescein diacetate (FDA) hydrolysis assay (Green et al., 2006), where 2 µL of FDA (dissolved in acetone) was mixed with 100 µL of dialysate, and incubated for two hours at 37 °C before reading absorbance of samples on a spectrophotometer at 490 nm. Stabilised pools were estimated as the difference between free and stabilised + free measurements, where the latter measurements were multiplied by give to account for dilution of enzymes from additional THAM buffer.

5.2.9 Estimation of total protein concentration

Total protein concentrations were determined using a modified Lowry assay as described by Redmile-Gordon et al. (2013) with some modifications. Briefly, 50 µL of sample was combined with 100 µL of an alkali reagent in a 96-well microplate. The alkali reagent comprised of 3.5 mg $CuSO_4 \cdot 4H_2O$ 100 mL^{-1}, 7 g sodium potassium tartrate 100 mL^{-1}, and 70 g Na_2CO_3 L^{-1} 0.35 N NaOH; mixed at a ratio of 1:1:100. Finally, 100 uL of Folin-Ciocalteu Phenol Reagent (20% v/v) was added to each sample, mixed thoroughly with an 8-channel multi-pipette, and incubated at room temperature for 30 minutes before measuring absorbance at 700 nm on a spectrophotometer. To estimate the interference of humic compounds in samples taken from soil, additional subsamples were prepared similarly as above, but using a modified alkali reagent with the volume of $CuSO_4$ substituted with deionised water (Redmile-Gordon et al., 2013).

5.2.10 Estimation of potential protease activity

Protease activity was determined as per (Kandeler, 1996), with some modifications. 0.5 g of soil was used measurements of soil enzyme activity, and with reaction volumes scaled to fit within a 300 µL well of a microtitre plate. For microdialysis samples, 75 µL of dialysate was combined with 75 µL of casein solution before incubation at 50°C; after which 75 µL of trichloroacetic acid (TCA) was added before centrifuging at 15,000 RPM for three minutes, after which supernatants were collected for analysis. The length of the casein incubation was modified according to expected rates of activity and requirement for sensitivity: for samples taken from solutions containing protease standard, incubations were run for between 30 minutes and one hour. For microdialysis samples of native enzyme activity (with expectations of low enzyme yields), samples were incubated with casein for up to three hours. For all incubations, casein blanks (using perfusate buffer) were used to estimate spontaneous casein degradation during incubation.

5.2.11 Visualisation of proteins using SDS-PAGE

Pooled samples representing the protease standard solution, and microdialysis samples from both solution and spiked soil, were qualitatively analysed using SDS-PAGE. After sampling, replicates were pooled and protein was precipitated similarly to Jiang et al. (2004). 100% (w/v) trichloroacetic

acid (TCA) was added to dialysates (20% TCA final concentration), incubating overnight at 4°C. Samples were then centrifuged at 10000 RPM for 30 minutes, with TCA supernatant removed. Residual TCA was washed from protein pellets by adding 500 µL of ice-cold acetone and incubated on ice for 15 minutes before centrifuging as above. The acetone supernatant was removed, and the pellet left to dry for approximately 15 minutes.

Pellets were resuspended in 25 µL of 2x Laemmli sample buffer (Bio-Rad Laboratories, product no. 1610737) and heated at 70°C in a water bath for 10 minutes. 25 µL of sample + buffer solutions were loaded into a 12% Mini-PROTEAN TGX Gel (Bio-Rad Laboratories, product no. 4561044) and run in an electrophoresis cell at 200V for approximately 30 minutes. All gels were run with a Precision Plus Protein Unstained Protein Standard (Bio-Rad Laboratories, product no. 1610363).

After electrophoresis, gels were stained using a Bio-Rad Stain Kit Plus (Bio-Rad Laboratories, product no. 1610449). Briefly, gels were fixed for 30 minutes in a 50% methanol 10% acetic acid (plus fixative agent), washed twice with milliQ water for 10 minutes each, before staining with silver solution for approximately 20 minutes. Gels were then stopped in 5% acetic acid, before a final rinse in milliQ water. Gels were immediately imaged on a Bio-Rad Gel Doc XR (Bio-Rad Laboratories).

5.2.12 Statistical analysis

Total protein concentrations and protease activities were analysed using one-way ANOVA, followed by Tukey's HSD post hoc test to determine significant differences between treatments (GraphPad Prism 6, Graphpad Software, Inc.). Differences between litter treatments were evaluated using a one-tailed Student's T-Test (GraphPad Prism 6, Graphpad Software, Inc.).

5.3 Results

5.3.1 Recovery of protease standard from solution and spiked soils

To evaluate the efficiency of the microdialysis technique to recover protease enzymes, we sampled directly from a 1 mg mL^{-1} standard protease solution, and then sampled a soil spiked with the same standard protease solution at three water-holding capacities (200, 100 and 70% WHC).

Relative recovery and relative protease activity were greatest when sampled directly from standard solution (total protein, 17 ± 0.6 %; protease activity, 71 ± 1.7 %) (Figure 5-3). Both recovery and protease activity significantly decreased (when compared to sampling from solution) in moist soil at 200% WHC (total protein, 11 ± 1 %; protease activity, 32.9 ± 0.9 %), and significantly declined in drier soils. Noticeable is the relatively larger change of enzyme activity and total protein between

sampling from solution and soil at 200% WHC. Protease activity was reduced by 38.6% after microdialysis from soil than from standard solution, while total protein recovery was only 5.7% lower from soil than solution.

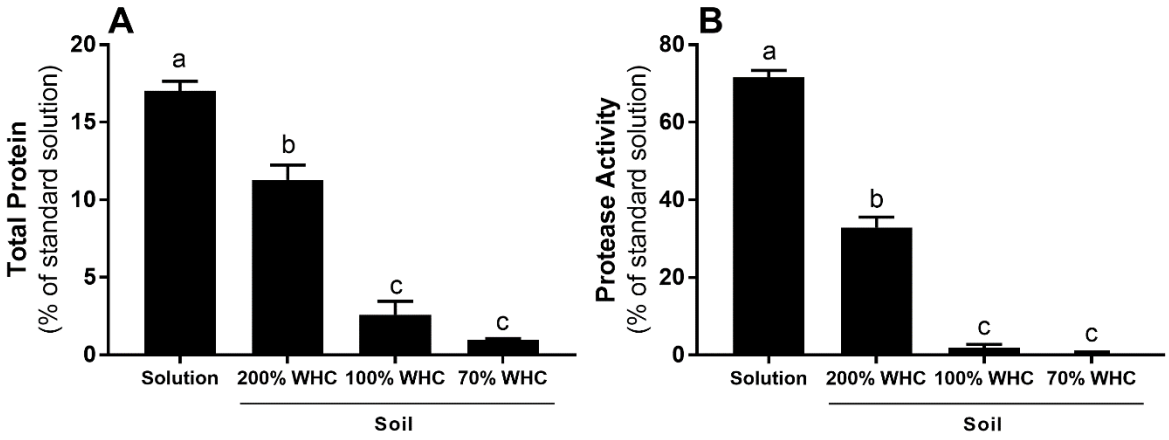

Figure 5-3. A – Relative recovery (% of standard solution) of a protease standard solution (1 mg mL^{-1}), sampled directly from solution, and from soil spiked with a similar protease standard solution, at three water-holding capacities (200%, 100% and 70%). **B** – Relative protease activity (% of standard solution) of dialysates sampled directly from protease solution, and spiked soils. Concentrations of protease solution were adjusted for each soil treatment to ensure the same dose of protein was added to each soil microcosm. For each treatment, $n \geq 4$; error bars represent ± 1 SEM.

5.3.2 SDS-PAGE of protease standards

Protease standards had many bands spanning 10 to 50 kDa, in addition to the expected bands between 16 and 27 kDa that represent protease x and y (Figure 5-4). Recoveries from the standard protease solution showed bands spanning a similar range, but with less distinct bands between 20 and 25 kDa, and 40 and 50 kDa.

Recovery of the standard solution from soil had noticeably reduced banding – however, a prominent band occurred at approximately 37 kDa (Figure 5-4 A), with minor bands at ~30 kDa (Figure 5-4 B) and 18 kDa (Figure 5-4 C). Protease solution samples prepared without the precipitation step with trichloroacetic acid (TCA) had strong protein fragmentation, with banding observed <10 kDa.

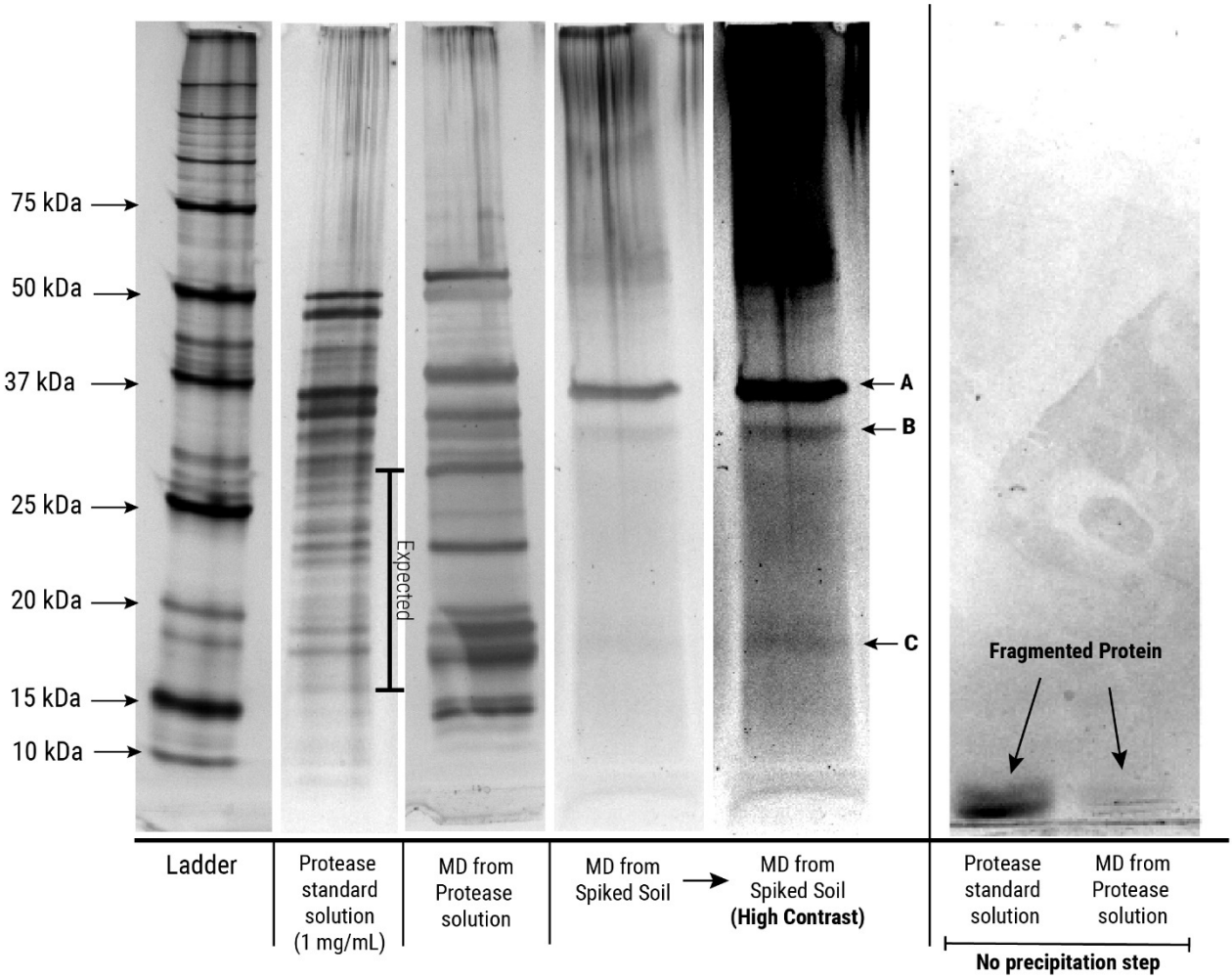

Figure 5-4. A composite photo of 1D SDS-PAGE gels using a silver stain, showing microdialysis (MD) recovery of a protease standard solution directly from the solution itself (MD from Protease Solution) and from a soil spiked with the protease solution (MD from Spiked Soil). A high-contrast version of the spiked soil lane is shown for greater clarity of bands (A, B and C). 'Expected' indicates molecular weights of enzymes in the protease standard, as per literature. Lanes are also shown for prepared samples which did not receive a trichloroacetic acid precipitation step, showing fragmentation of proteins.

5.3.3 Protease activity in soils amended with soybean litter

Protease activity in two litter-amended soils (sandy and clay) were compared using conventional extraction assays or microdialysis (Figure 5-5). Conventional extractions showed no differences between litter treatments in the sandy soil (litter: 253 ± 18 µg tyrosine h^{-1}; no litter: 262 ± 43 µg tyrosine h^{-1}; $t_6 = 0.2$, $p = 0.8$), but in clay soils, the litter treatment had greater activity than no-litter soils (litter: 363 ± 131 µg tyrosine h^{-1}; no litter: 83 ± 57 µg tyrosine h^{-1}; $t_6 = 1.9$, $p < 0.05$).

Protease activity in microdialysis samples was approximately an order magnitude smaller than extractions. However, in the sandy soil, litter amendments had significantly greater protease activity than soil with no-litter (Litter: 22.3 ± 5.3 µg tyrosine h^{-1}; no litter: 8.5 ± 2.3 µg tyrosine h^{-1}; $t_6 = 2.4$,

$p < 0.05$). Similarly, litter amendments in clay soil were significantly greater than no-litter soil (Litter: 20.8 ± 3 µg tyrosine h^{-1}; no litter: 3.8 ± 1.3 µg tyrosine h^{-1}; $t_6 = 5.3$, $p = 0.001$)

The addition of Na-Azide as a bacteriostatic agent (10% of total volume) to dialysates during sampling had no effect on subsequent measurements on protease activity (with Na-Azide: 53 ± 20 µg tyrosine h^{-1}; without Na-Azide: 61 ± 28 µg tyrosine h^{-1}; $t_{13} = 0.2$, $p = 0.8$).

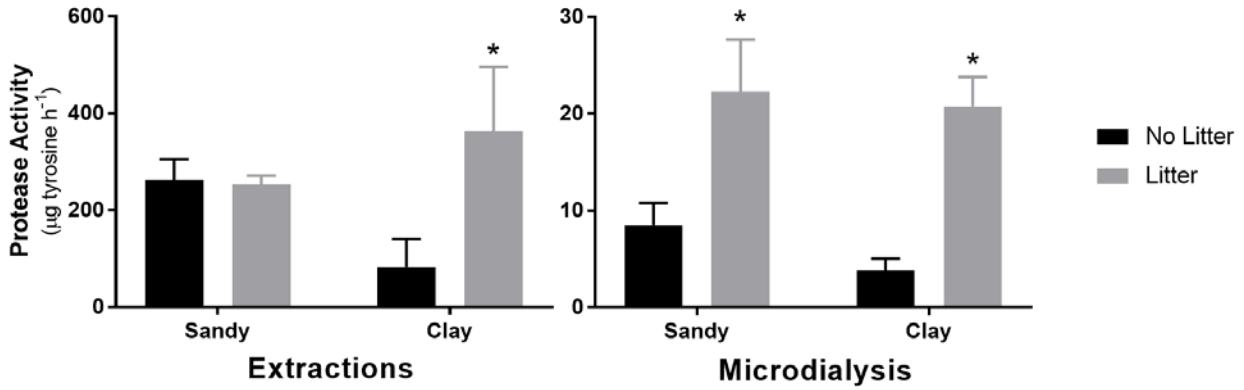

Figure 5-5. Potential protease activity as measured by conventional extractions and microdialysis, in two soils (sandy, clay) amended with soybean litter. Error bars represent ± 1 SEM; asterisks denote significant differences between treatments within each soil type and sampling method.

5.3.4 Estimating free and stabilised enzyme pools

Free and stabilised pools were estimated using a rapid fluorescein diacetate hydrolysis assay, in a sandy soil with and without soybean litter (Figure 5-6 A). Activity in stabilised enzyme pools were found to be no different between litter treatments (Litter: 1.12 ± 0.5 µg fluorescein h^{-1}; no litter: 1.24 ± 0.6 µg fluorescein h^{-1}; $t_6 = 0.9$, $p > 0.2$), but activity in free pools was significantly greater in litter-amended soils (litter: 0.9 ± 0.04 µg fluorescein h^{-1}; no litter: 0.07 ± 0.01 µg fluorescein h^{-1}; $t_6 = 16.4$, $p < 0.001$).

In litter-amended soils, free enzymes contributed 46 ± 11% of total enzyme activity; in contrast to no-litter soils, where free enzymes contributed 9 ± 2% of total enzyme activity (Figure 5-6 B).

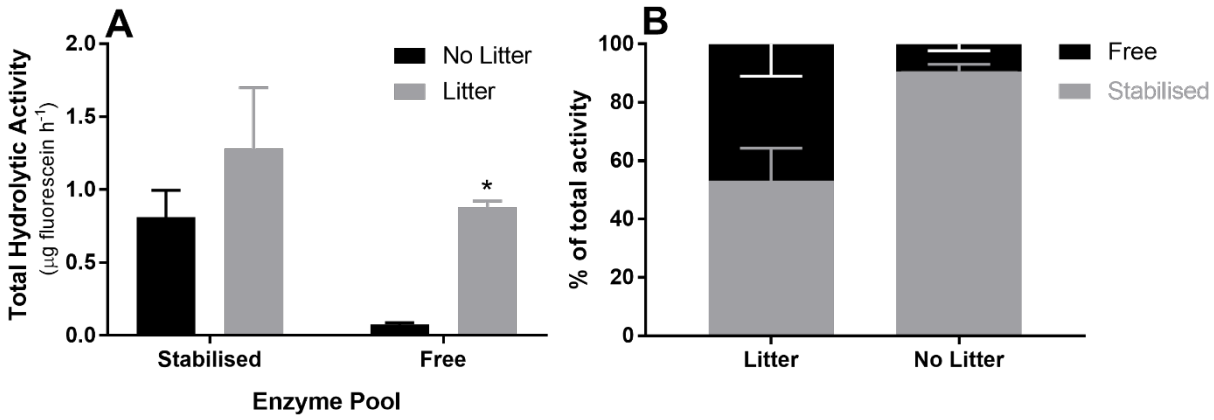

Figure 5-6. A) FDA hydrolysis activity (µg fluorescein h^{-1}) of putative stabilised and free enzyme pools, in a clay soil amended with soybean litter. Error bars represent ± 1 SEM; asterisk denotes significant difference between litter treatments in free enzyme pool. **B).** Proportions (%) of total enzyme activity (FDA hydrolysis) as represented by free enzymes (in black) and stabilised enzymes (in grey). Error bars represent ± 1 SEM.

5.4 Discussion

We show that microdialysis is a promising *in situ* alternative of sampling enzymes from soils, with potential to provide a way of measuring activity within distinct pools – enzymes free in soil solution versus enzymes stabilised to soil surfaces. The ability to differentiate between these pools has been a longstanding technical challenge (Wallenstein and Weintraub, 2008, Burns et al., 2013), and our research provides a basis for future work to explore the roles of both pools in soil nutrient cycling.

5.4.1 Enzyme recoveries in soil environments

Here, we show that although microdialysis can sample enzymes, there may be a requirement for moist soil conditions which optimises protein diffusion through complex soil environments. Our study also shows that total protein recoveries will be low. Under ideal conditions, 11% of external concentrations were recovered in a protein-spiked over-saturated soil, and much less with lower soil water content. Soils provide substantial resistances to protein diffusion – through interactions with charged soil surfaces which can adsorb protein molecules, and through physical hindrances of small soil pores (Tinker and Nye, 2000). By increasing water availability, solid-phase interactions may be reduced, improving both protein diffusion and microdialysis recoveries. These observations will inform future uses of microdialysis for enzyme research in soils to balance recovery rates and alterations to the soil environment.

The magnitude of change between solution and soil environments (at 200% WHC) was much greater for enzyme activity, possibly due to the lower recovery of enzymes at predicted enzyme molecular

weights, especially between 16 and 27 kDa. However, there was an obvious recovery of higher molecular weight proteins (likely non-enzyme related) at approximately 30 to 37 kDa. Although smaller solutes are thought to be preferentially sampled due to higher rates of diffusion (Inselsbacher et al., 2011), this may not be true for all protein – for instance, Kjellström et al. (1999) recovered insulin (MW 5.7 kDa) from solution at similar rates as larger proteins of 12.4 to 14.4 kDa. The reasons for this are likely a complex combination of differential interactions with the external environment, dependent on charge and hydrophobicity (Saxena et al., 2002, Rillig et al., 2007), and interactions with the microdialysis membrane surface, which could preferentially bind some proteins and prevent passage through the membrane (Torto et al., 1998). The increased banding at 30 to 37 kDa observed in our study in spiked soils may suggest a bias towards proteins which have limited interaction with soils or are more mobile in soil solution. Alternatively, these proteins may represent proteins less susceptible to degradation by soil microorganisms, or by autolysis from the standard protease solution itself.

5.4.2 The contribution of stabilised and free enzymes to measures of soil enzyme activity

We show that microdialysis sampling is sufficiently sensitive to detect differences in *in situ* enzyme activity with plant litter addition, even when conventional EEA assays do not. We also show that the breakdown of substrates in our modified assays are from soluble enzymes in dialysates, and not from the responses of any extant microbial contaminants. A noteworthy difference between microdialysis and conventional EEA assays is that the former is likely to sample the free enzyme pool and exclude the soil matrix- or membrane-bound enzyme pool. Conventional EEA assays quantify both pools with the stabilised (bound) pool thought to contribute more to EEA (Kandeler, 1990, Wallenstein and Weintraub, 2008), and future research can now validate this notion.

Enzyme stabilisation is thought to play a critical role in extending the lifespan of soil enzymes – where adsorption to soil surfaces provides protection from depolymerisation, but may not necessarily reduce activity (Wallenstein and Burns, 2011); for this reason, it is thought that stabilised enzymes contribute much of the potential activity measured in conventional EEA assays (Kandeler, 1990, Nannipieri, 2006). Our preliminary work which estimates the activity of free and stabilised enzyme pools suggests that under limiting substrate conditions (without significant addition of organic matter such as plant litter), stabilised pools are likely represent the most significant pool of active enzymes – responsible for 91% of the total activity (FDA hydrolysis) in our experimental soil. In contrast, adding soybean litter induced a greater representation of free enzymes. Fresh plant litter can induce microbial exoenzyme production (as observed in chapter 3), which is likely to be accompanied by transient increases in free enzyme pools as seen here. How long this state of elevated free enzymes would remain is unclear, but it is probable that the production and availability of free enzyme pools

may be greatly influenced by the rise and fall of nutrient demand within the microbial community that produces them (Sinsabaugh and Follstad Shah, 2012).

Taken together, the research presented here demonstrates the potential for microdialysis to explore a challenging aspect of soil enzyme activity, where the innate bias towards free solutes allows discrimination between bound and stabilised enzyme pools. However, there some caveats to this method that should be considered. Firstly, the extraction method used here would likely solubilise loosely-stabilised enzymes, and thus only a small proportion of the total stabilised pool that is studied using conventional assays. Secondly, microdialysis may be biased towards smaller, water-soluble enzymes; as molecular weight has a significant effect on the diffusive properties of solutes, large proteins will be subject to much slower diffusion rates – even when discounting interactions with the soil environment (Kjellström et al., 1999, Tinker and Nye, 2000, Schutte et al., 2004). Thirdly, the microdialysis membrane may discriminate against larger proteins, depending on membrane cut-offs used. However, we feel there is an opportunity to explore fundamental questions regarding the locations of enzyme pools, and their relationships with nutrient bio-availability.

5.4.3 Further avenues for optimising protein recovery from soil

Protein recoveries from the soil solution are clearly possible with microdialysis, but further optimisation may improve recoveries. For instance, push/pull pump techniques which promote a mass flow of external water (and solutes it contains) over the membrane may allow for increased recoveries (Kjellström et al., 2000, Rosenbloom et al., 2005). Additionally, membranes with larger molecular weights cut-offs (> 100 kDa) will allow for a greater diversity of proteins to be sampled – but may come with a risk of microbial contamination, or capture of interfering humic compounds (Miró and Frenzel, 2005). Other membrane modifications – such as larger membrane surface areas – may provide a further optimisation step (see Chapter 6).

Assessments of soil protein concerned with identification (SDS-PAGE and mass spectrometry pathways) rather than absolute concentrations may benefit from using faster fluxes which are achieved with faster flow rates – for example, 5 µL min^{-1} (Inselsbacher et al., 2011). Flow rate modifies the pace at which perfusate passes behind the membrane, and at faster flows an equilibrium with external concentrations is discouraged, resulting in a constant net influx, and greater protein mass transport per sampling time. Although this will give a more dilute sample (as more perfusate is pumped through the system), protein could be concentrated using precipitation techniques (e.g. Jiang et al., 2004) to generate greater protein loads for visualisation and identification.

5.5 Conclusions

As the complex nature of soil interfaces and their interactions are examined (van Dam and Bouwmeester, 2016), sensitive tools capable of monitoring multiple processes are advantageous. Recent functional studies of complex soil microbial communities are analysing metaexoproteomes, or metasecretomes, enabled by sensitive instrumentation and bioinformatics tools (Johnson-Rollings et al., 2014, Alessi et al., 2017). The increasing recognition that green plants are mixotrophs with an autotrophic shoot and a heterotrophic root (reviewed by Selosse et al., 2017, Schmidt et al., 2013) has also focused much attention on root exoenzymes and their roles in plant nutrition (reviewed by Paungfoo-Lonhienne et al., 2012). There is great practical relevance for expanding knowledge of exoenzymes in soil environments across a diversity of enzyme types such as proteases, phosphatases, hydrolases and chitinases – particularly, how nitrogen and phosphorus can be mobilised from complex organic structures. This will further inform efforts to reintroduce organic wastes as fertilisers for more nutrient efficient cropping systems (Paungfoo-Lonhienne et al., 2012). Here, we show that microdialysis is a useful method for not only exploring nutrient fluxes of such environments, but also directly observing the products of microbial activity.

6 Improving in situ recovery of soil nitrogen using the microdialysis technique

The following chapter represents a manuscript which was submitted to, and published in *Soil Biology and Biochemistry* in July, 2017. The reference is as follows:

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Co-authors were collaboratively involved with initial conception of the research, and design of the experiments. Data was collected by R. Brackin and myself, with assistance from S. Jämtgård with the field work component (Materials and Methods, section 6.3.3.3).

Data analysis and the majority of the interpretation of data was performed by myself, with some assistance with interpretation given from co-authors on specific points.

The manuscript was solely drafted by myself, including with graphs and diagrams, and with minor corrections from co-authors before submission to *Soil Biology and Biochemistry.*

Improving *in situ* recovery of soil nitrogen using the microdialysis technique

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Keywords

Soil nitrogen availability; N flux; inorganic N; amino acids; organic nitrogen

6.1 Abstract

Microdialysis is a technique that can be used to sample fluxes of nitrogen (N) in soils with minimal disturbance. To advance our understanding of the technique and improve N recovery, we compared a common membrane type (10 x 0.5 mm probe length and width, 20 kDa molecular weight cut-off; MWCO) with alternative length and MWCO configurations (30 mm; and 100 kDa MWCO). We hypothesised that the alternative membranes would improve recovery of low molecular weight N *via* increased surface area and membrane pore size. The test environments, sampled at fixed pump flow rates, were: (i) stirred 100 µM N standard solution containing organic (amino acid) and inorganic (ammonium, nitrate) N; (ii) soil spiked with 100 µM standard N solution; and (iii) *in situ* boreal forest soil. In general, long membranes recovered more N, but the magnitude of improved recovery varied with test environment. Long membranes recovered more inorganic N regardless of flow rate, except ammonium in stirred solution, where length had no effect at slow flow rates. Long membranes also recovered more organic N from stirred solution regardless of flow rate, and recovered most N at slow flow rates in spiked soil. Longer membranes recovered more amino acids *in situ* in forest soil, with improved resolution of individual amino acids, but were biased towards soluble, mobile forms. MWCO did not affect N recoveries, indicating that in the test conditions, membrane length had greater control than pore size. We discuss the bottlenecks of microdialysis application in soil research and conclude that optimised membrane configurations will advance its use as a tool for quantifying nutrient fluxes in soils.

6.2 Introduction

Microdialysis is a novel method for sampling solutes, initially developed for biomedical research to sample or deliver solutes within living tissue *via* diffusion, with minimal impact or disruption (Nandi and Lunte, 2009). Use of microdialysis has increased in environmental monitoring (Miró and Frenzel, 2004; Öhlund and Näsholm, 2004; Miró and Frenzel, 2005; Sulyok et al., 2005; Inselsbacher et al., 2011), and the low-impact nature of the technique is suitable for examining processes in undisturbed soil and nutrient availability at small spatial scales, such as the rhizosphere (Inselsbacher et al., 2011).

In-depth descriptions of the microdialysis technique have previously been presented (Miró and Frenzel, 2005; Nandi and Lunte, 2009; Inselsbacher et al., 2011). Briefly, soil solutes are sampled by passive diffusion across a small semi-permeable membrane, positioned in the soil with minimal disturbance to the surrounding soil structures. Diffusion is induced by the slow perfusion of water behind the membrane, allowing solutes to move across the membrane along a concentration gradient. The solute/water mixture (termed 'dialysate') is collected for analysis. Subsequent measures of solutes are termed a diffusive flux; *i.e.* the amount of solute which has passed across the membrane over the sampling period (often expressed in nmol m^{-2} s^{-1}). The technique is still considered novel for soil research, but has already provided valuable information on N availability and potential N acquisition by plants in natural and agricultural soils (Inselsbacher and Näsholm, 2012a; Inselsbacher et al., 2014; Oyewole et al., 2014; Shaw et al., 2014; Brackin et al., 2015; Buckley et al., 2016; Oyewole et al., 2016). As microdialysis avoids many of the disruptions that are introduced by aqueous soil extractions (Jones and Willett, 2006; Ros et al., 2009; Rousk and Jones, 2010; Hobbie and Hobbie, 2013; Inselsbacher, 2014), it may provide better estimates of plant-available N in soils – especially since estimates of soil nutrient fluxes can be related to both surface area and nutrient uptake capacity of roots (Brackin et al., 2015; Oyewole et al., 2016).

However, microdialysis sampling often provides low recoveries of target molecules from soil. Of the N compounds tested so far, organic N (in the form of amino acids) can constitute a considerable proportion of the low molecular weight N fluxes in soils (Inselsbacher et al., 2011; Inselsbacher and Näsholm, 2012a; Inselsbacher et al., 2014; Brackin et al., 2015), but concentrations for individual amino acids in dialysates are often near the detection limits of analysis. To address this issue, this study explores ways the microdialysis technique can be optimised for increased N recovery and improved sensitivity.

Recovery of a solute (E_d) is a function of resistances to solute movement imposed by the soil environment (R_{ext}), the membrane itself (R_m) and the dialysate flowing behind the membrane (R_d) as follows (Bungay et al., 1990):

$$E_d = 1 - \exp\left(-1 / Q_p \left(R_d + R_m + R_{ext} \right)\right) \qquad (1)$$

where Q_p is the perfusate flow rate. Improvements to recovery can be made by decreasing these resistances. Similarly, slower flow rates can improve recoveries, but at the cost and practicality of longer sampling times (Inselsbacher et al., 2011).

For low molecular weight compounds in soil, $R_{ext} >> R_m >> R_d$; that is, the resistances to solute movement within the soil have greater control over solute recovery than membrane or dialysate resistances (Hsiao et al., 1990; Miró et al., 2010). R_{ext} includes environmental factors such as impedances to solute movement by the soil solid phase (Tinker and Nye, 2000), and biological processes which dictate the production and removal of compounds from the matrix; for instance, microbial immobilisation and mineralisation (Schimel and Bennett, 2004). However, whilst aiming for minimal soil disturbance, it is undesirable to modify the soil matrix to reduce R_{ext}. R_d includes resistances introduced by the perfusate, such as viscosity, temperature and solutes already present in the perfusate (Miró et al., 2010), but these generally have minor effects on recovery (Bungay et al., 1990). R_m remains as a means of increasing solute recoveries, achievable by modifying physical attributes of the membrane.

R_m can be described as follows (Bungay et al., 1990; Hsiao et al., 1990):

$$R_m = ln\left(r_o / r_i\right) / 2\pi L D_m \varnothing_m \qquad (2)$$

where r_o is the membrane's outer radius, r_i is the membrane's inner radius, L is the membrane length, $D_m \varnothing_m$ is the diffusion coefficient of the membrane for a specific solute. From this equation, physical and practical characteristics of the membrane, including radius, length (L), and diffusion coefficient ($D_m \varnothing_m$), could be modified to reduce R_m. The effect of greater membrane length on solute recovery has been shown (Tossman and Ungerstedt, 1986; Eliasson, 1991; Kjellström et al., 2000; Miró and Frenzel, 2005); yet few studies use membranes longer than 10 mm – particularly in environmental sampling – leading to the assumption that longer membranes may lack robustness for field use (Miró and Frenzel, 2005). Here, we compare the effectiveness of conventional membranes (10 mm length, 20 kDa MWCO) to a custom-made 30 mm membrane with the same aperture (Figure 6-1).

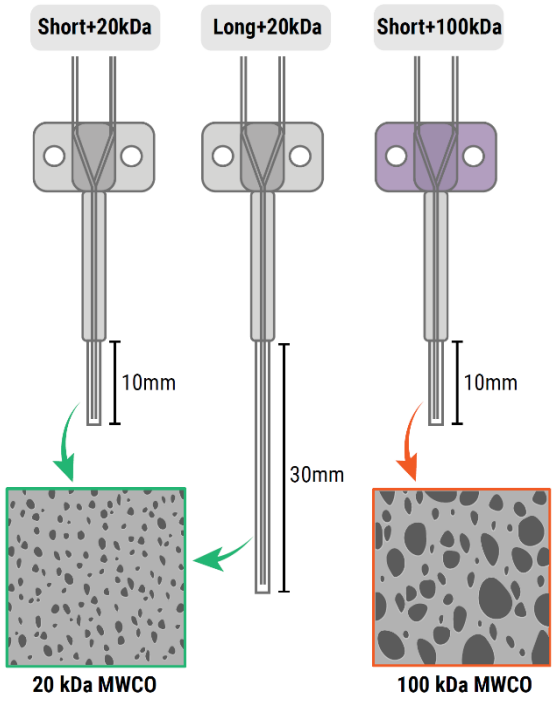

Figure 6-1. Three types of membranes with different molecular weight cut-off (MWCO) or length were used. Short+20kDa membranes represent a common configuration used in soil N sampling.

Increasing D_m $ø_m$ should also increase recoveries, particularly when – for a membrane of a fixed radius and length – D_m $ø_m$ will have the greatest influence over R_m (Bungay et al., 1990). D_m $ø_m$ is modified by membrane porosity and tortuosity, but both are difficult parameters to measure directly so that studies empirically derive an effective diffusion coefficient (D_{eff}) for a given membrane and compound (Torto et al., 1999). Quantifying D_{eff} can be made more difficult by membrane 'fouling', the adsorption of solutes (most typically larger organic molecules such as proteins) to membranes, which block or interfere with the passage of smaller solutes (Rosenbloom et al., 2005). Fouling can affect diffusion of target solutes over time (Torto et al., 1999; Snyder et al., 2001), and is likely a phenomenon in environmental microdialysis sampling where heterogeneous solutions of diverse organic molecules predominate (Torto et al., 1998).

Although 20 kDa MWCO membranes would not typically hinder low molecular weight (LMW) solutes during sampling (Bungay et al., 1990), fouling may decrease membrane functionality. While we do not directly measure D_m $ø_m$, or the effect of fouling on R_m, we hypothesize that a larger membrane pore size (characterised by a larger MWCO) may improve N recoveries through decreased solute hindrance, and/or tortuosity of solute movement, during exposure to soil environments likely prone to membrane fouling. Most recent N studies have utilised ≤ 20 kDa MWCO membranes, but 100 kDa MWCO membranes have also been used to estimate N fluxes across a grassland soil gradient (Shaw et al., 2014). We therefore investigate the effectiveness of a commercially-available 100 kDa

MWCO membrane to recover N from soil (Figure 6-1). Three test environments were chosen to represent different levels of experimental control: a stirred solution, homogenised soil spiked with organic and inorganic N, and forest soil *in situ*.

6.3 Materials and Methods

6.3.1 Soil collection and storage

Soil was collected in June 2016, from the O Horizon of a Scots pine heath forest at the Rosinedal Research area, near Umeå, Sweden (64°10′20″N, 19°44′30″E; see Lim et al. (2015) for a detailed description of the site). The annual mean precipitation is 587 mm and the annual mean air temperature is 1.9 °C. Soil was taken from Plot 3, a nutrient-poor forest soil, classified as a sandy glacial till Haplic podzol (FAO, 2006). Soil samples were taken from the uppermost organic soil layer (0-10 cm) and transferred to the laboratory for further processing. Soil were sieved (2 mm mesh) to remove large debris, mixed, and stored at 4 °C until use. Soil contained 1.04 ± 0.03 % N, 38.67 ± 1.07 % C (C/N = 37.2), with a pH_{H2O} of 4.5.

6.3.2 Microdialysis system setup

Four infusion pumps (CMA 4004; CMA Microdialysis AB, Solna, Sweden) were equipped with a total of 16 micro-syringes (2.5mL; CMA Microdialysis AB, Solna, Sweden). Each syringe was connected to a microdialysis probe, through-connected to a 1.5 mL collection tube kept on ice during sample collection.

Microdialysis probes with three distinct membrane configurations were used in this study (Figure 6-1); i) 10 mm x 0.5 mm (surface area 0.159 cm^2) with a 20 kDa molecular weight cut-off (MWCO), referred to as ‘Short+20kDa’; ii) 10 mm x 0.5 mm (surface area 0.159 cm^2), with a 100 kDa MWCO, referred to as ‘Short+100kDa’; and iii) 30 mm x 0.5 mm (surface area 0.4732 cm^2), with a 20 kDa MWCO, referred to as ‘Long+20kDa’. Short+20kDa and Short+100kDa membranes (CMA 20) are commercially available from CMA Microdialysis AB, Solna, Sweden; Long+20kDa probes were custom-made by the same supplier. Short+20kDa and Long+20kDa membranes are constructed of polyarylethersulphone (PAES); the Short+100kDa membrane is constructed from polyethersulphone (PES).

6.3.3 Microdialysis sampling

Soil N recovery of each membrane type was compared by microdialysis sampling of soil N pools in three environments – a stirred standard N solution; moist soil spiked with a standard N solution; a minimally disturbed heath forest, *in situ*.

6.3.3.1 *Nitrogen recovery from standard nitrogen solution*

12 probes (four of each membrane type) were placed in a glass flask containing standard N solution with 100 µmol N L^{-1} of the following compounds: ammonium (NH_4^+), nitrate (NO_3^-) and 18 amino acids (alanine, arginine, asparagine, aspartic acid, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, valine). The solution was kept at 21 °C and was slowly stirred using a magnetic stirrer throughout the sampling period. Probes were perfused with high-purity deionised water (MilliQ), at five flow rates: 1, 3, 5, 7 and 10 µL min^{-1} for one hour. Sample tubes were frozen at -20 °C until analysis, when dialysate samples, standard N solution and blanks were analysed for NO_3^-, NH_4^+ and amino acids using Ultra-Pressure Liquid Chromatography (UPLC) and colourimetric analysis (see 6.3.4).

6.3.3.2 *Nitrogen recovery from soil spiked with a standard nitrogen solution*

Approximately 25 g (DW) of heath forest soil was packed into a custom-made microcosm, consisting of a modified 50 mL centrifuge tube (Inselsbacher et al., 2009). Soils were air-dried to approximately 0% water-holding capacity (WHC), and then moistened to 100% WHC using a 100 µmol N L^{-1} solution (approximately 35 mL) consisting of NH_4^+, NO_3^- and four amino acids (glycine, glutamine, glutamic acid, and lysine). Insertion holes were made using an introducing needle (for longer membranes, a longer needle matching the increased length was used) and 12 probes (four of each membrane type) were inserted into individual microcosms. Probes were perfused with MilliQ water at five flow rates: 1, 3, 5, 7 and 10 µL min^{-1} for one hour. Freshly prepared microcosms (packed with soil, and recently moistened to 100% WHC with the standard N solution) were used for each flow rate, to avoid the creation of depletion zones around the membranes and to minimise microbial transformation of standard N solution. Samples were frozen at -20 °C until analysis, when N pools within samples, standard N solution and blanks were analysed using UPLC and colourimetric analysis (see 6.3.4).

6.3.3.3 *In situ sampling of heath forest soil*

In June 2016, a field site was selected at the Rosinedal Research area, near the soil collection site referenced in 6.3.1. A small trench (15cm x 100cm) was dug by hand into the O horizon, exposing a vertical soil facing with intact vegetation. Using introducing needles to create insertion holes, 12

probes (four of each membrane type) were placed horizontally into the exposed O horizon soil face. Probes were perfused with MilliQ water for one hour at 5 µL/min. This was performed three times in succession, at different parts of the exposed soil face to decrease sample variability, and to avoid the creation of depletion zones around the membranes during successive sampling. Sample tubes were placed on ice and returned to the lab the same day, and frozen at -20 °C for later analysis of N pools using UPLC and colourimetric analysis (see 6.3.4).

6.3.4 Determination of nitrogen pools

Microdialysis samples were analysed for NH_4^+ and amino acids using reverse-phase liquid chromatography, using a Waters Ultra High Performance (UPLC) system equipped with a Waters Tunable UV (TUV) detector. Samples were prepared as per Inselsbacher et al. (2011). NO_3^- was determined via the reduction of NO_3^- to NO_2^- with vanadium (III) chloride, followed by the Griess reaction, as described by Miranda et al. (2001).

6.3.5 Calculation of relative recovery and diffusive flux

Relative recovery (*EF*) represents the proportion of the standard N solution recovered in dialysates, and is calculated as follows:

$$EF\ (\%) = 100 \times C_{dial} / C_{std} \quad (3)$$

where C_{dial} is the concentration of the measured N compound in the dialysate, and C_{std} is the concentration of the compound in the standard solution. Diffusive flux (D) represents the flux rate of N compounds diffusing across the membrane, accounting for the total surface area of the membrane and the sampling time, and is given as nmol N m^{-2} s^{-1}, and is calculated as follows:

$$D = C_{dial} / A_m \times t \quad (4)$$

where A_m is the surface area of the membrane, and t is sampling time.

6.3.6 Permeability factors

Permeability factors (*PF*) can be used as an indicator of membrane resistance to solute movement (Torto et al., 1998), and is defined by the following equation (Bungay et al., 1990):

$$PF = 1 / (R_d + R_m + R_{ext}) \quad (5)$$

In the stirred standard N solution, $R_{ext} = 0$ (Bungay et al., 1990), and in aqueous solutions $R_m >> R_d$. Therefore, in the stirred N solution:

$$PF = 1 / R_m \quad (6)$$

In the spiked soil environment, R_{ext} is expected to be significant as the soil solid phase and microbial interaction impedes solute movement. If we assume that (R_{ext} + R_m) >> R_d, then:

$$PF = 1 / (R_m + R_{ext}) \quad (7)$$

PF for NO_3^-, NH_4^+, glycine, glutamine, glutamic acid and lysine were derived by linearising equation (1), and taking the slope of a plot of -ln(1-*EF*) vs. $1/Q_d$, (Bungay et al., 1990; Torto et al., 1998). PF was determined for both the stirred N solution, and the spiked soil.

6.3.7 Statistical Analysis

Data was analysed using multi-factorial ANOVA followed by Tukey's HSD post-hoc test, using R Studio (0.99.92), running the R statistical package (v3.3.2). When required, data was transformed to meet assumptions of normality required of ANOVA. Statistical significance was placed at $p \leq 0.05$. Linear and non-linear regressions, including comparisons of slopes (for PF values) were calculated in GraphPad Prism 7.01 (GraphPad Software, Inc.). Significant differences of amino acid sampling frequencies (Figure 6-6, B) were calculated using a Chi-squared test (R Studio), based on the categorical variables of presence or absence of individual amino acids within each dialysate.

6.4 Results

As impedances to solute movement from soil can make it difficult to distinguish R_m and R_{ext} in soil, we firstly compared membrane configurations in an environment where $R_{ext} = 0$ with a stirred standard N solution commonly used as a configuration step (Bungay et al., 1990; Inselsbacher et al., 2011). As $R_d << R_m$, any impedance to solute recovery can be attributed to the membrane alone, and serves as a direct comparison of membrane performance. However, environmental impedances are important to practical field sampling, so we further compared membrane recoveries of a known N standard concentration from soil microcosms. As a standard N solution of known concentration was sampled from both environments at five increasing flow rates, we calculated a permeability factor (PF; for stirred solution, Appendix Table A1; for spiked soils, Appendix Table A2), which provides a relative measure of individual solute permeability for each membrane and environment (Bungay et al., 1990; Torto et al., 1998; Torto et al., 1999). Finally, we compared *in situ* recoveries by each membrane configuration in a practical sense, sampling native N from a Swedish heath forest soil.

6.4.1 Relative N recovery and diffusive fluxes in solution

When compared to Short+20kDa and Short+100kDa membranes, Long+20kDa membranes provided a greater relative recovery of N compounds from the standard solution – a pattern which was consistent across all flow rates and N forms, except for NH_4^+ where all three membranes recovered similar proportions for flow rates 1-3 µL min^{-1} (NH_4^+, NO_3^-, glycine, glutamine, glutamic acid and lysine are shown as representative N forms in Figure 6-2; exponential and linear functions describing recovery for inorganic N and 18 amino acids are provided Appendix Table A1). Short+20kDa and Short+100kDa generally recovered similar standard N proportions, with exceptions at 3 µL min^{-1}, where Short+100kDa membranes consistently recovered more amino acids (Figure 6-2). Some differences in NO_3^- recoveries were also noted between Short+20kDa and Short+100kDa membranes (greater recovery by Short+20kDa at 1 µL min^{-1}, and likewise with Short+100kDa at 5 µl min^{-1}).

Relative N recoveries of each membrane type decreased as flow rate increased, fitting an exponential decrease for most compounds (Figure 6-2), as previously described by Inselsbacher et al. (2011). This was not the case for Long+20kDa and Short+100kDa membranes for both NO_3^- and NH_4^+, where a negative linear relationship best explained their decrease in recovery with increasing flow rate (Appendix Table A1). Permeability factors (PF) Long+20kDa membranes were significantly greater (ANCOVA, $p < 0.05$) than other membranes for most compounds, except for NH_4^+ and glycine (Appendix Table A1); PF values for Short+100kDa membranes were generally no different to Short+20kDa membranes (ANCOVA, $p > 0.05$), with the exception for alanine, histidine, and lysine, where Short+20kDa < Short+100kDa < Long+20kDa (ANCOVA, $p < 0.05$) (Appendix Table A1).

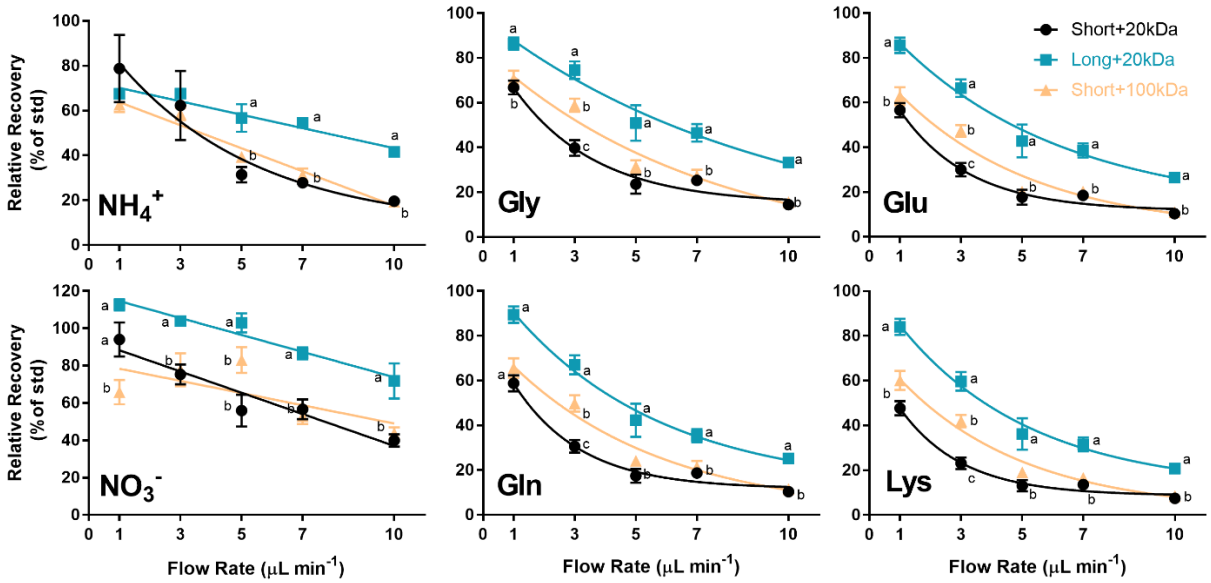

Figure 6-2. Relative recovery (% of standard solution) from a 100 µmol N standard solution, derived from three membrane types – Short+20kDa (in black), Long+20kDa (in blue), and Short+100kDa (in orange) – showing recoveries of six N compounds: ammonium (NH_4^+), nitrate (NO_3^-), glycine (Gly), glutamine (Gln), glutamic acid (Glu), and lysine (Lys) at five flow rates. Letters represent statistical significance between membrane types at each flow rate (one-way ANOVA, $p < 0.05$). For each data point, $n \geq 7$.

For diffusive fluxes (accounting for N recovery per unit surface area of the membrane), Long+20kDa membranes typically sampled less N per surface area, regardless of flow rate and compound, although the effect was greatest for NO_3^- and NH_4^+ (Figure 6-3). Short+100kDa often sampled greater amino acid fluxes, particularly at 3 – 7 µL min^{-1}, but these differences from Short+20kDa membranes decreased with increasing flow rate. Fluxes for all compounds generally increased exponentially with flow rate, with the greatest fluxes at 10 µL min^{-1} as previously described by Inselsbacher et al. (2011). However, this model was statistically ambiguous for recoveries of amino acids by Short+100kDa membranes, and NH_4^+ by Short+20kDa membranes, with alternating increases and decreases in recovery (dependent on compound and flow rate) leading to an ambiguous exponential fit (Figure 6-3, and Appendix Table A2).

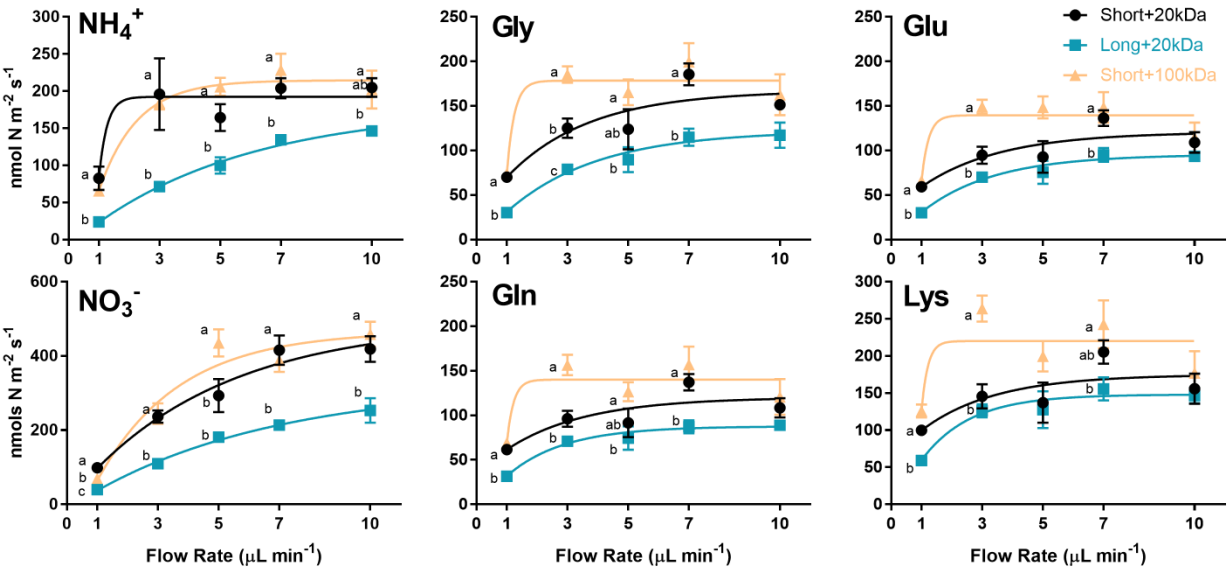

Figure 6-3. Diffusive fluxes (nmol N m^{-2} s^{-1}) of six N compounds: ammonium (NH_4^+), nitrate (NO_3^-), glycine (Gly), glutamine (Gln), glutamic acid (Glu), and lysine (Lys) at five flow rates, sampled from a 100 µmol N standard solution with three membrane types – Short+20kDa (in black), Long+20kDa (in blue), and Short+100kDa (in orange). Letters represent statistical significance between membrane types at each flow rate (one-way ANOVA, $p < 0.05$). For each data point, n ≥ 7.

6.4.2 Relative recovery and diffusive fluxes in spiked soil

Relative recoveries from spiked soil could be described by an exponential decrease with increasing flow rate (Figure 6-4 and Appendix Table A3), but with much weaker curve-fitting than recoveries from solution (Appendix Table A1). Long+20kDa membranes recovered significantly more NH_4^+ and NO_3^- than other membranes at all flow rates (Figure 6-4, left column), and were highest at slower flow rates (1-3 µl min^{-1}), recovering 104.4 ± 3.5 % NO_3^-, and 91.5 ± 2.5 % NH_4^+ at 1 µL min^{-1}, which was a 41.5 % and 38 % increase (*c.f.* Short+20kDa membranes) for NO_3^- and NH_4^+ respectively. Although a similar trend of increased recoveries at 1-3 µL min^{-1} was observed for amino acids, this was generally not significant (one-way ANOVA, $p > 0.05$) (Figure 6-4), and overall relative recoveries of amino acids were much lower than inorganic N, with the highest recoveries observed with glycine (17.3 ± 4.3 %, at 1 µL min^{-1} with Long+20kDa membranes; Figure 6-4). Lysine (Figure 6-4, bottom-right) was most poorly recovered from spiked soils, and was detected in only 20% of the combined dialysates across membrane types. Short+100kDa membranes recovered similar N proportions to Short+20kDa membranes across all flow rates and N forms – with exception to NH_4^+, where recoveries at 1 µL min^{-1} were significantly less than Short+20kDa recoveries by approximately 10.6% (Figure 6-4). PF values for each membrane (Appendix Table A3) were smaller compared to their counterparts derived from solution recoveries (Appendix Table A1), however, NO_3^- and NH_4^+ PF values for Long+20kDa membranes (2.74, and 2.51 respectively) were considerably larger from

spiked soil than from solution (0.43, and 1.28 respectively). Amino acids provided relatively small PF values (Gln – 0.03-0.08; Glu – 0.05-0.11, Gly – 0.08-0.18), particularly so for lysine (0.001 – 0.003). This is in comparison to relatively large PF values for inorganic N (NH_4^+ – 0.57-2.74; NO_3^- – 0.62-2.51) (Appendix Table A3). Although PF values were generally greatest for Long+20kDa membranes, these were only significant (ANCOVA, $p < 0.05$) for NH_4^+ and NO_3^- (Appendix Table A3).

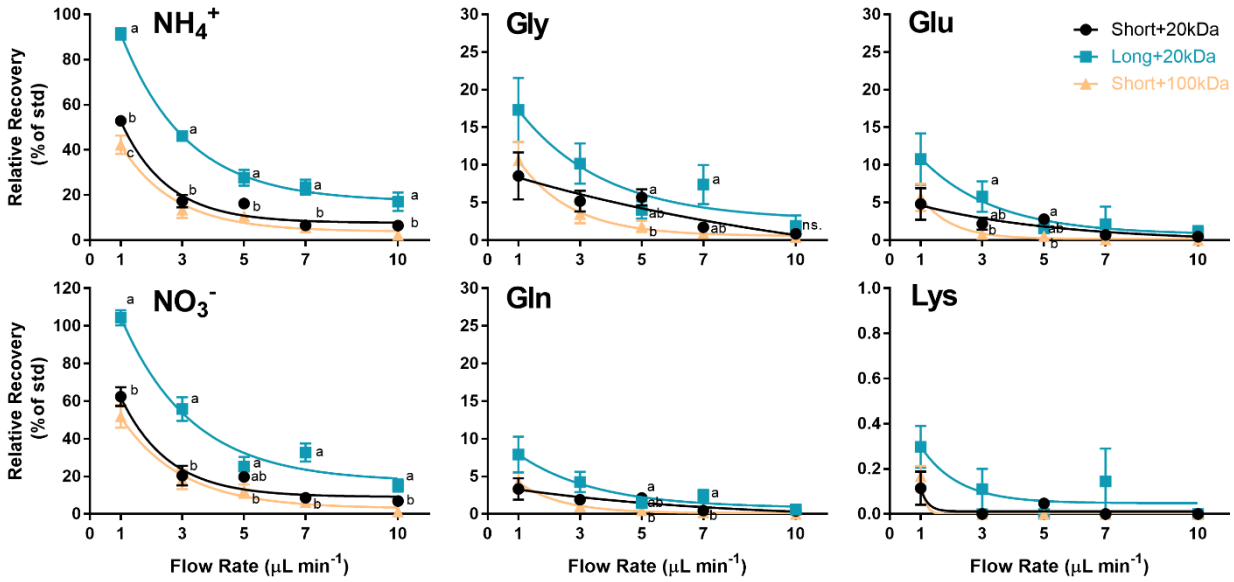

Figure 6-4. Relative recovery (% of standard solution) from a soil spiked with a 100 µmol N standard solution, derived from three membrane types – Short+20kDa (in black), Long+20kDa (in blue), and Short+100kDa (in orange) – showing recoveries of six N compounds: ammonium (NH_4^+), nitrate (NO_3^-), glycine (Gly), glutamine (Gln), glutamic acid (Glu), and lysine (Lys), at five flow rates. For each data point, n = 6. Letters represent statistical significance between membrane types for each flow rate (one-way ANOVA, $p < 0.05$).

In contrast to diffusive fluxes from solution, those from spiked soil showed no clear exponential pattern with increased flow rate, however fluxes measured with Short+100kDa membranes significantly decreased between flow rates 5 and 10 µL min^{-1} for all compounds except lysine, which was not detected in dialysates at flow rates greater than 1 µL min^{-1} (Appendix Figure A13). At most flow rates, there was little difference in diffusive fluxes between membranes, with some exceptions. Significantly greater fluxes were observed with Short+20kDa membranes at 5 µL min^{-1} across all compounds except lysine (Appendix Figure A13). Significant differences in diffusive fluxes between membranes were also observed at 1 µL min^{-1} for NH_4^+ and NO_3^- (Appendix Figure A13), with Short+20kDa > Short+100kDa > Long+20kDa membranes, and at 10 µL min^{-1}, with similar fluxes

for NH_4^+, NO_3^- and glutamine between Short+20kDa and Long+20kDa membranes, but significantly smaller fluxes using Short+100kDa membranes.

Despite these differences, relative contributions of NH_4^+, NO_3^- and amino acids to total N concentrations in samples from spiked soils did not differ between membranes for any flow rate (one-way ANOVA, $p > 0.05$) (Appendix Figure A14).

6.4.3 Sampling of native N from in situ heath forest soil

Total amino acid-N (TAA-N) represents the summed total of all amino acid-N detected in each dialysate sample. Long+20kDa membranes significantly increased concentrations of NH_4^+-N and TAA-N in dialysates, by 42% and 293% respectively, compared to Short+20kDa membranes (Figure 6-5, A), whereas Short+20kDa and Short+100kDa membranes did not significantly differ. NO_3^- was recovered at high concentrations regardless of membrane used, with mean dialysate concentrations ranging from 0.93 (Short+100kDa) to 1.81 µM N (Short+20kDa); noticeable was the high variability across samples, with standard deviations of ±1.96 µM N (Short+100kDa) and ±2.87 µM N (Long+20kDa). Diffusive N fluxes did not differ between membrane types, regardless of N form (Figure 6-5, B), with each membrane estimating greater TAA-N fluxes than NH_4^+ ($p < 0.05$), with between 0.457 – 0.464 nmols N m^{-2} s^{-1} for TAA-N; 0.20 – 0.32 nmols N m^{-2} s^{-1} for NH_4^+. NO_3^- fluxes also accounted for large proportions of total N fluxes, with the highest average fluxes – 1.23 nmols N m^{-2} s^{-1} – reported in Short+20 kDa dialysates. However, high variation meant that fluxes of NO_3^- for all membrane types were not significantly different from either TAA-N or NH_4^+ fluxes (one-way t-test, $p > 0.05$).

Membrane length increased recoveries of glutamine, aspartic acid, glutamic acid, and alanine (Figure 6-6 A), and increased detection frequencies (*i.e.* the detectable presence of a given amino acid in individual samples) of asparagine, glutamine, aspartic acid and glutamic acid (Figure 6-6 B). Dialysates derived from Long+20kDa membranes had a greater number of identifiable amino acids per sample (7.83 ± 0.73), compared to short membranes (Short+20kDa, 4.83 ± 0.74 amino acids; Short+100kDa, 4 ± 1 amino acids; Figure 6-7). Molecular weight cut-off did not affect recovery or sampling frequency of individual amino acids (Figure 6-6 A, B), or identifiable amino compounds (Figure 6-7).

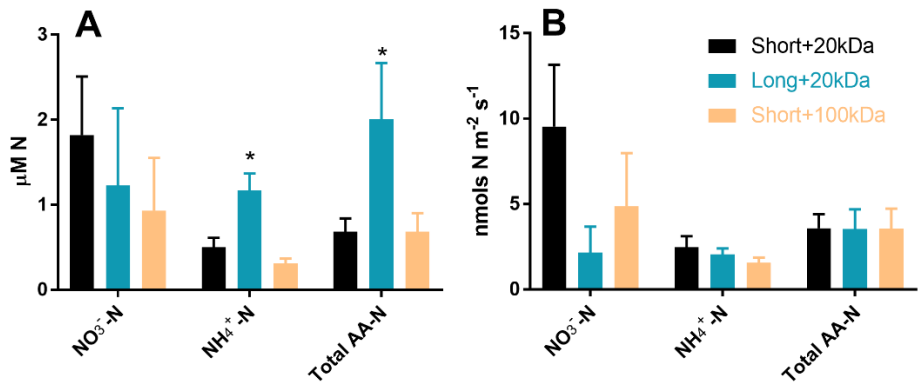

Figure 6-5. Dialysate concentrations (A; in µM N) and diffusive fluxes (B; in nmols N m^{-2} s^{-1}) of NO_3^-, NH_4^+ and total amino acids from a heath forest soil *in situ* near Umeå, Sweden, by three membrane types – Short+20kDa (in black), Long+20kDa (in blue) and Short+100kDa (in orange). For each mean, n ≥ 10. Bars represent ± SE. Asterisks denote statistical significance between membrane types for each compound (one-way ANOVA, $p < 0.05$).

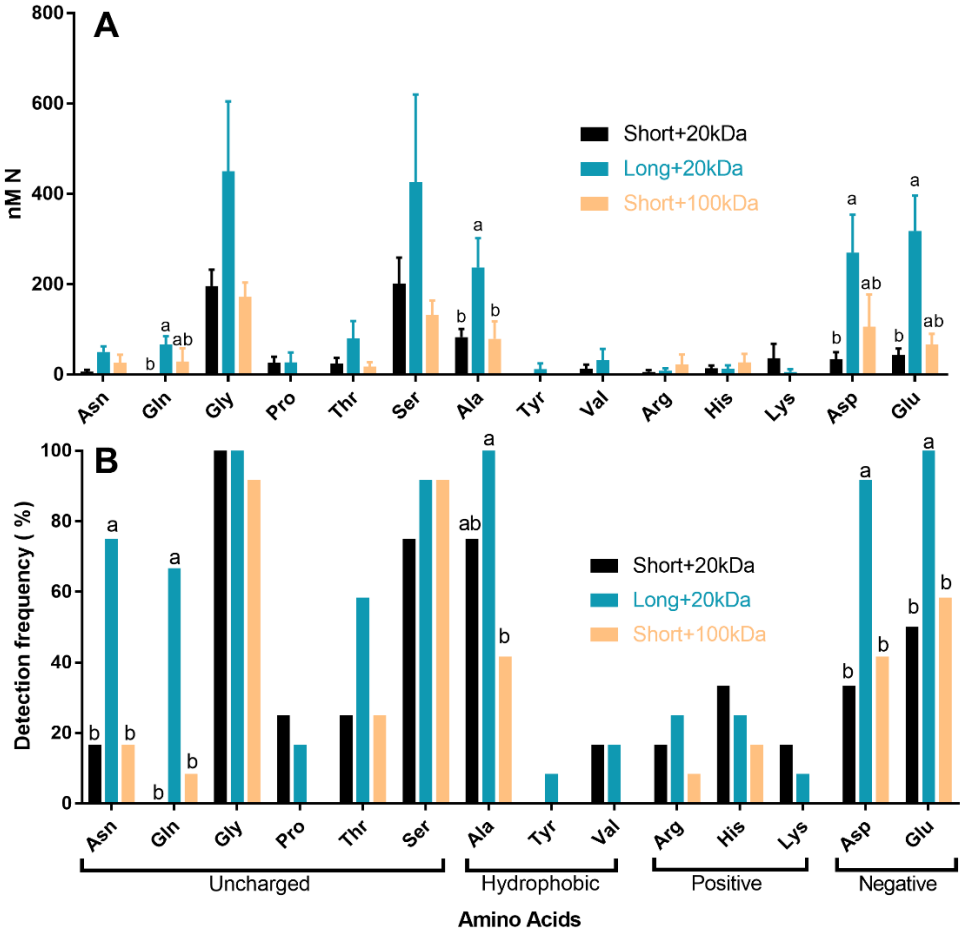

Figure 6-6. Dialysate concentrations (A; in nM N), and frequency of detection (B) of individual amino acids, sampled from boreal heath forest soil in situ using three membrane types – Short+20kDa (in black); Long+20kDa (in blue); Short+100kDa (in orange). For each mean, n = 12. Error bars in graph A represent ± SE. Letters on each graph represent statistical significance between membrane types for each compound (for graph A – one-way ANOVA, $p < 0.05$; for graph B – Chi-squared test, $p < 0.05$).

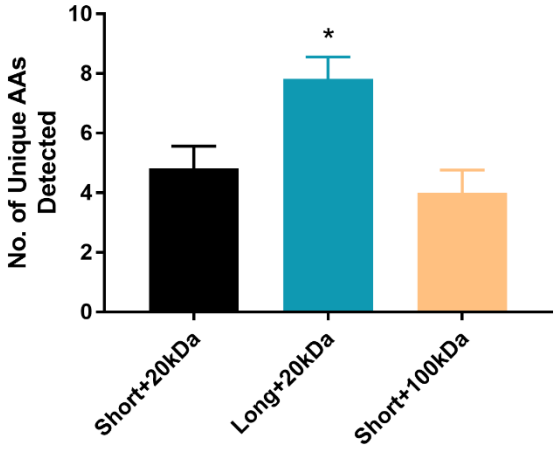

Figure 6-7. The mean number of unique amino acids (AAs) detected in dialysates derived from three membrane types – Short+20kDa (in black), Long+20kDa (in blue), and Short+100kDa (in orange). For each mean, n = 12. Error bars represent ± SE. Asterisk denotes statistical significance (one-way ANOVA, $p < 0.05$).

6.5 Discussion

Tools to investigate soluble soil N pools with minimal structural disturbances are essential to grow understanding of N processes. While microdialysis has promise as a research tool, low N recovery, especially for the chemically-diverse organic N pool, can hamper detection. Reducing R_m – that is, resistances to solute movement across the microdialysis membrane – is a logical pathway to improving *in situ* recoveries, achieved by modifying membrane characteristics. Our study shows that increasing the length of microdialysis membranes can significantly improve quantity and diversity of organic and inorganic N in samples collected *in situ* in forest soil; increasing mean dialysate concentrations of NH_4^+ and total amino acids by 135% and 193% respectively. Testing the technique in soil spiked with N, we show that soil impedances can significantly affect the recovery of inorganic and organic N to a similar degree regardless of membrane type, but that longer membranes allow for greater recovery overall. Long+20kDa membranes also performed well in laboratory and field environments without breakage, with the added length providing a level of flexibility which resisted minor shear stresses during insertion. Although careful use is important, this allays certain concerns that longer membranes are less robust (Miró and Frenzel, 2005). In contrast, higher MWCO membranes did not significantly improve N recoveries from spiked or *in situ* soils, suggesting that membrane length has a greater influence on R_m than membrane pore size – at least for the LMW-N and soil environments evaluated.

The poor recoveries of amino acids from spiked soils, and small permeability factors (compared to those from stirred solution) was an expected outcome, and demonstrates an established understanding

of the buffering capacity of soil, occurring most notably through the physical adsorption of N compounds to the soil aggregate, or through immobilisation by the microbial biomass (Tinker and Nye, 2000). Immobilisation and mineralisation of amino acid solutions by microbes can be rapid, with average half-lives of 1.8 hours (Jones et al., 2009); but given the high concentrations added to spiked soils (100 µM of each compound), and the immediacy of sampling, we conclude that adsorption processes also contributed to their apparent disappearance from the soil solution. The poor recovery of lysine from spiked soils is likely a good example of this, given it is a strong cation that can displace other soil-bound cations and rapidly adsorb to the soil surfaces (Bartlett and Doner, 1988; Vieublé Gonod et al., 2006; Rothstein, 2010; Inselsbacher et al., 2011). In field soil, Long+20kDa membranes did not significantly improve recoveries of lysine and arginine (both strong cations), but did improve those of more mobile amino acids such as glycine, aspartic acid, glutamic acid and alanine (Figure 6-6), highlighting an important aspect of microdialysis: any improvements to soil N recoveries using microdialysis will innately bias analyses of available N towards soluble forms with better mobility through the soil matrix. However, as the soluble N pool is likely the most relevant for acquisition by plants and microbes, this bias may in fact reflect the bio-available N fraction in soil. Experimental assumptions must consider this bias when using microdialysis, taking care to interpret data accordingly.

High spatial variability of NO_3^- in field soil (regardless of membrane type) also raises questions regarding the availability of NO_3^- in boreal forest soils – often found at low concentrations using aqueous soil extraction methods (Jerabkova et al., 2006; Kranabetter et al., 2007; Inselsbacher and Näsholm, 2012a; Inselsbacher et al., 2014). Detection of low NO_3^- concentrations are not necessarily the result of low nitrification rates in boreal ecosystems; in fact, high microbial consumption of NO_3^- could obscure true rates (Stark and Hart, 1997). Heterogeneous availability of C and moisture likely provide high-nitrification micro-sites (Gross et al., 1995; Stark and Hart, 1997), which may be detectable by *in situ* microdialysis sampling, but blurred by bulk soil extractions. Microdialysis may therefore provide a useful tool for testing assumptions of nitrification in boreal soils.

It must be noted that the Long+20kDa membranes in our study measured smaller N fluxes in standard solution, in comparison to Short+20kDa and Short+100kDa membranes; a counterintuitive finding which initially suggests membranes with larger surface areas may lead to contrasting estimates of diffusive fluxes to previous microdialysis work. N fluxes from the spiked soils, estimated by Long+20kDa membranes, were also significantly less than other membranes, but only for inorganic N at 1 µl min^{-1} (Appendix Figure A13); furthermore, N fluxes from the field site showed no differences between membranes (sampled at 5 µl min^{-1}). We may then conclude that the confounding effect of membrane length on diffusive flux measurements may only be significant in sampling

conditions which allow a perfusate to approach equilibrium with external concentrations, such as a highly homogeneous, labile system like the stirred standard N solution, or a moist high N soil sampled at very slow flow rates (for instance, spiked soils at 1 µL min^{-1}).

These findings are consistent with models developed by Bungay et al. (1990), where in a stirred homogenous solution, convective movement of solutes provide a constant recharge of solutes to the membrane surface, and as such, $R_{ext} = 0$. As perfusate is pumped within the inner cannula (behind the membrane), diffusional influx of external solutes decrease concentration gradients between the internal and external solutions. A longer membrane provides a longer flow path for the perfusate, and thus greater solute exchange times to approach equilibrium – or at least a steady-state – with the external solution, and slower flow rates would further facilitate this process. Net inward fluxes would then effectively cease for much of the membrane, decreasing N recovery per unit surface area (Figure 6-8). The opposite is likely true for a heterogeneous soil environment below water-holding capacity, where solute movement is restricted by soil moisture, adsorption and microbial interactions (Tinker and Nye, 2000), and therefore R_{ext} becomes far more influential. Depletion zones, disconnected areas of soil water and localised regions of high and low N availability may all work in unison to effectively prevent a steady-state forming; as such longer membranes provide a similar estimate of flux to other membranes with smaller surface areas (Figure 6-8), but with the advantage of greater N concentrations in subsequent samples. Previous work comparing commercially-available microdialysis probes of variable length and design for biomedical sampling found that observable differences between membranes *in vitro* were much less pronounced *in vivo*, where impedances from tissues formed the largest resistance to solute recovery (Hsiao et al., 1990). We therefore conclude that the use of Long+20kDa membranes in the field will not lead to differing estimates of N flux than previously reported – particularly at flow rates greater than 1 µL min^{-1}.

Given the potential for long membranes to approach equilibrium with moist environments, there may be circumstances where inducing a steady-state flux could be useful. Determining *in situ* concentrations of compounds in solution is one such example, and may be estimated by the 'no-net flux' method (Lonnroth et al., 1987); by perfusing at a fixed flow rate, with a gradient of concentrations of a target solute, and measuring final concentrations in subsequent dialysates, *in situ* concentrations of a solute can be estimated by calculating a perfusate concentration which induces no influx or efflux. With

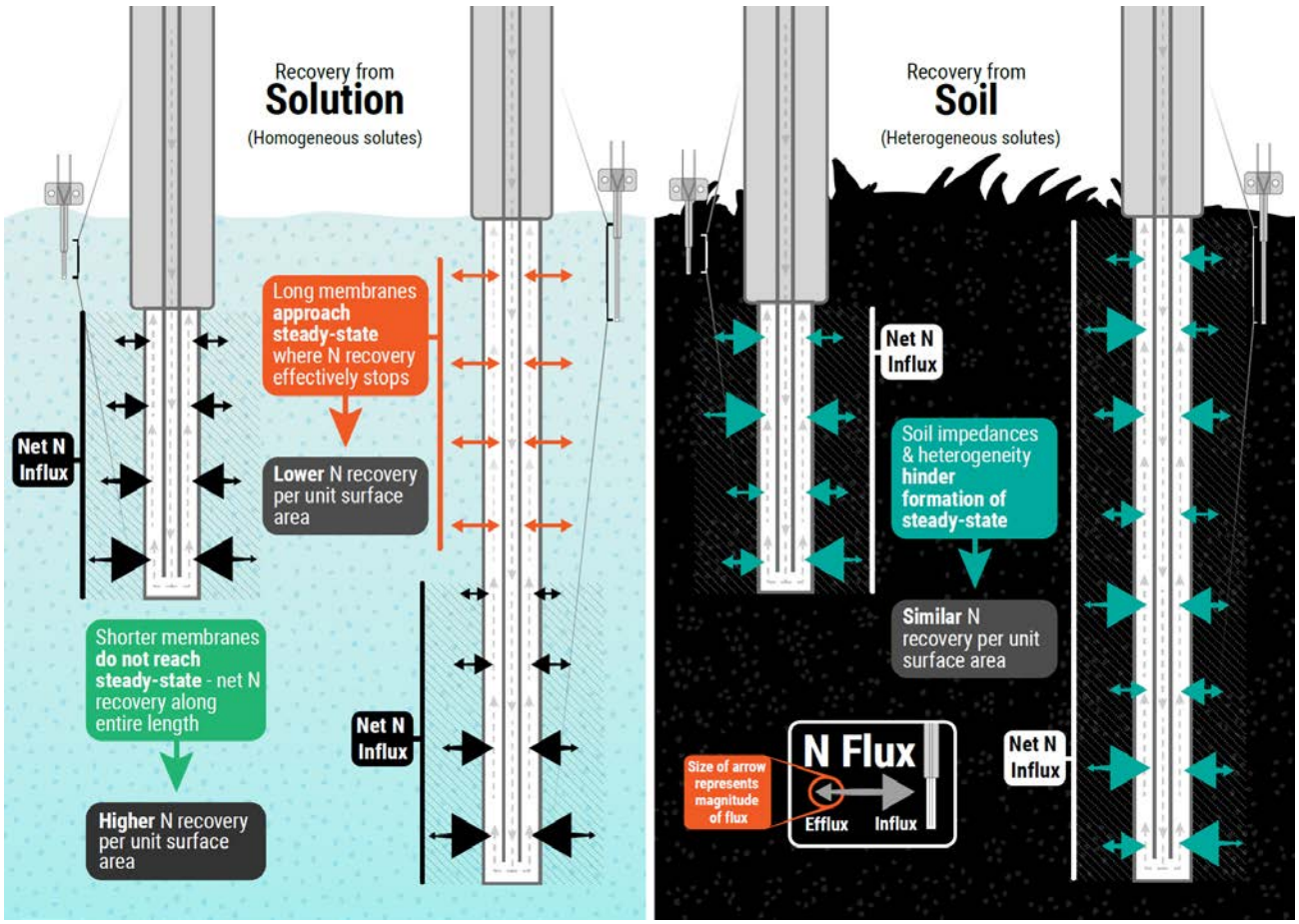

Figure 6-8. A conceptual illustration of the differences in N recovery by short and long membranes between solution and soil environments. Membranes depicted are not to scale.

approximately 100% recoveries of inorganic N obtained from the spiked soil at a flow rate of 1 µL min^{-1}, the Long+20kDa membranes may provide an avenue for finally determining absolute N concentrations in soil solutions – at least in moist soils. Very slow rates (< 1 µL min^{-1}) are frequently used by neurological microdialysis studies to investigate such steady-state conditions (Jacobson et al., 1985; Parsons and Justice, 1992; Hutchinson et al., 2000; Steuerwald et al., 2006); combined with longer membranes, we may also be able to approach a steady-state for labile amino acids in soil solution, and thus an approximation of absolute concentrations, *in situ*.

There were few differences in performance between Short+100kDa and Short+20kDa membranes in the spiked soil, or the *in situ* boreal forest soil. However, fluxes of NO_3^-, NH_4^+ and glutamine sampled with Short+100kDa membranes from the spiked soil decreased significantly at higher flow rates (7-10 µL min^{-1}); additionally, higher fluxes of amino acids were observed using Short+100kDa in stirred solution (particularly at 3 µL min^{-1}, Figure 6-3). These unexpected deviations in performance may be explained by ultrafiltration, which is the loss of perfusate water through the membrane during sampling (Rosenbloom et al., 2005; Chu et al., 2014; Jadhav et al., 2016). Although we did not

account for this possibility, ultrafiltration is known to affect solute recoveries with higher MWCO membranes (Gonzales et al., 1998; Snyder et al., 2001). As such, these probes have often been deployed with push/pull pump techniques (Kjellström et al., 2000; Jadhav et al., 2016), or with negative hydrostatic pressure (Rosenbloom et al., 2005; Chu et al., 2014) to counteract water loss and should be considered with any future use of larger MWCO membranes; however, such measures may impose practical restrictions on their use – particularly in the field.

Although larger MWCO membranes may not improve recoveries of LMW solutes from environments with high external impedances and membrane fouling potential (also briefly noted by Torto et al. (1998)), estimates of diffusive fluxes made with Short+100kDa membranes will be comparable to previous soil microdialysis work; particularly at slower flow rates ($\leq$ 5 μL min^{-1}), and when ultrafiltration is accounted for. Furthermore, higher MWCO membranes may enable sampling of larger compounds such as peptides and proteins from soil solution, as has been done in biomedical research (Kjellström et al., 2000; Schutte et al., 2004; Rosenbloom et al., 2005; Steuerwald et al., 2006; Jadhav et al., 2016), highlighting the potential to simultaneously sample soil N compounds across a greater spectrum of molecular weights.

6.5.1 Conclusions

We show that longer membranes can improve recoveries of N from forest soils, with greater accuracy and precision than possible with shorter membranes. This is an important step in transitioning microdialysis from its laboratory-based application to a robust *in situ* soil sampling method. As the sampling applications of microdialysis extend well beyond N compounds, the increased sensitivity provided by longer membranes provides a basis for exploring the availability of many other solutes related to plant nutrition, rhizosphere signalling and microbial cycling, many of which exist at low concentrations in soils.

6.6 Acknowledgements

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7 General Discussion

There is a dire need to develop a deeper understanding of soil processes controlling N cycling and availability in agricultural systems, to better match nutrient supply with plant demand, and ultimately reduce environmental losses. However, a lack of sensitive tools providing useful perspectives of soil environments have limited progress towards this goal.

This thesis presents research which demonstrates the potential of microdialysis to explore multiple aspects of N availability in soil environments, and to provide new perspectives of challenging questions in soil science. In Chapter 3 and 4, microdialysis fluxes are shown to have a greater context than extractions with the dynamic soil environment, where microbial consumption and soil adsorption play substantial roles in controlling availability of compounds like NH_4^+. In contrast, the potential of extractions to mobilise bound sources of NH_4^+ establishes the danger in relying on extractions to estimate plant-available N, but also the potential for using both techniques to derive a greater perspective of soil N availability.

In Chapter 5, microdialysis is shown as a promising tool for exploring challenging aspects of *in situ* enzyme activity – particularly the contribution of free enzymes to soil processes; a question which has previously been difficult to investigate. The technique may also open doors to directly sampling and identifying proteins and enzymes related to nutrient cycling and plant-microbe signalling in highly-sensitive zones such as the rhizosphere (van Dam and Bouwmeester, 2016).

Lastly, in Chapter 6, we show that by increasing the surface area of microdialysis membranes, the technique can be advanced for soil environments by improving recovery of soil N. This is particularly useful for low concentration compounds such as amino acids, which can often be close to detection limits. Increasing the membrane surface area is shown not to change absolute fluxes obtained during field sampling, suggesting that their use will allow for comparable measurements with other studies using membranes with smaller surface areas.

Taken together, this thesis establishes microdialysis as a valuable tool for soil researchers, but also concludes that promising aspects of the technique remain to be explored. The following discussion will examine consequences of the technique for measuring N availability, as well as directions and critical considerations for moving the technique forward.

7.1 Re-defining N availability with microdialysis

Many questions surrounding plant-available N in soil are influenced by how we define and measure it. For instance, if a compound (such as NH_4^+) represents a large proportion of the total extractable pool, is that compound more 'plant-available' than others? A true measure of availability requires additional context regarding both production (release of new N into a soil system) and immobilisation (the removal of N by microbes, or adsorption to soil particles), but is difficult to estimate with coarse bulk sampling methods like extractions and mineralisation assays. Microdialysis fluxes may provide contrasting views of soil N simply because they represent just that – a flux, integrating both production and immobilisation by the adjacent microbial community (Chapter 3), and solute mobility under given soil parameters (Chapter 4).

Microdialysis may then offer a better perspective of bioavailable N, although logically this may also mean that what we measure with microdialysis is the 'leftovers' – what remains after microbial communities have exploited available N forms, and what hasn't been adsorbed to soil surfaces. Independently, fluxes also provide a singular perspective of soil N – albeit one we have yet to truly explore – but may inevitably lead to similar unanswered questions regarding bioavailability. For instance, Figure 7-1 (taken from Chapter 3) describes two outcomes for NH_4^+ which may lead to the small fluxes observed whilst measuring N released during litter decomposition – rapid consumption by native microbes, and adsorption to soil surfaces.

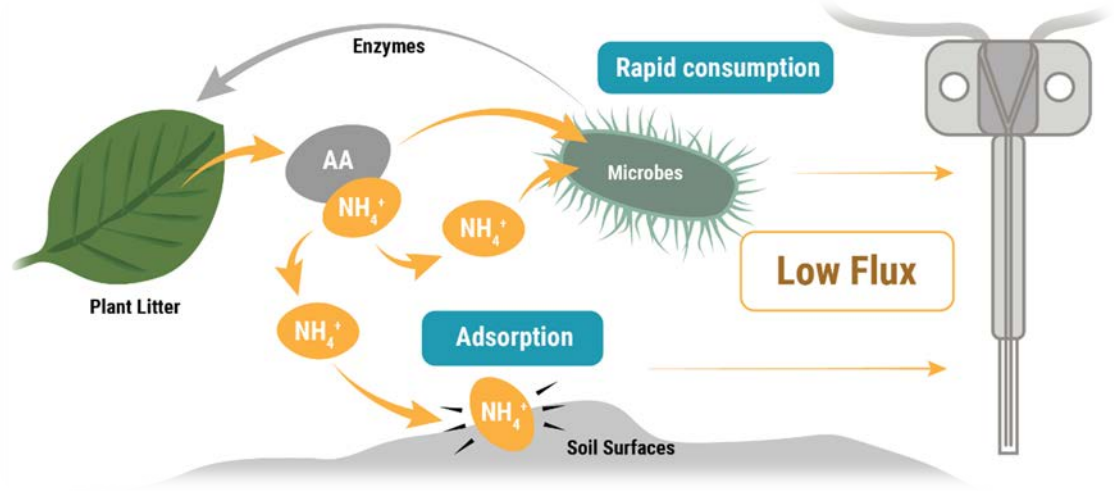

Figure 7-1. Two pathways which may result in low NH_4^+ diffusive fluxes as measured by microdialysis. Rapid consumption by microbes may result in immobilisation of NH_4^+, reducing fluxes; alternatively, abiotic adsorption to soil surfaces may also reduce NH_4^+ fluxes. Diagram taken from Chapter 3.

Each pathway implies two different outcomes for bioavailability: rapid consumption suggesting high bioavailability (with equally high microbial utilisation), and adsorption suggesting low bioavailability. Although the most realistic scenario is a combination of both processes, this highlights the complexity of determining the bioavailability of a solute with just one sampling technique alone. Salt extractions – with all their faults in disrupting the soil environment and introducing artefacts (Jones and Willett, 2006, Rousk and Jones, 2010, Inselsbacher, 2014) – may still provide a useful perspective of total N – particularly of adsorbed fractions within a soil system – with microdialysis offering measures of dissolved pools which are integrated with biotic and abiotic factors.

The concept that microdialysis represents what remains after microbial consumption and physical adsorption represents a point of divergence with models of uptake in plants, which are active competitors for soil N with their own regulated mechanisms for N exploitation and root uptake (Lambers et al., 2008, Kraiser et al., 2011, Schmidt et al., 2013, Zemunik et al., 2015). However, microdialysis is capable of exploring some of these mechanisms as well, with recent studies examining the influence of transpiration-driven mass flow (Oyewole et al., 2014, Oyewole et al., 2017) and root exudation (Demand et al., 2017) on nutrient availability in the rhizosphere. Depletion zones formed during sampling can also mimic similar nutrient depletions around roots during nutrient uptake (Li et al., 1991, Gahoonia et al., 1994, Tinker and Nye, 2000). Additionally, our work describing the use of microdialysis to sample free enzymes *in situ* (Chapter 5) may open an additional perspective of microbial and plant-derived enzymatic influences on nutrient availability, particularly as both enzymes and the products of their *in situ* activity can be sampled simultaneously. Although microdialysis is *not* a perfect artificial model for nutrient root uptake, it does provide an avenue to explore rates of nutrient supply to a root surface under relevant soil conditions – a useful perspective to have available.

7.2 Future directions: challenges and opportunities

As the technique continues to be developed for soil research, microdialysis will likely find its niche investigating questions that previously were considered too challenging or sensitive to be explored by other methods – and this is exciting. Nevertheless, as research interest grows, the research community must discuss the best use of the technique – particularly, ways of standardising setup and deployment. This will help ensure studies are comparable that are carried out in different research groups and soil environments.

7.2.1 Considerations for measuring absolute fluxes

Diffusive fluxes have proven a useful measure of soil solute availability, particularly given they are measured in similar units to other relevant fluxes, such as solute uptake by roots (Brackin et al., 2015, Demand et al., 2017) and emissions of greenhouse gases from soil (Leitner et al., 2017b, Leitner et al., 2017a), and are commonly quoted in microdialysis soil studies (Inselsbacher et al., 2011, Shaw et al., 2014, Oyewole et al., 2014, 2016, 2017, Brackin et al., 2017, Ganeteg et al., 2017). However, there are some considerations regarding the deployment and interpretation which may influence findings and make comparisons between studies challenging – but these influences could be minimised through standardisation of the technique.

As previously discussed in Chapter 2, the use of diffusive flux measurements is more common in soil research than in the biomedical origins where microdialysis was first developed. This is in part due to diffusive fluxes having a non-linear relationship with flow rate (Figure 7-2 – orange line), generally reaching a plateau at higher flow rates. This dependent relationship with flow rate will therefore make it difficult to compare fluxes between studies that employ different flow rates.

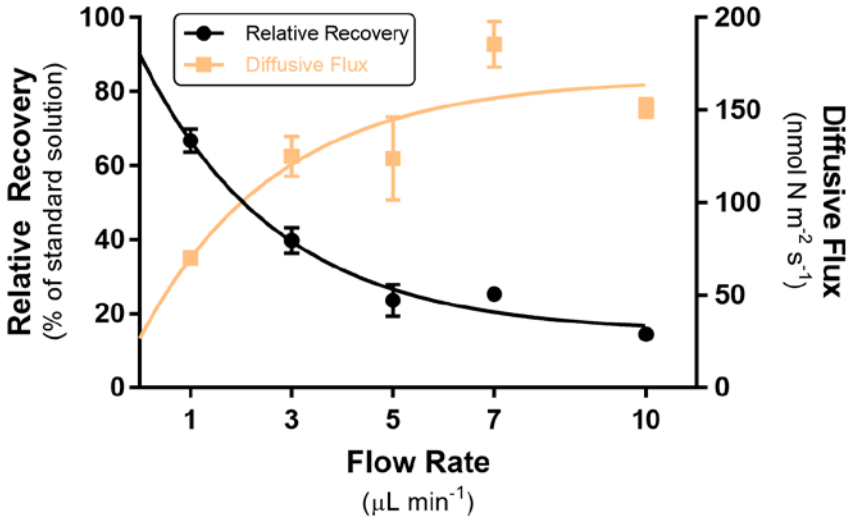

Figure 7-2. Effect of flow rate on the recovery of glycine from stirred standard solution containing 100 µM glycine; relative recovery (black circles) and diffusive fluxes (orange squares) are shown. Error bars represent ±1 SEM, and lines represent non-linear equations approximating each measure versus flow rate. Data is taken from Chapter 6.

Although a non-linear model can be derived by sampling a known solute standard from soils at a diversity of flow rates (Chapter 6, Jämtgård et al., 2018), the characteristics of the model are likely to be dependent on individual qualities of a given soil and solute. This appears to be particularly relevant for positively-charged solutes, for instance, amino acids such as lysine, as these solutes bind to the

mostly negatively-charged soil matrix (Chapter 6, Vieublé Gonod et al., 2006, Inselsbacher et al., 2011), which means that such models may be unsuitable for predicting fluxes between different soils and flow rates.

Standardising the flow rate across investigations may be a potential solution, and many studies have used 5 µL min^{-1} as per recommendations by Inselsbacher et al. (2011). This is a useful flow rate which allows for ample collection volumes and sampling precision whilst minimising sampling time, and we would advise the same for future studies. However, users should also recognise there is a trade-off; slower flow rates have significantly greater relative recoveries (Figure 7-2 – black line) as solute exchanges between the inner membrane and the external environment approach equilibrium, and as such provide a better representation of *in situ* fluxes, but at the cost of smaller collection volumes and longer sampling times. Clearly there is no easy answer to choosing a flow rate, but as it directly influences our measurements, we recommend it be of paramount importance in experimental designs, and particularly when aiming to compare microdialysis fluxes across studies.

Depletion of solutes around the probe is another consideration when quantifying solute fluxes – particularly when sampling continuously over longer time frames (hours to days). As briefly described in Chapter 3, depletion occurs as solutes immediately adjacent to the membrane are removed from solution, decreasing solute concentrations in this zone, creating a concentration gradient which extends to regions further away from the membrane. Replenishment of this zone is limited by diffusion from higher concentration zones further away from the membrane so that over time, successive measures of fluxes will decrease, generally in a non-linear manner (Figure 7-3, A). The degree of depletion is likely affected by the concentration and mobility of a solute, combined with the resistive forces from the soil matrix, but has been a common feature in continuous sampling studies featuring time fractions (Inselsbacher et al., 2011, Jämtgård et al., 2018, Warren, 2018, Brackin et al., 2016).

Given that depletion may be a significant factor for some solutes, it is important to note that the length of the sampling period will likely affect the final flux measurement for that solute. For instance, in the example shown in Figure 7-3 A, by sampling solutes for 60 and 120 minutes and measuring only at the end of these periods, we would arrive at different flux measurements for the tested solutes ammonium and nitrate, approximated here by the mean of the fluxes over those time (Figure 7-4 B). As end-point measurements of flux (rather than time-fractions) are commonly used in soils studies, it would be wise to also standardise sampling times, but similarly to standardising flow rates, this may enforce impracticalities, particularly for downstream analysis.

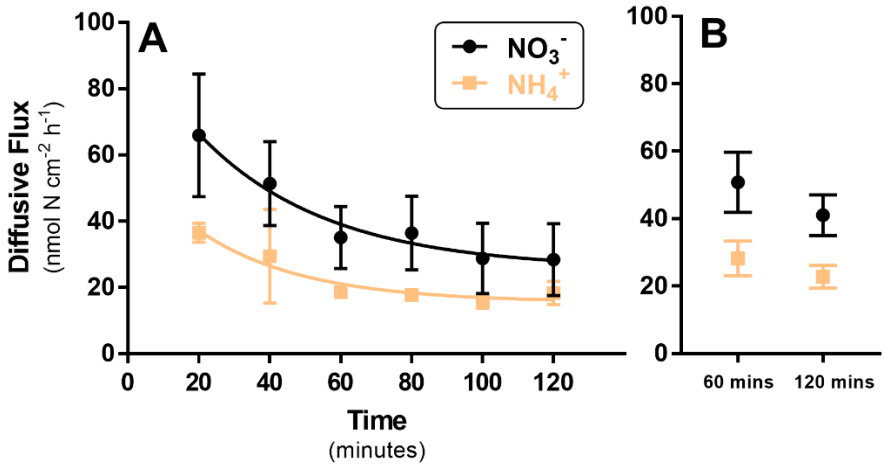

Figure 7-3. A – Fluxes of nitrate (NO_3^-, black circles) and ammonium (NH_4^+, orange squares) sampled from a laboratory-incubated agricultural soil at 20-minute intervals over 120 minutes at a flow rate of 1 µL min^{-1}; For each point n = 4. **B –** Mean fluxes over first 60 minutes, and 120 minutes. Error bars represent ± 1 SEM. Data is taken from Chapter 4.

There is also some potential for depletions to be misinterpreted – for instance, as uptake by microbes or adsorption to soil particles; although such processes may be difficult to differentiate from sampling depletion. Consideration should be given to the impact of depletion zones when interpreting short-term time course measurements (minutes to hours), in context of overall dynamics of the soil system, including likely soil concentrations and solute mobility (Leitner et al., 2017a).

A final practical consideration is membrane type. In Chapter 6, we have shown that longer membranes (30 mm) can significantly improve recoveries of low-concentration N compounds. As many soil solutes of interest can also be low in concentration, we recommend their use to improve measurement precision.

7.2.2 Alternative approaches to quantification

An alternative approach may be to shift our focus towards estimating *in situ* concentrations using calibration techniques, as done in many biomedical studies (Kho et al., 2017, Bourne, 2003, Hammarlund-Udenaes, 2017). Given that most conventional methods of sampling soil solutes provide absolute concentrations (not fluxes), estimating *in situ* solute concentrations may provide an avenue for better comparisons across soils studies.

In Chapter 6, we observed that longer probes (30 mm length) allowed for nearly 100% relative recovery of an inorganic N standard from a spiked soil at a flow rate of 1 µL min^{-1}, suggesting that estimates of absolute concentrations *in situ* could be possible with such membranes, at least in moist soil environments. However, we also found that recoveries of amino acids, although improved by using longer membranes, were significantly lower than inorganic N. As adsorption processes and

microbial utilisation likely play a role with such compounds, it may be difficult to estimate *in situ* concentrations for using calibration – and this may be similar for many other soil solutes with low mobility, such as phosphate (Demand et al., 2017).

Interestingly, Miró et al. (2010) and Demand et al. (2017) were able to calculate the external concentration of target solutes by estimating soil resistances (R_{ext} – Eqn. 2 in Chapter 2) using calculations of effective diffusion coefficients for each ionic species based on mathematical models by Olesen et al. (2001), with potential to map solute depletions at distances from the probe over time (Demand et al., 2017). These methods require information regarding volumetric soil moisture content, soil texture and bulk density, parameters commonly quantified in soil studies. A potential drawback may be the requirement to destructively harvest soils to obtain these parameters, which may not be feasible for time-course studies. Nevertheless, these studies stand as useful examples of how soil concentrations could be estimated in future studies.

Innate features of depletion zones formed during sampling, such as flux plateaus (explored in Chapter 4) could provide an avenue for exploring the influences of molecular characteristics and soil parameters on the transport of solutes, as well as giving greater insight into the capacity of soils to sustain nutrient diffusion to a root surface after initial depletion around a root. Such processes are difficult to study empirically, and direct observations of solute movement using microdialysis may allow for improved models of nutrient supply at small scales (Brackin et al., 2017).

7.2.3 Wider application of the technique

To date, only a handful of compounds have been explored in soils using microdialysis. In reality, any soluble compound could be explored given sufficiently sensitive analyses to quantify low concentrations in dialysates. The nature of microdialysis particularly lends itself to monitoring sensitive, fragile environments such as the rhizosphere. Recent developments in metabolomics research have illuminated many of the compounds plant roots exude, and there is much interest in how these exudates shape microbial communities (van Dam and Bouwmeester, 2016). Sensitive measurements of absolute fluxes, as well as retrodialysis techniques (Demand et al., 2017) delivering model exudates over root-relevant timeframes may provide interesting and fruitful avenues for exploring these rhizosphere relationships.

7.3 Conclusion

Combining our work with a growing body of soil microdialysis research, we can now view microdialysis as a holistic tool for exploring soil N dynamics, with potential to quantify of wide range

of soluble N compounds, from high molecular weight N such as protein (including enzymes) and peptides (Jämtgård et al., 2018), to low molecular weight N such as quaternary ammonium compounds (Warren, 2013), amino acids, NH_4^+ and NO_3^-, albeit from the perspective of compounds dissolved in soil solution.

We show that microdialysis offers a means for researchers to track N from its depolymerisation as organic N (including associated enzymatic bottlenecks), through to mineralisation as NH_4^+ and NO_3^-, all without the need for destructive manipulation of soil environments (Figure 7-4). If we further consider pairing microdialysis with online instruments for high resolution temporal measurements (e.g. Warren, 2018), we may be afforded an unparalleled view of N cycling previously inaccessible to soil researchers.

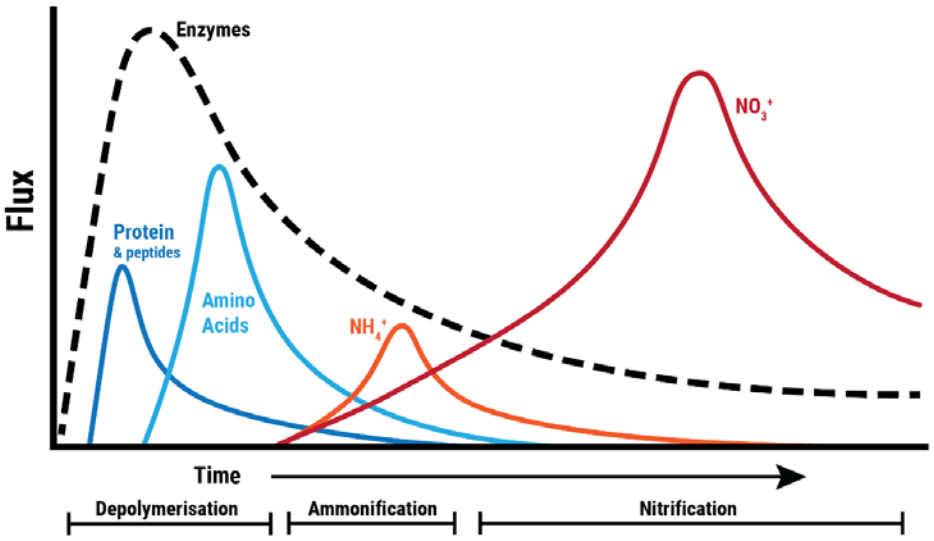

Figure 7-4. Microdialysis is a holistic technique, capable of exploring a range of N compounds and processes which contribute to soil N cycling and availability.

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Appendix A: Supplementary Data (Chapter 6)

Table A1. Exponential and linear functions of relative recoveries (EF) of ammonium (NH_4^+), nitrate (NO_3^-), and 18 amino acids, recovered by three membrane types from a stirred solution containing 100 µmol of each N compound. Equations are expressed as functions of flow rate (Q_p). Permeability factors (PF) are given for each membrane and compound; letters represent significant differences between each membrane.

	Short+20kDa			**Short+100kDa**			**Long+20kDa**		
Compound	**Eqn**	**R^2**	**PF**	**Eqn**	**R^2**	**PF**	**Eqn**	**R^2**	**PF**
NH_4^+	EF = 91.28 × exp(-0.22 × Q_p)	0.43	0.76[a]	EF = -5.143 × Q_p + 68.9	0.96	0.67[a]	EF = -2.992 × Q_p + 73.1	0.93	0.38[b]
NO_3^-	EF = 5.694 × Q_p + 93.99	0.90	1.14[a]	EF = -3.221 × Q_p + 79.38	0.63	0.22[b]	EF = -4.532 × Q_p + 119.2	0.95	1.28[a]
Ala	EF = 71.18 × exp(-0.39 × Q_p)	0.81	0.66[c]	EF = 84.6 × exp(-0.17 × Q_p)	0.82	0.96[b]	EF = 93.06 × exp(-0.14 × Q_p)	0.76	1.56[a]
Arg	EF = 72.71 × exp(-0.52 × Q_p)	0.85	0.68[b]	EF = 78.07 × exp(-0.24 × Q_p)	0.82	0.89[b]	EF = 94.78 × exp(-0.21 × Q_p)	0.76	1.96[a]
Asn	EF = 72.35 × exp(-0.43 × Q_p)	0.84	0.78[b]	EF = 82.44 × exp(-0.18 × Q_p)	0.82	0.94[b]	EF = 93.51 × exp(-0.18 × Q_p)	0.75	1.70[a]
Asp	EF = 70.64 × exp(-0.43 × Q_p)	0.84	0.75[b]	EF = 79.88 × exp(-0.19 × Q_p)	0.83	0.91[b]	EF = 87.17 × exp(-0.22 × Q_p)	0.71	0.64[a]
Gln	EF = 74.07 × exp(-0.46 × Q_p)	0.84	0.78[b]	EF = 81.06 × exp(-0.2 × Q_p)	0.80	0.93[b]	EF = 96.27 × exp(-0.20 × Q_p)	0.77	2.27[a]
Glu	EF = 69.68 × exp (-0.44 × Q_p)	0.83	0.72[b]	EF = 76.82 × exp(-0.23 × Q_p)	0.81	0.86[b]	EF = 89.42 × exp(-0.18 × Q_p)	0.74	1.49[a]
Gly	EF = 75.13 × exp(-0.37 × Q_p)	0.82	0.92[b]	EF = 91.54 × exp(-0.14 × Q_p)	0.82	1.01[ab]	EF = 98.36 × exp(-0.11 × Q_p)	0.69	1.41[a]
His	EF = 59.73 × exp(-0.21 × Q_p)	0.73	0.68[c]	EF = 68.9 × exp(-0.39 × Q_p)	0.75	0.91[b]	EF = 3.803 × Q_p + 20.05	0.72	0.61[a]
Ile	EF = 72.14 × exp(-0.45 × Q_p)	0.84	0.75[b]	EF = 78.31 × exp(-0.53 × Q_p)	0.82	0.89[b]	EF = 91.69 × exp(-0.19 × Q_p)	0.75	1.81[a]
Leu	EF = 71.7 × exp(-0.46 × Q_p)	0.84	0.74[b]	EF = 77.78 × exp(-0.21 × Q_p)	0.82	0.88[b]	EF = 91.54 × exp(-0.19 × Q_p)	0.75	1.80[a]
Lys	EF = 63.44 × exp(-0.49 × Q_p)	0.84	0.57[c]	EF = 75.51 × exp(-0.24 × Q_p)	0.82	0.84[b]	EF = 91.95 × exp(-0.23 × Q_p)	0.77	1.50[a]
Met	EF = 71.24 × exp(-0.49 × Q_p)	0.84	0.75[b]	EF = 75.51 × exp(-0.24 × Q_p)	0.82	0.88[b]	EF = 92.33 × exp(-0.18 × Q_p)	0.75	1.86[a]
Phe	EF = 72.49 × exp(-0.46 × Q_p)	0.85	0.76[b]	EF = 76.65 × exp(-0.22 × Q_p)	0.82	0.87[b]	EF = 91.71 × exp(-0.19 × Q_p)	0.75	1.80[a]
Pro	EF = 73.34 × exp(-0.42 × Q_p)	0.83	0.82[b]	EF = 83.87 × exp(-0.17 × Q_p)	0.82	0.94[b]	EF = 91.68 × exp(-0.17 × Q_p)	0.82	1.64[a]
Ser	EF = 70.17 × exp (-0.41 × Q_p)	0.83	0.77[b]	EF = 82.95 × exp(-0.18 × Q_p)	0.83	0.96[b]	EF = 91.72 × exp(-0.14 × Q_p)	0.70	1.41[a]
Thr	EF = 74.34 × exp(-0.47 × Q_p)	0.82	0.76[b]	EF = 80.25 × exp(-0.19 × Q_p)	0.83	0.91[b]	EF = 91.23 × exp(-0.18 × Q_p)	0.75	1.67[a]
Tyr	EF = 73.61 × exp(-0.45 × Q_p)	0.84	0.76[b]	EF = 76.02 × exp(-0.22 × Q_p)	0.83	0.85[b]	EF = 92.25 × exp(-0.19 × Q_p)	0.75	1.90[a]
Val	EF = 73.26 × exp(-0.44 × Q_p)	0.84	0.77[b]	EF = 78.52 × exp(-0.21 × Q_p)	0.82	0.90[b]	EF = 91.73 × exp(-0.18 × Q_p)	0.75	1.74[a]

Table A2. Exponential and linear functions of diffusive fluxes (D) of NH_4^+, NO_3^-, and 18 amino acids, recovered by three membrane types from a stirred solution containing 100 µmols of each N compound. Equations are expressed as functions of flow rate (Q_p).

	Short+20kDa		**Short+100kDa**		**Long+20kDa**	
Compound	**Eqn**	**R^2**	**Eqn**	**R^2**	**Eqn**	**R^2**
NH_4^+	Ambiguous		D = -119.7 x exp(-0.8 x Q_p)	0.70	D = -66.96 x exp(-0.19 x Q_p)	0.91
NO_3^-	D = -175.1 x exp(-0.21 x Q_p)	0.65	D = -120.5 x exp(-0.16 x Q_p)	0.88	D = -209.5 x exp(-0.40 x Q_p)	0.77
Ala	D = -43.47 x exp(-0.38 x Q_p)	0.34	Ambiguous		D = -41.05 x exp(-0.37 x Q_p)	0.64
Arg	D = -68.48 x exp(-0.37 x Q_p)	0.22	Ambiguous		D = -99.36 x exp(-0.56 x Q_p)	0.50
Asn	D = -34.99 x exp(-0.38 x Q_p)	0.31	Ambiguous		D = -35.5 x exp(-0.48 x Q_p)	0.54
Asp	D = -35.14 x exp(-0.38 x Q_p)	0.31	Ambiguous		D = -33.17 x exp(-0.33 x Q_p)	0.53
Gln	D = -30.98 x exp(-0.38 x Q_p)	0.29	Ambiguous		D = -34.11 x exp(-0.53 x Q_p)	0.54
Glu	D = -32.46 x exp(-0.38 x Q_p)	0.29	Ambiguous		D = -35.47 x exp(-0.42 x Q_p)	0.60
Gly	D = -50.55 x exp(-0.37 x Q_p)	0.43	Ambiguous		D = -45.75 x exp(-0.33 x Q_p)	0.60
His	D = -48.49 x exp(-0.45 x Q_p)	0.19	Ambiguous		D = -68.33 x exp(-0.61 x Q_p)	0.50
Ile	D = -31.59 x exp(-0.38 x Q_p)	0.30	Ambiguous		D = -34.96 x exp(-0.45 x Q_p)	0.58
Leu	D = -31.22 x exp(-0.38 x Q_p)	0.30	Ambiguous		D = -34.66 x exp(-0.45 x Q_p)	0.57
Lys	D = -40.96 x exp(-0.41 x Q_p)	0.19	Ambiguous		D = -59.35 x exp (-0.62 x Q_p)	0.45
Met	D = -32.88 x exp(-0.38 x Q_p)	0.30	Ambiguous		D = -35.72 x exp(-0.44 x Q_p)	0.58
Phe	D = -33.54 x exp(-0.34 x Q_p)	0.35	Ambiguous		D = -35.31 x exp(-0.44 x Q_p)	0.59
Pro	D = -39.61 x exp(-0.38 x Q_p)	0.33	Ambiguous		D = -39.23 x exp(-0.39 x Q_p)	0.63
Ser	D = -38.58 x exp (-0.39 x Q_p)	0.33	Ambiguous		D = -39.25 x exp(-0.40 x Q_p)	0.59
Thr	D = -30.91 x exp(-0.26 x Q_p)	0.25	Ambiguous		D = -35.93 x exp(-0.43 x Q_p)	0.59
Tyr	D = -31.78 x exp(-0.37 x Q_p)	0.30	Ambiguous		D = -35.11 x exp(-0.45 x Q_p)	0.58
Val	D = -33.74 x exp(-0.38 x Q_p)	0.31	Ambiguous		D = -36.24 x exp(-0.43 x Q_p)	0.60

Table A3. Exponential and linear functions of relative recoveries (EF) of NH_4^+, NO_3^-, glutamine (Gln), glutamic acid (Glu), glycine (Gly) and lysine (Lys), recovered by three membrane types from a soil microcosm spiked with 100 µmols of each N compound. Functions were calculated from Figure 3, and are expressed as functions of flow rate (Q_p). Permeability factors (PF) are given for each membrane and compound; letters represent significant differences between each membrane.

	Short+20kDa			**Short+100kDa**			**Long+20kDa**		
Compound	**Eqn**	**R^2**	**PF**	**Eqn**	**R^2**	**PF**	**Eqn**	**R^2**	**PF**
NH_4^+	$EF = 86.91 \times \exp(-0.65 \times Q_p)$	0.93	0.75^b	$EF = 67.7 \times \exp(-0.57 \times Q_p)$	0.87	0.57^c	$EF = 118.8 \times \exp(-0.47 \times Q_p)$	0.93	2.74^a
NO_3^-	$EF = 101.2 \times \exp(-0.65 \times Q_p)$	0.83	0.76^b	$EF = 80.43 \times \exp(-0.50 \times Q_p)$	0.79	0.62^b	$EF = 135 \times \exp(-0.44 \times Q_p)$	0.87	2.51^a
Gln	$EF = 4.7 \times \exp(-0.81 \times Q_p)$	0.28	0.03^b	$EF = 8.3 \times \exp(-0.71 \times Q_p)$	0.56	0.05^{ab}	$EF = 10.59 \times \exp(-0.40 \times Q_p)$	0.41	0.08^a
Glu	$EF = 6.01 \times \exp(-0.22 \times Q_p)$	0.27	0.05	$EF = 15.09 \times \exp(-0.15 \times Q_p)$	0.56	0.07	$EF = 15.44 \times \exp(-0.42 \times Q_p)$	0.36	0.11
Gly	$EF = 16.01 \times \exp(-0.08 \times Q_p)$	0.34	0.08	$EF = 17.96 \times \exp(-0.58 \times Q_p)$	0.63	0.12	$EF = 20.98 \times \exp(-0.36 \times Q_p)$	0.40	0.18
Lys	*Ambiguous*		0.001	*Ambiguous*		0.002	$EF = 0.57 \times \exp(-0.05 \times Q_p)$	0.18	0.003

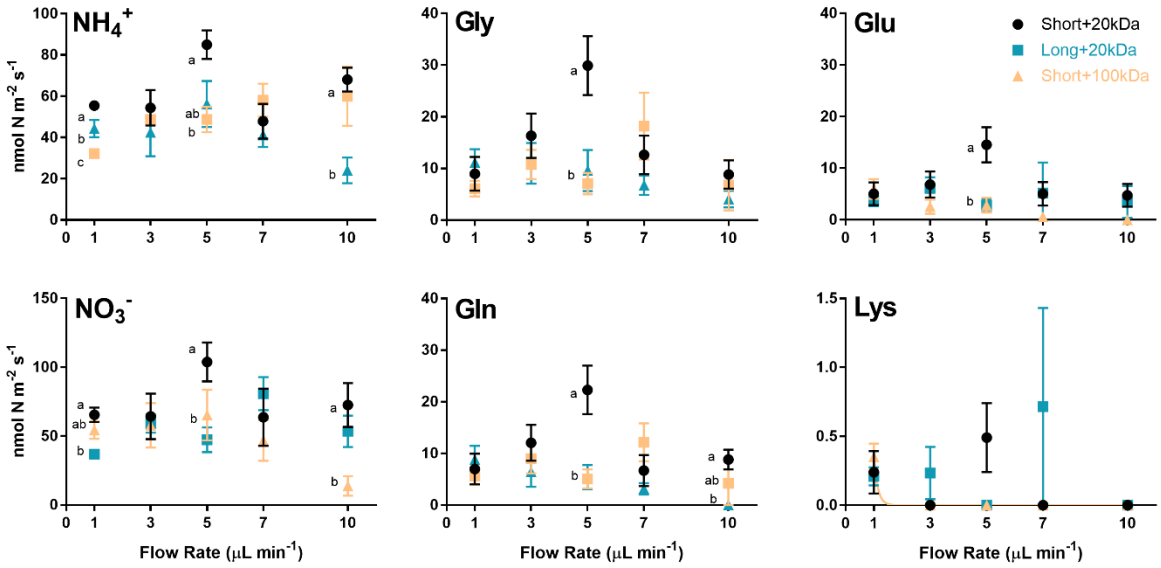

Figure A5. Diffusive fluxes (nmol N m^{-2} s^{-1}) of six N compounds: ammonium (NH_4^+), nitrate (NO_3^-), glycine (Gly), glutamine (Gln), glutamic acid (Glu), and lysine (Lys) at five flow rates, sampled from a soil spiked with 100 µmol N standard solution with three membrane types – Short+20kDa (in black), Long+20kDa (in blue), and Short+100kDa (in orange). Letters represent significant differences between membranes at each flow rate ($p \leq 0.05$). For each data point, n = 6.

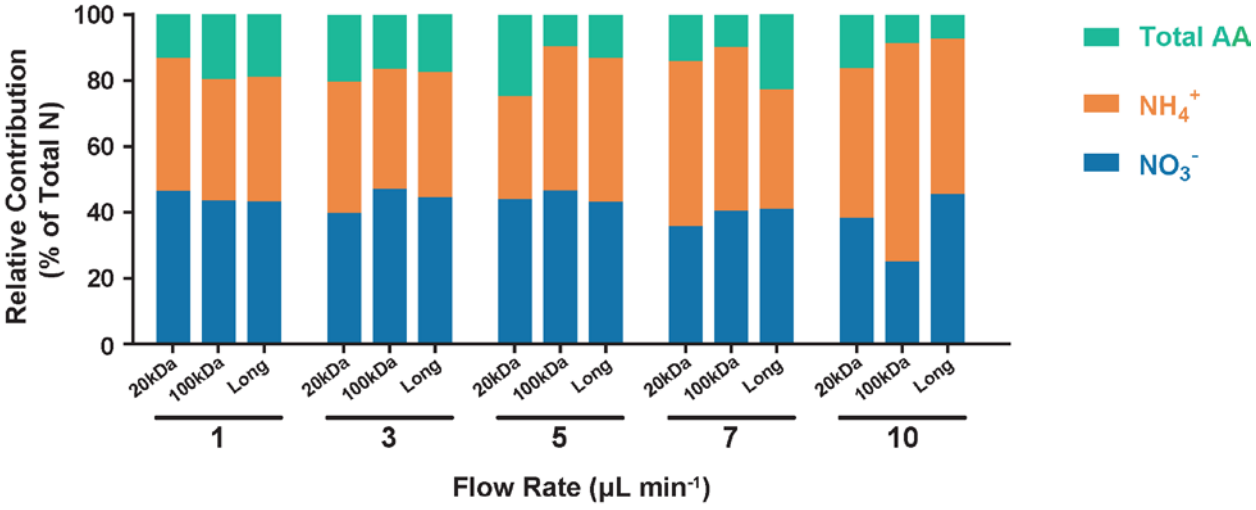

Figure A6. Mean relative contributions of total amino acids (Total AA, in green), ammonium (NH_4^+, in orange) and nitrate (NO_3^-, in blue) to total nitrogen recoveries from a soil spiked with a 100 µmol N standard solution, by three membrane types – Short+20 kDa (20kDa); Short+100 kDa (100kDa) and Long+20kDa (Long) at five flow rates. For each mean, n = 6. No statistical differences in N proportions were observed between membrane types at each flow rate (one-way ANOVA, $p > 0.05$).