DOCTOR OF PHILOSOPHY

Developing in-situ and real-time methods of soil nitrogen determination

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Award date:
2015

Awarding institution:
Bangor University

Developing in-situ and real-time methods of soil nitrogen determination

A thesis submitted to Bangor University by

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In candidature for the degree

Philosophiae Doctor

March 2015

School of Environment, Natural Resources and Geography

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“A gem cannot be polished without friction, nor a man perfected without trials.”

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Table of contents

Thesis Summary

The efficient use of nitrogen (N) fertilisers in agriculture is of great concern as diffuse losses, where N has been applied in excess of crop demand, may lead to significant environmental pollution and contribute to global warming. This thesis investigated a range of novel and emerging techniques to better enable the real-time and in-situ determination of soil N, in order to increase our fundamental understanding of soil N dynamics and improve management of agricultural soils. Microdialysis is an emerging technique which has been used for in-situ and minimally-invasive sampling of soil solution solutes. In *Article 1,* the use of microdialysis for the assessment of soil N status was investigated. Diffusive-flux measurements of 8 soils along a catena sequence were compared to conventional soil core batch extractions (using 0.5 M K_2SO_4 or distilled H_2O). The percentage contribution that amino acids, NH_4^+, and NO_3^- made to total plant-available N, were most similar to distilled water extractions. However, the relative magnitude of the diffusive-flux measurements did not always reflect the pool sizes as estimated by the soil extractions, which indicates the role that differing chemical and physical soil properties have in the control of plant N availability. In *Article II*, microdialysis was used for the in-situ sampling of amino acids, NH_4^+, and NO_3^- from the rhizospheres of *Zea mays* L. seedlings grown in soil filled rhizotubes. The results showed a significant decrease in soil solution [NO_3^-] as the root tip grew past the probe. Net amino acid exudation from root tips had been identified using direct sampling from root surfaces of seedlings grown in a sterile nutrient solution but this exudation was not evident in the microdialysis sampling, which was attributed to rapid microbial uptake. *Article III* investigated the use of commercially available NO_3^- ion-selective electrodes (ISEs) and dual-wavelength UV spectroscopy for the rapid on-farm measurement of soil N. Our results showed that manual extraction using distilled H_2O, combined with either NO_3^- ISEs or UV spectroscopy could accurately determine the NO_3^- concentration of the extracts. As such, both of these methods have the potential to be used as on-farm quick tests. In *Article IV*, the use of novel NO_3^- ISEs for in-situ and real-time monitoring of an agricultural soil, both in a field trial and under controlled conditions in the laboratory, was demonstrated. Results from the ISEs were found to be statistically similar to conventional laboratory analysis of contemporaneous soil samples on 16 out of 19 occasions. These novel NO_3^- ISEs provide a new opportunity for in-situ and real-time measurement of soil N dynamics, which represents a significant step forward for analytical soil science and environmental monitoring. *Article V,* investigated the spatial variation of soil N in a grazed grassland field in order to optimise the spatial and economic configuration of an in-situ sensor network. It was established that at least 61% of the total accumulated variance in amino acids, NH_4^+ and NO_3^- occurred at scales < 2 m, with significant variation occurring at the sub 1-cm scale. This data was used to demonstrate how an in-situ sensing network could be optimised on a cost-accuracy basis. Future work needs to focus on how data derived from in-situ soil N sensors can be used to improve fertiliser recommendations and the efficiency of N-use in agriculture.

Acknowledgements

I would like to acknowledge the funding for this PhD from Eblex, Hybu Cig Cymru (HCC), DairyCo and Quality meat Scotland (QMS). I hope these organisations will continue to recognise the importance of soil for livestock production and provide funding for future research. Thanks especially go to Lynfa Davis, Debbie McConnell and Liz Genever for support, guidance and good times at the annual PhD conference.

This project was supervised by Prof. DL Jones and Dr AP Williams from Bangor University and Prof. A Miller from the John Innes Centre, Norwich. I am very grateful for all their support and good humour. Thanks especially to Davey for taking me on after 7 years in the academic wilderness and to Prysor for dealing with the Six Nations defeat by England so well this year. Thanks also go to Dr. Paul Hill for taking the time to help with experimental techniques and for the many corridor discussions on microdialysis and ion-selective electrodes. I am also indebted to Dr. Murray Lark, of the British Geological Survey for collaborating on the final chapter of this thesis.

I very much enjoyed my fieldwork at Henfaes, the University farm, which was supported by Llinos Hughes and Mark Hughes. Thanks also to ECW technical staff – Sarah, Jonathan and Gordon for putting up with me in the lab and helping out where ever they could. I have not been alone in my PhD quest and the support, both moral and intellectual, from fellow PhD students has been invaluable. Thanks especially to John Hyland, Darren Owen, Kara Marsden and Al-Mario Casimir. Thanks also to Helen Glanville who always had time to help out and make me a brew.

Finally, thanks to my mother for supporting and encouraging me in whatever I have chosen to do in life, and to Jen, for all her love.

List of articles

This thesis is based on the following articles:

I. Shaw, R.; Williams, A.P; Jones, D.L. (2014). Assessing soil nitrogen availability using microdialysis-derived diffusive flux measurements. Soil Science Society of America Journal. 78: 1797-1803.

II. Shaw, R.; Hill, P.W.; Williams, A.P; Jones, D.L. (2015). Nitrogen dynamics in the rhizosphere. *Unpublished manuscript.*

III. Shaw, R.; Williams, A.P; Miller, A.; Jones, D.L. (2013). Assessing the potential for ion selective electrodes and dual wavelength UV spectroscopy as a rapid on-farm measurement of soil nitrate concentration. Agriculture. 3: 327-341

IV. Shaw, R.; Williams, A.P; Miller, A.; Jones, D.L. (2015). In-situ monitoring of soil nitrate in real-time using ion-selective electrodes. *Unpublished manuscript.*

V. Shaw, R.; Lark, R.M.; Williams, A.P; Chadwick, D.R.; Jones, D.L. (2015). Characterising the within-field scale spatial variation of different N forms in a grassland soil and the implications for in-situ N sensor technology and precision agriculture.
A version of this manuscript has been submitted to Agriculture, Ecosystems and Environment (July 2015) and is currently under review.

Chapter 1

Introduction and thesis objectives

1. General introduction and rationale

This body of research was jointly funded by the agricultural levy boards EBLEX, Hybu Cig Cymru (HCC), Quality Meat Scotland (QMS) and DairyCo as part of their drive to improve on-farm economics, increase the efficiency of agricultural production and reduce the environmental footprint of farming operations.

Agriculture faces a challenging future, where increasing production to meet demand from an ever growing global population is set against the need to reduce its environmental impact. Of particular concern is the diffuse loss of reactive nitrogen (N) from agricultural land where N fertilisers and manures are frequently applied in excess of crop demand. These losses have resulted in perturbation of natural ecosystems and enrichment of the atmosphere, hydrosphere and biosphere (Vitousek et al., 1997). It is currently estimated that on average, 50% of manure and fertiliser N applied to agricultural land in Europe is lost to the environment resulting in an economic cost in the range of €13 - €65 billion per year (Sutton et al., 2011). As such, improving the efficiency of N-use represents a major goal of sustainable farming systems from both an economic and environmental standpoint.

Improving our knowledge of both spatial and temporal variation in soil N availability and embracing the precision agriculture paradigm may bring about improvements in fertiliser N management and N-use efficiency (NUE). However, current methods of soil testing are time consuming and expensive and there is an over reliance on semi-official fertiliser recommendations and modelling approaches which have limited accuracy (Cuttle and Jarvis, 2005; Sylvester-Bradley et al., 2008). Significant improvements in NUE may be realised by the development of methods and sensors that allow on-site or in-situ monitoring of soil N in real time (Sylvester-Bradley et al., 1999; Adamchuk et al., 2004; Kim et al., 2009). Furthermore, our fundamental understanding of soil N dynamics, and hence, our ability to infer accurate fertiliser recommendations from soil measurements, is frequently limited by a lack of non-destructive and minimally-invasive in-situ techniques.

This thesis attempts to address these issues by investigating and developing a range of emerging and novel techniques for the determination of soil N.

2. Plan of thesis

This thesis is divided into a further 7 chapters, starting with a literature review which chronicles the issues surrounding N use in agriculture and explores different approaches to improve NUE

through better methodologies of soil N determination. In particular, this chapter evaluates current sensing technologies that have the potential to be used for real-time, in-situ soil N monitoring.

The experimental work is presented as five stand-alone scientific articles; as such, there is some unavoidable repetition of some introductory material, methodology and references. The first two experimental chapters detail studies into the use of microdialysis sampling for the in-situ assessment of soil N. More specifically, chapter 3 investigated the use of microdialysis sampling for assessing the availability of amino acids, NH_4^+ and NO_3^- in a range of agricultural soils. Chapter 4 describes a study to investigate N dynamics in the rhizosphere of *Zea mays* L. seedlings. Microdialysis sampling was used to monitor in-situ changes in soil N concentration within the developing rhizosphere of *Zea mays* L. seedlings. This approach complemented parallel experiments which assessed root exudation of amino acids and bacterial uptake of exogenously applied amino acids from soil.

The use of UV spectroscopy and commercially available ion-selective electrodes (ISE) for the on-farm rapid testing of soil NO_3^- was investigated in chapter 5. In chapter 6, the use of a novel NO_3^- ISE, which was constructed in our laboratory, for the in-situ monitoring of soil NO_3^- in real-time is demonstrated. As an extension to this study, chapter 7 investigated how to optimise the configuration of an in-situ sensor network for soil N monitoring in an agriculture field. This was achieved using a geo-statistical approach to characterise soil N spatial variance at within-field scales.

Chapter 8 includes a general discussion, within the context of the aims and objectives of this thesis, of the results from all the experimental chapters. Conclusions are drawn and areas of further work identified. Finally, the appendices include conference paper abstracts that were presented orally at the 18th Nitrogen Workshop, Lisbon, Portugal (2014), and the European Grassland Federation Conference, Aberystwyth, UK (2014). Also included is a detailed protocol for building the NO_3^- ISEs used in chapter 6 and a selection of photographs of experimental work.

3. Thesis aims and objectives

The aims and objectives of this thesis were decided upon within the contextual framework described above and to satisfy the funding body's research needs. They were:

1) To chronicle issues related to the use of N in agriculture and to review new approaches to soil N determination with a focus on in-situ monitoring (chapter 2);

2) Investigate the use of microdialysis based sampling for the determination of plant available N and in-situ monitoring of soil N dynamics (chapters 3 & 4);
3) Develop farmer operated tools and methodologies which are user-friendly and could be used for the on-farm determination of soil N (chapters 5 & 6).
4) Construct, develop and test a NO_3^- ISE for the real-time, in-situ monitoring of soil N (chapter 6).
5) Investigate how to optimise the field-scale configuration of an in-situ sensor network and to facilitate both accurate and economical soil N monitoring (chapter 7).

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Chapter 2

Literature review: Nitrogen use in agriculture; exploring the potential of in-situ and real-time soil measurements to improve nitrogen-use efficiency

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Unpublished manuscript

Author contributions:

RS wrote the first draft of the manuscript with all authors contributing to the final version.

1. The future of farming

Global agriculture faces a challenging future. The population of our planet is growing rapidly and in 2050 it is predicted to reach 9 billion (Godfray et al., 2010). In addition, the demand for food with a high protein and calorific content is increasing. It is estimated that this demand equates to a 100%-110% increase in food production from 2005 to 2050 (Tilman et al., 2011); with a subsequent large increase in fertiliser use (Tilman et al., 2002). To meet this demand in production, agriculture is faced with the dilemma of whether to expand crop production into natural ecosystems – extensification – or increase yields from existing farmland by further intensification. "Sustainable intensification" refers to altering agricultural systems so that yields from existing cropland are increased, whilst the delivery of other ecosystem services from agriculture are maintained or increased. The practicalities of how this may be achieved has been one of the main areas of agricultural research for the past two decades.

Central to the concept of sustainable intensification is improving nitrogen-use efficiency (NUE) (Cassman et al., 2002; Robertson and Vitousek, 2009). Currently, it is estimated that 50% of manure and fertiliser N applied to agricultural land in Europe is lost to the environment, resulting in an economic loss in the range of €13-65 billion per year (Sutton et al., 2011b). Improving NUE would therefore be beneficial to both the farmer, as it will allow a greater return for each unit of N that is used, and to the environment, as pollution per unit of food produced and per unit of N applied is reduced. However, if predicated increases in crop yields occur, it is likely that N pollution on a land area basis will continue to increase unless significant advances are made in fertiliser-use efficiencies and reductions in crop requirements are achieved (Sylvester-Bradley and Withers, 2011). This review summarises the issues of agricultural N pollution, the pathways by which fertiliser N may be transformed and subsequently lost from the soil-plant system and approaches that may lead to improved NUE. Specifically, new methods for soil N determination, with an emphasis on real-time, in-situ sensing, are explored.

2. The nitrogen problem

One hundred years ago, the Haber-Bosch process, by which ammonia (NH_3) is synthesised from its constituent elements, was commercialised. This invention is arguably one of the most important in human history as it has enabled the mass production of inorganic N fertilisers. In turn, this has bought about large increases in crop yields (i.e. the Green Revolution), driving the exponential growth of the human population that has occurred over the past century. The

benefits of inorganic N fertilisers to agriculture and the development of society are irrefutable. However, these benefits are not without their associated costs, and as the world population grows and production of reactive N (Nr) continues to increase, these costs are becoming more apparent. The European Nitrogen Assessment estimates that the costs of excess Nr in the environment are more than double the value that nitrogen fertilisers are estimated to add to European farm income (Sutton et al., 2011a). Annual Nr fixation (which includes fixation from the burning of fossil fuels) is now double that of natural, pre-industrial levels (Galloway et al., 1995) and our planet has become highly enriched with Nr. This considerable anthropogenic disturbance of the N cycle is having large and far-reaching, adverse effects on terrestrial, freshwater and marine ecosystems, whilst also changing the composition of the atmosphere (Vitousek et al., 1997; Sutton et al., 2011b). Exacerbating this problem is a concept known as the Nr cascade. One atom of N can have multiple deleterious effects on different ecosystems and to human health as its moves from one environment to another and is transformed to different forms of Nr, before it is eventually recycled back to N_2 (Galloway et al., 2003).

Nr pollution from agriculture and its effects on ecosystems and human health has been well documented in the literature. Of particular concern is diffuse agricultural pollution of aquatic ecosystems, both freshwater and marine, by nitrate (NO_3^-) leached from fields where inorganic fertilisers or manures have been applied in excess (Iversen et al., 1998; Grizetti, 2011). These ecosystems, in many areas of the world, are being negatively affected by increasing concentrations of NO_3^- (Edwards et al., 2003; Smith, 2003; Conley et al., 2009). This can lead to eutrophication, which may cause toxic algal blooms, water anoxia, fish kills and biodiversity loss (Grizetti, 2011). Elevated concentrations in drinking water sources, especially aquifers, has been a concern for human health and has been linked to increased risk of gastro-intestinal cancers and methaemoglobinaemia, although this remains controversial and disputed (Addiscott and Benjamin, 2004; Powlson et al., 2008). Concerns over high levels of NO_3^- in both surface and ground waters led to the implementation of the European Nitrates Directive (EEC, 1991). In accordance with this directive, areas where concentrations of NO_3^- in surface or ground waters exceed or have the potential to exceed 50 mg NO_3^- l^{-1}, and where waters are eutrophic or likely to become eutrophic, must be identified as NO_3^- vulnerable zones (NVZs). Within these NVZs, farmers are required to conform to strict guidelines regarding use of both inorganic and organic N fertilisers, have adequate storage facilities for slurries and manures and keep records of the amount of N applied to their land in order to reduce N losses to ground and surface waters (Defra, 2011).

Agriculture is also responsible for increased emissions of nitrous oxide (N_2O) from soils, a potent greenhouse gas with a global warming efficacy 310 times that of CO_2 (IPCC, 2007). Thomas et al. (2011), estimates that in 2009, N_2O emissions for England and Wales constituted 7.1% and 12.5% of total greenhouse gas emissions, of which, 75.8% and 87% were attributed to agricultural sources. The increasing inputs of N to agricultural land via manure, fertilisers and atmospheric deposition are responsible for the increase in the emissions of N_2O (Kroeze et al., 1999). N_2O is also currently the single most important ozone-depleting pollutant and is expected to remain so for the duration of the 21st century (Ravishankara et al., 2009). Emissions of NH_3 and, to a lesser extent NO_2, can also cause significant tropospheric atmospheric pollution in the form of particulate matter, formation of ozone and photochemical smog and direct foliar damage (Vitousek et al., 1997; Moldanova et al., 2011). Increasing concentrations of Nr in the atmosphere also cause increased atmospheric deposition of Nr, which is recognized as one of the most important threats to global biodiversity (Sala et al., 2000) and is one of the main drivers for biodiversity loss in Europe (Dise, 2011).

This agricultural pollution is having a damaging effect on some ecosystem services, i.e. the provision of clean drinking water and air, loss of biodiversity, all of which has a direct or indirect economic cost. As these externality costs increase, the economic benefit gained from N-fertilisation of agricultural land is diminished. It is likely, therefore, that if these costs were taken into consideration, the economic optimal N-fertilisation rate in Western Europe would be reduced by at least 50 kg ha^{-1} (Brink and van Grinsven, 2011).

3. N cycling in Agri-ecosystems

3.1. The fate of fertiliser N

Understanding the cycling and dynamics of N in soil is fundamental to improving NUE. In natural ecosystems, with low Nr inputs, the cycling of N is tightly conserved and hence losses are minimal (Schimel and Bennett, 2004; Huygens et al., 2007). In agricultural systems, characterized by large Nr inputs, the N cycle is very leaky as excess Nr, not used by plants or immobilized by microbes, may be lost from the system in significant quantities (Cassman et al., 2002). The relative importance of each loss pathway depends upon many factors including the form of fertiliser N which is applied, the fertiliser strategy used, soil type and weather. Figure 1 illustrates the main biogeochemical transformations of N in soil and summarised in the following sections (section 3.2 – 3.5) are the mechanisms via which fertiliser N may be transformed and lost from the soil–plant system.

3.2. Immobilisation

Immobilisation of N occurs when heterotrophic microorganisms soil take up and assimilate NH_4^+ and NO_3^-, and hence N is removed from the plant-available pool. Whether this can be considered as a pathway for N loss in debatable, as much of the immobilised N will be remineralised and become plant available as the bacterial pool turns over. A study on the fate of fertiliser N applied to spring wheat, across seven sites in East Anglia, UK, suggested that immobilisation of fertiliser N was the greatest cause of fertiliser use inefficiency (King et al., 2001). Immobilisation may be a particular problem if it occurs during times of high plant demand and if re-mineralisation occurs during periods of low demand or following harvest, which may lead to environmental losses. Essentially, the importance of immobilisation relates to the dynamics of plant-microbe competition, as plant-available N is also microbial-available N. The dynamics of this competition are complex; it is thought that in the short term, microbes will outcompete plants, but the unidirectional flow of nutrients from the soil to the roots means that plants are the winner in the long run (Kuzyakov and Xu, 2013). Microbial uptake of NO_3^- in bulk soil has been shown to be minimal compared to NH_4^+ and amino acid (AA) uptake (Abaas et al., 2012), which suggests that use of nitrate fertilisers may be a way to reduce immobilisation 'losses'. However, these experiments were conducted in bulk soil rather than in the rhizosphere, where root exudation of labile carbon may stimulate increased microbial NO_3^- uptake (Kuzyakov, 2002).

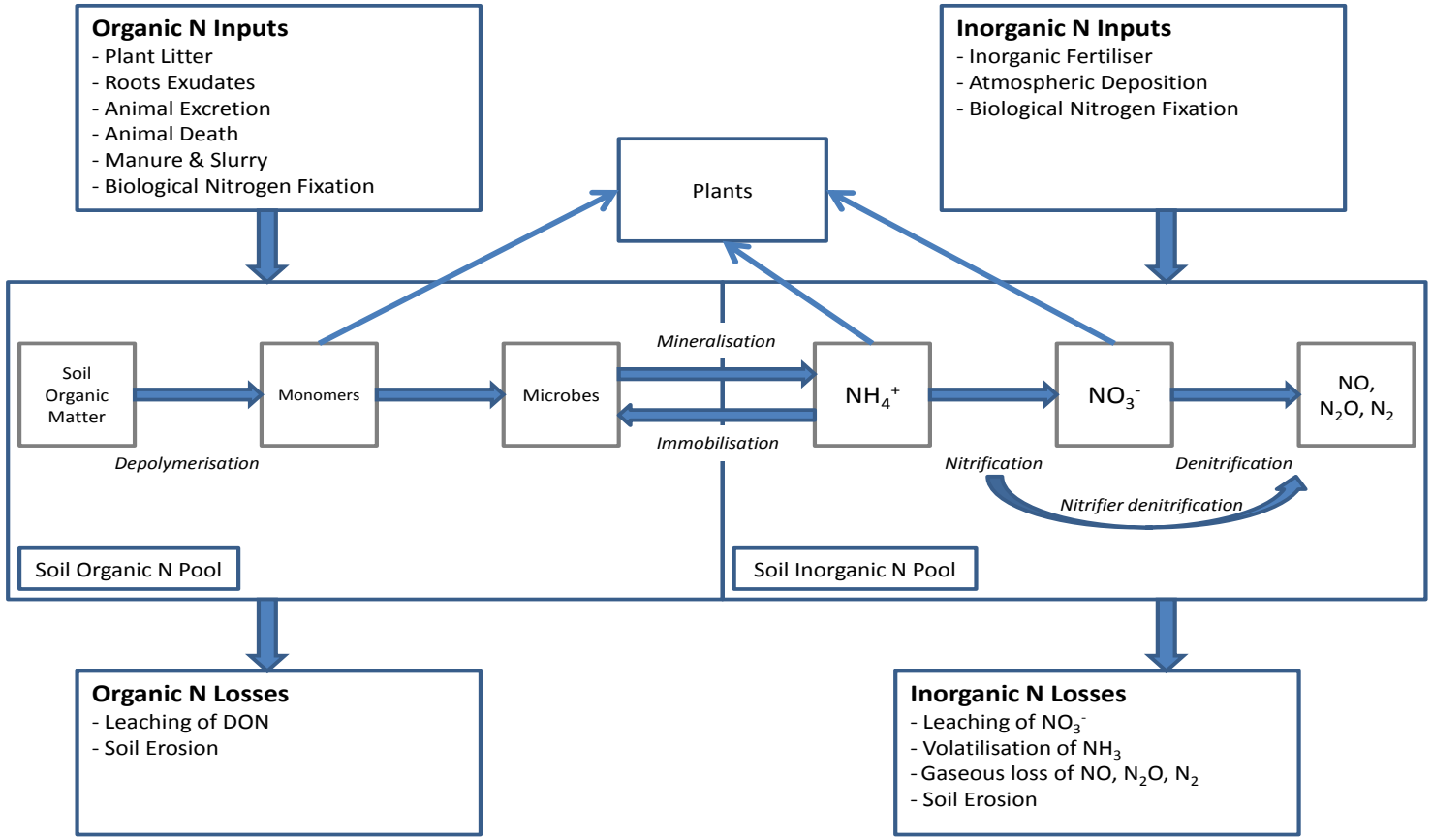

Figure 1. The nitrogen (N) cycle within agricultural systems, showing the major inputs and losses of reactive N and the main biogeochemical transformations of N in the soil.

3.3. Ammonia volatilisation

Ammonia (NH_3) volatilisation is a major pathway of N loss, especially from livestock farms, where manures and slurries are stored and applied to land as fertiliser (Oenema et al., 2007). In addition, application of urea (Soares et al., 2012) and ammonium-based synthetic fertilisers to warm, dry and alkaline soils (Bouwman et al., 1997), may result in large emissions of NH_3. There is scope for large improvements in the N efficiency of manure and slurry storage (Jarvis, 2011), but here we focus on the use of manures and slurries as N fertiliser. Global emissions of NH_3 from application of synthetic fertilisers and manures/slurries has been estimated at 78 and 33 million tons N yr^{-1}, respectively, accounting for approximately 14% and 23% of total global fertiliser and manure/slurry N (Bouwman et al., 2002).

NH_3 (and other N forms) can also be lost from the plant during photorespiration, with the main factor for this loss being the imbalance between plant N uptake and assimilation (Xu et al., 2012). Losses via this pathway have been shown to be significant, with excess of 40 kg ha^{-1} being reported for soybean and maize. It is rare, however, that direct plant N losses are included in N budgets, which may lead to an overestimation of N losses from the soil and an underestimation of plant N uptake.

3.4. Nitrate leaching

Nitrification transforms relatively immobile NH_4^+, into a highly mobile form – NO_3^-. This has a large effect on the ability of the ecosystem to retain inorganic N, as NO_3^- can be quickly lost from the system by leaching, gaseous losses during the nitrification process and from subsequent denitrification (see section 3.5 below). Nitrification also has an acidifying effect on the soil as hydrogen ions are released during conversion of NH_4^+ to NO_3^-. The process is facilitated by obligate aerobic autotrophic bacteria which oxidise NH_4^+ by a two-step process to NO_3^- as shown in figure 2.

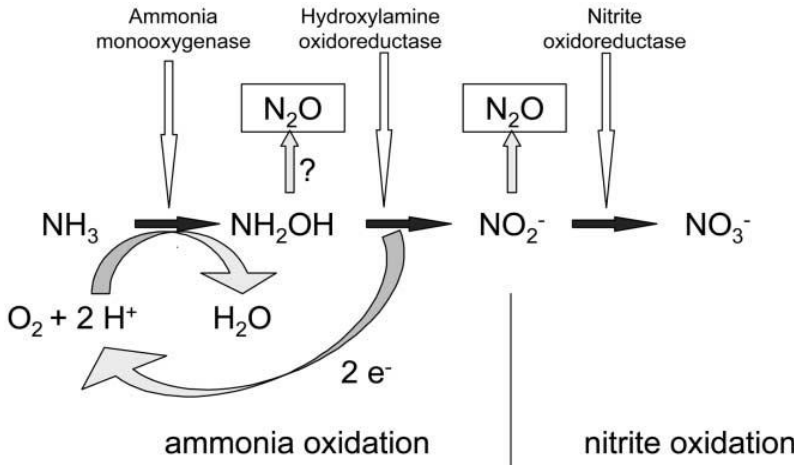

Figure 2. The nitrification process, showing enzymes utilised to catalyse the reactions. Taken from Wrage et al. (2001).

The most important factor regulating the rate of nitrification is the supply of NH_4^+ (Booth et al., 2005). Rates of nitrification are likely to be high in most aerobic agricultural soils where carbon limitation favours autotrophic NH_4^+ use (nitrification) over heterotrophic use of NH_4^+ (immobilisation) (Jones et al., 2004; Booth et al., 2005); as such, NO_3^- is typically the most important N source for crops. The key environmental controls of nitrification are soil water status and temperature. Soil pH was thought to be a key controlling variable; however, a global scale synthesis showed that pH has little influence on nitrification rates (Booth et al., 2005). In soils below field capacity, water availability will limit key physiological and metabolic processes and the supply of substrate to the microbes and hence rates of nitrification will be reduced. In water saturated soils, the supply of O_2 will be limited and as nitrifying bacteria are obligate aerobes, nitrification rates will be reduced (Norton and Stark, 2011). During the growing season, loss of nitrate via leaching is normally low as fertiliser is generally applied as plant uptake is increasing and soil moisture deficits are developing (Sylvester-Bradley and Withers, 2011). However, it is possible that in wet summers and in areas of high rainfall the field capacity of soils could be exceed, resulting in leaching of NO_3^-. Of greater concern is the residual NO_3^- remaining in the soil after harvest. This will happen when N fertilisers have been applied in excess of crop requirement. This residual NO_3^- is particularly vulnerable to leaching as there are no plants to take it up and increasing autumn precipitation, combined with a lack of transpiration, will induce movement of water and solutes down the soil profile.

3.5. Denitrification

Denitrification is the stepwise process, shown in figure 3, by which NO_3^- and nitrite (NO_2^-) are reduced to gaseous NO, N_2O and N_2, all of which can be the end product of the process depending upon the prevailing environmental conditions.

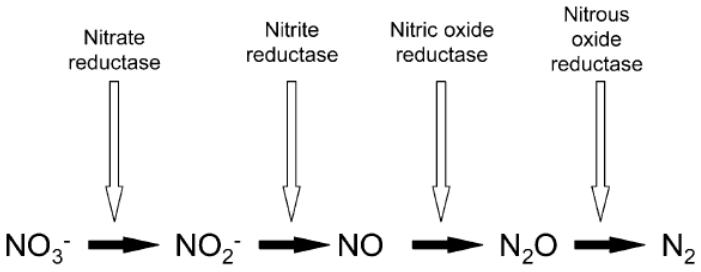

Figure 3. The stepwise reactions of the denitrification process, showing the enzymes which catalyse each stage. Taken from Wrage et al. (2001).

The process is carried out by a wide range of heterotrophic microbes, the majority of which are facultative aerobes. In aerobic conditions, it is more energy efficient to use O_2 rather than reduced forms of N as an electron acceptor. Consequently, denitrification will only occur when O_2 is limited. Because of this, it was initially thought that denitrification was only important in wetlands and fully saturated soils. However, it has been shown that nitrification and denitrification can occur concurrently in unsaturated soils due to localised anoxic conditions caused by the soil structure or areas where demand for oxygen is higher than can be met by diffusion – such as around a highly labile fragment of organic matter. These anoxic zones will become 'hotspots' for denitrification (Parkin, 1987).

The rate and final product of denitrification, often expressed as the N_2O:N_2 or N_2O:(N_2 + N_2O) ratio, depends upon an number of environmental conditions. A reduction in pH and an increase in O_2 availability both reduce the rate of denitrification but increase the N_2O:N_2 ratio (Knowles, 1982). It also appears that the N_2O:N_2 ratio increases as the concentration of NO_3^- increases as it is preferred as an electron acceptor over N_2O (Weier et al., 1993). Water filled pore space (WFPS) is often cited as a key environmental control on denitrification. Increasing the WFPS has been shown to increase total gaseous production from denitrification, with the highest values observed at 100% and 120% (soil submerged) and the greatest loss of N_2O at 80% (Weier et al., 1993). The N_2O:(N_2 + N_2O) ratio has been shown to decrease with increasing WFPS (Ciarlo et al., 2007).

N_2O may also be produced during the nitrification process under environmental conditions that are sub-optimal for heterotrophic denitrification (Bremner and Blackmer, 1978) and can comprise a significant proportion of total N_2O production (Kool et al., 2011). This process has been termed nitrifier denitrification and should be considered separate to conventional denitrification. The two processes are facilitated by different organisms and respond differently to environmental conditions, with conventional denitrification favoured by anaerobic conditions and nitrifier denitrification favoured by more aerobic conditions (Kool et al., 2011).

4. Improving nitrogen-use efficiency (NUE) in arable and grassland systems

4.1. Concepts for improving NUE

In arable systems, improving NUE will come primarily through increasing efficiency of fertiliser use (Raun and Johnson, 1999). Livestock systems are inherently more complicated and additional improvements in NUE may come from improved grassland management (reducing spoilage during grazing and silage cutting), increased N assimilation during digestion (Calsamiglia et al., 2010), dietary control (Jonker et al., 2002), improved management of manures and slurries (Oenema et al., 2007), modification of the agricultural system used (Gourley et al., 2012) and integration with arable farming (Watson et al., 2005).

Central in attempts to improve NUE of the soil-plant system is the fact that as the pool of plant available N in the soil increases, the proportion of this N which is subsequently lost to the environment by the pathways described above also increases (Cassman et al., 2002; Cardenas et al., 2010). N use by crops is most efficient at the lowest N application rates as there is a strong linear relationship between inputs of inorganic N and percentage recovery of that N by plants (Scholefield et al., 1991). Improvement in NUE can therefore be achieved by simply reducing inputs (Sylvester-Bradley et al., 2008). However, given the same crop management and climatic conditions, this will also reduce yields, which is economically undesirable for the farmer and counter to the concept of sustainable intensification. The challenge lies in the development and implementation of a system which gives improved efficiency for the same total input of N over the growing period. One way this may be achieved is by improved breeding and genetic modification of crops. Traditionally, crops have been bred for increased yields and/or disease resistance in conditions where N is not limiting to growth. This has meant that in order to support these higher yielding varieties, more fertiliser N is required and hence environmental losses have increased and NUE decreased. Breeding and genetic engineering

must now focus on improving NUE as well as yields. This may be achieved by improving N uptake efficiency, especially at low soil N availability, increasing N assimilation into plant tissue, improving N harvest index and reducing N losses due to photorespiration (Xu et al., 2012). Such genetic manipulations are however, very complex and metabolic feedback systems means there are often trade-offs to be made. In the short term therefore, it is likely that improvements in NUE will come from improved N fertiliser and soil management (Xu et al., 2012). Agronomists must concentrate on improving fertiliser N strategies to reduce environmental losses and hence increase the proportion of the applied fertiliser which is uptaken by crops. In theory, this can be achieved by synchronizing soil N supply with plant N demand, both spatially and temporally. This will entail the maintenance of the pool of plant available N at the minimum size required to meet crop N requirements and hence minimize losses to the environment (Cassman et al., 2002; Robertson and Vitousek, 2009). Interestingly, this is often purported to be a major way to reduce N losses in the UK, however, this strategy would be expected to have limited impact on losses in the non-growing season (i.e. winter N leaching losses).

4.2. Synchronising N availability with crop uptake

Theoretically, synchronising soil N availability with predicted crop N uptake sounds a fairly simple concept, although achieving this in practice is challenging due to the complex nature of the soil-plant system. Currently, most fertiliser recommendations for arable crops attempt to synchronise N supply and demand, but at very coarse temporal and spatial resolutions. They are often calculated by predicting crop yield, given optimum N fertilisation, based on soil, climatic and topographical variables of that field, and from this it is possible to calculate the total N requirement over the growing season. The indigenous N supply, from mineralization of soil organic matter, is then estimated – soil N supply index (SNS) - and deducted from the total crop N requirement; the resulting figure is the amount of N that must be supplied from fertilisers and/or legumes. This is certainly the case in the UK, where fertiliser recommendations are detailed in The Fertiliser Manual (RB209) (Defra, 2010). For grasslands, the system is slightly different. Farmers estimate the amount of herbage they require to support a specific production intensity and use RB209 for guidance on how much fertiliser N is needed to achieve the desired yields. The guidance in RB209 is based on the best available evidence from a large number of field trials but experimental work has shown that both the method to estimate SNS and overall N fertilisation recommendations remain seriously inaccurate (Sylvester-Bradley et al., 2008).

Precision agriculture (PA) attempts to address this issue by reducing uncertainties surrounding the measurement of key variables in order to determine optimum N fertiliser management (Pierce and Nowak, 1999; Dobermann et al., 2004). Key to the success of PA is the accurate assessment of within-field soil N status at a high spatial and temporal resolution to enable the variable rate application of N fertiliser. This approach allows areas of N deficiency and surplus to be addressed as well as in-season adjustment of fertiliser rates in accordance with current and predicted growing conditions. It has been argued that crop N status is the best indicator of soil N supply. Crop canopy sensing techniques for determination of plant N status are now in commercial use and can be used to inform variable rate fertiliser application (Raun and Johnson, 1999; Diacono et al., 2013). Whilst the advantages of this approach in some situations have been evidenced (Diacono et al., 2013), plant N status and resulting yield is the product of many variables and is not controlled solely by soil N availability (Devienne-Barret et al., 2000; Gastal and Lemaire, 2002). As such, accurate inference of soil N availability from plant N status may not be possible. Therefore, accurate calculation of fertiliser applications are likely to require a direct assessment of soil N to be made. However, conventional soil sampling techniques, coupled with laboratory analysis are expensive, labour-intensive, and time-consuming and cannot provide data of sufficient resolution to accurately inform PA management (Sylvester-Bradley et al., 1999; Kim et al., 2009). Destructive sampling, transport and storage of the sample, prior to laboratory analysis, may lead to changes in the chemistry of the soil samples. In addition, significant changes to the soil in-situ may occur during the time period between sampling and receiving the results. Such sampling is often done at the start of the growing season, but it is rare for in-season sampling to occur. One of the main limitations to the development of PA is a lack of appropriate techniques and sensors to enable the on-site, and ideally, in-situ quantification of soil N in real time (Sylvester-Bradley et al., 1999; Adamchuk et al., 2004; Kim et al., 2009). Some of the attempts to address this issue and the potential for in-situ soil N sensing are discussed below.

5. Soil N determination for precision agriculture

5.1. On-farm rapid testing of soil N status

Farmer-operated diagnostic tests for soil N removes the need for soil samples to be sent away for laboratory analysis. As such, information on soil N availability is directly available on-site in a rapid and, by avoiding the costs of analyses, economically effective way. This may improve the spatial and temporal resolution of soil N determination, with subsequent improvements in

fertiliser N management, although taking a large number of samples will still be fairly labour intensive. Previous work on rapid soil tests has involved manual soil extraction, using a variety of solutions, followed by subsequent analysis of NO_3^- by colorimetric strips combined with a handheld reflectometer (Jemison and Fox, 1988; Roth et al., 1991; Hartz, 1994; Wetselaar et al., 1998; Schmidhalter, 2005) or ion selective electrodes (ISEs) (Hartz et al., 1993; Hartz, 1994). Whilst this approach may represent an improvement over conventional sampling, there have been issues raised as to the accuracy and detection limits of these tests (Schmidhalter, 2005). Furthermore, these analyses must be coupled with a determination of soil moisture content to convert the NO_3^- concentration of the extract into soil NO_3^- concentration. These rapid tests may provide an alternative to laboratory analysis for a one-off soil testing event e.g. that which is performed at the start of the growing season prior to fertiliser application. However, it is unlikely, due to the effort required, that this approach would be used to obtain high resolution data on soil N status throughout the growing season to inform PA management.

5.2. On-the-go testing

The concept of the rapid-test has been extended by coupling NO_3^- ISEs with vehicle mounted, automated soil sampling and rapid extraction platforms (Adsett and Zoerb, 1991; Adsett et al., 1999; Sibley et al., 2009; Sibley et al., 2010). Sibley et al. (2009) developed an on-the-go system that took a soil sample (0–15 cm depth) and mixed it vigorously with distilled water to extract the NO_3^- (6 s per sample). The extract was immediately analysed using a NO_3^- ISE for to enable the determination of soil NO_3^- concentration in near real time. Sibley et al. (2009) found an excellent correlation ($r^2 > 0.9$) between their on-the-go rapid extraction/ISE measurement and their standard lab method (2 M KCl extraction/colourimetric analysis (Keeney and Nelson, 1982)). Each sample was geo-referenced, enabling soil NO_3^- maps to be produced, as shown in figure 4, which could be used to inform variable rate application of fertiliser.

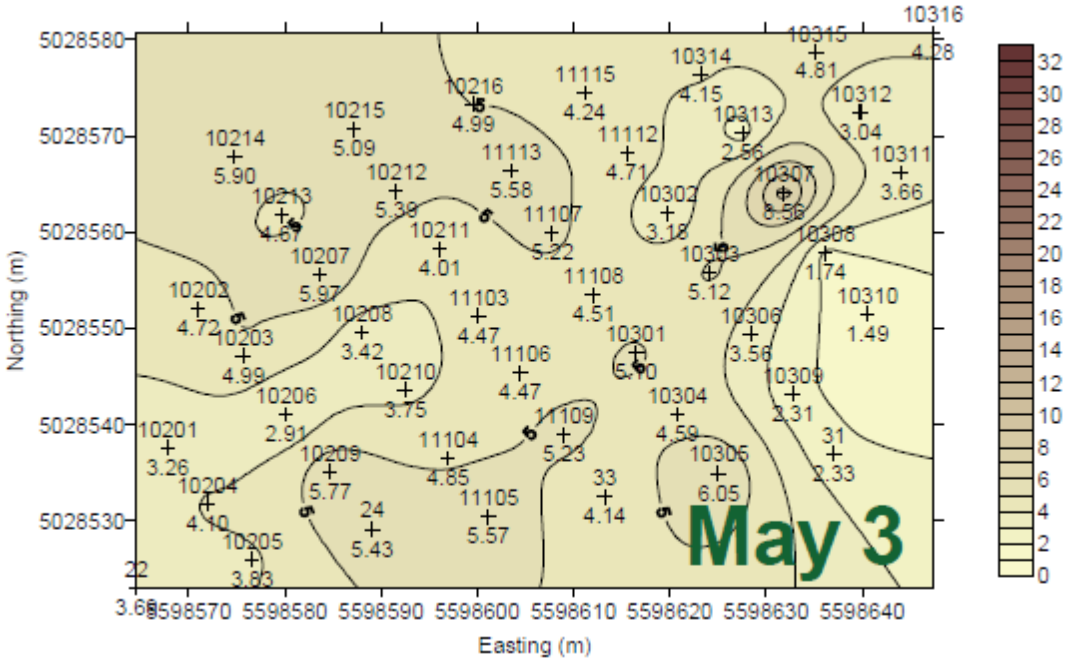

Figure 4. Soil NO_3–N concentration (mg kg^{-1}) contour map of a wheat field produced by on-the-go sampling. Numbers above the crosses are sample location codes. Numbers below the posts are NO_3–N concentration (mg kg^{-1}). Adapted from (Sibley et al., 2009).

A similar methodology for on-the-go soil pH testing using ISEs has been developed (Adamchuk et al., 1999) and subsequently commercialised. Modelling work has suggested that using this approach, combined with variable rate application of lime may result in significant economic benefits compared to conventional soil sampling and a fixed rate of lime application (Adamchuk et al., 2004). Whether this approach could bring about an improvement in NUE has yet to be investigated.

Whilst on-the-go sampling represents an improvement from on-farm rapid-tests, each sampling event entails an economic cost and crucial changes in soil N status maybe occur between sampling events. Essentially, there is a tradeoff to be made between the temporal resolution of sampling events and cost; the optimum balance between the two will depend upon the economic benefit accrued by each additional sampling event.

The extent of farmer adoption of both on-the-go testing and rapid-tests is not clear, and in general there is a lack of information in the literature detailing how and when farmers and extension services perform soil sampling/analysis. It is likely, especially given that on-the-go platforms for N testing have not been commercialised, that use of these methods within commercial farming is low.

5.3. Towards in-situ, real-time soil N determination

The development of sensors capable of in-situ determination of soil N in real time may represent an ideal solution for PA. Sensor networks deployed in-field during the growing season may enable continual monitoring of soil N, allowing a dynamic approach to fertiliser management that responds quickly to changing agronomic conditions. In-situ, real-time approaches have the advantage that following sensor deployment, no further effort is required for data assimilation. Sensor networks could also be wireless enabled to facilitate remote access to the data stream and for developing "smart agriculture", where data would feed directly into models to generate fertiliser recommendations. However, at present there has been little development of suitable sensing technologies. As such, the challenges for sensor development and sensors which have the potential for in-situ, real-time soil N determination are discussed below.

Challenges for development of in-situ sensors

For sensors to undertake in-situ, real-time monitoring, they must be able to perform direct soil measurement (DSM), which presents a number of challenges that need to be overcome:

- Sensors must be able to operate under a wide range of environmental conditions and soil types. Soil temperature and moisture contents will vary widely throughout the growing season, which will make the production of agronomic relevant results particularly challenging.
- The sensor needs to be physically robust and durable to cope with a period of extended burial within an agricultural environment. They also need to resist both microbial and chemical contamination of the sensing element.
- Its calibration parameters must remain constant for an extended period of time so that reliable measurements can be collected without the need for sensor recalibration.
- The sensor must be coupled to a data logging system capable of storing large quantities of data. Ideally, these data need to be accessible remotely through a wireless system.
- The data logging system and possibly the sensor will require power in some form.

N sensors which have been tested for DSM, or have the potential to be used in this way are discussed below.

Ion-selective electrodes (ISEs)

ISEs in general are cost effective, relatively accurate and offer a short response time (<60 s), all within a small portable package (Sinfield et al., 2010) and are therefore potentially very

useful for in-situ monitoring in the field. However, using ISEs for analysis in complex environments, such as lakes, seawater and soils, does present a challenge due to a range of potential problems including electrode fouling, drift, instability, dissolution and cross contamination (De Marco et al., 2007).

ISEs work by measuring the potential difference between a reference electrode and an electrode that contains a membrane which selects for the ion of interest, as shown in figure 5.

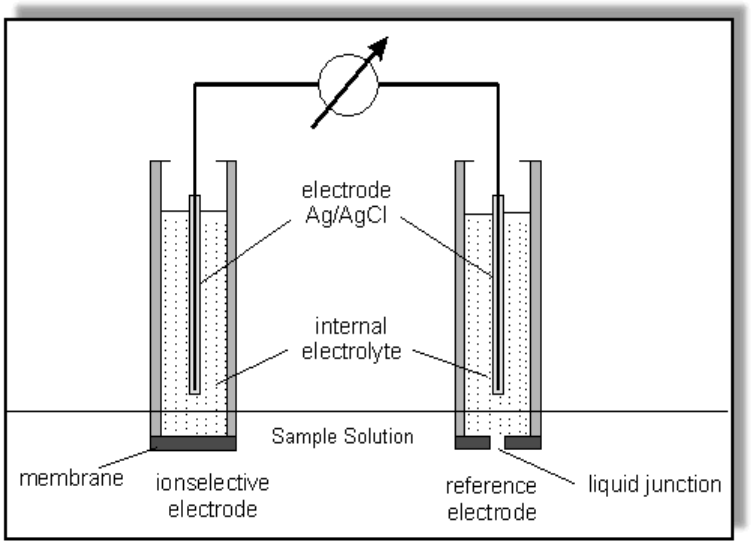

Figure 5. Schematic of an ISE system showing ISE and reference electrode linked by a mV meter.

The potential difference is linearly related to the logarithm of the activity of the ion selected for and is described by the Nernst Equation (Eqn. 1). This states that at 25 °C a 59.1 mV change in electrode potential should result from a 10-fold change in activity of the selected monovalent ion.

$$E = K + (2.303RT/z_iF)\log a_i \quad \text{(Eqn. 1)}$$

Where E is the potential, z_i and a_i are the charge and activity of the ion of interest, K is a constant which includes all sample-independent potential contributions (influenced by the design of the ISE), *R* is the gas constant, T is temperature in Kelvin and F is the Faraday constant.

ISEs respond to the activity of the specified ion, which differs from the absolute concentration. The activity can be thought of as the biologically available concentration, i.e. the concentration sensed by plant and microbes. The use of ISEs for determination of NO_3^- activity or concentration in the soil extracts has been well researched and documented over the past 40 years (Keeney et al., 1970; Davies et al., 1972; Hansen et al., 1977; Adsett and Zoerb,

1991; Adamchuk et al., 2005) and have been used for on-farm rapid tests and on-the-go sampling, as described previously. NO_3^- ISEs are commercially available with response times <10 s and limits of detection (LOD) in the range 1.0 to 7.0×10^{-7} M (NICO 2000, 2012; Vernier, 2012; Omega, Undated). Most ISEs for NO_3^- determination feature a PVC polymer matrix selective membrane which holds a water immiscible sensing chemical cocktail (Sinfield et al., 2010).

Using ISEs for soil NO_3^- determination normally requires the extraction of NO_3^- ions into a solution or sampling of the soil water. However, DSM has been shown to be possible, albeit with varying success. NO_3^- ISEs were used during experiments on winter wheat at Rothamsted during the 1970's to determine NO_3^- concentration in soils (Nair and Talibudeen, 1973; Page and Talibudeen, 1977; Page et al., 1978). The method used involved adding 1 ml of distilled water to an in-situ hole (maintained by polythene tubes) in the soil. This was left for 10 minutes to allow it to equilibrate with the soil and then the ISE was used to directly measure the soil NO_3^-. At times when the soil was dry and cracked, a soil sample was removed then mixed with water to form a paste, which was then measured with the ISE. It is hard to determine the accuracy of this method as no r^2 values were presented in comparison with standard laboratory methods although they found that using "a '1:0.5', soil:water, ratio, recovery of added N by the electrode and chemical measurements was 87.0% and 95.5% respectively" (Nair and Talibudeen, 1973). Although the method used above is not strictly DSM, it did show the potential for ISEs to be used in this way. Adamchuck et al. (2005) examined the possibility of using NO_3^- ISEs for DSM under laboratory conditions. The ISEs were coupled with an automated measurement system that had been previously used for on-the-go soil pH testing (Adamchuk et al., 1999). The procedure was performed on 15 soils with varying textures, organic content and gravimetric water contents. Samples (n = 6) of each soil were sent to a range of commercial laboratories for conventional soil NO_3^- determination. The NO_3^- results obtained with the ISEs compared poorly with those obtained by commercial laboratories (r^2 = 0.24 – 0.35). Ito et al. (1996) developed a novel type of NO_3^- ISE, which utilised an Urushi matrix (a type of latex) membrane, rather than a more conventional PVC membrane. They claimed that this improved the hardness, mechanical strength, lifespan and response time of the membrane whilst retaining similar selectivity and detection range. All of these are obviously useful attributes for an ISE which will be used in field. These ISEs and conventional ISEs with a PVC membrane were tested on an agricultural andosol, with a gravimetric water content of 80%. Soil – electrode contact was maintained for 1-3 minutes

until the output stabilised. They found that the Urushi membrane, in comparison to manual laboratory determination (Bremner method) had r^2 values of 0.994.

As well as being able to perform DSM, any in-situ sensor must be able to perform accurate measurements over an extended period of time. Whilst this has yet to be demonstrated for soils, monitoring of NO_3^- concentration of water bodies has been successfully achieved. ISEs, with a membrane containing *N*,*N*,*N*-triallyl leucine betaine as the sensor covalently attached to polystyrene-*block*-polybutadiene-*block*-polystyrene matrix, were tested for continuous in-situ monitoring of NO_3^- in an agricultural drainage ditch and a small moorland stream. The NO_3^- results obtained with the ISEs at hourly intervals compared very favourably with those obtained with laboratory automated chemical determinations made on contemporaneous samples ($r^2 \geq 0.9$) (Le Goff et al., 2002; Le Goff et al., 2003). Most importantly, the ISEs required no re-calibration and no fouling of the membrane or deterioration of performance was observed during the monitoring period (4 months (2002) and 40 days (2003)). These electrodes where subsequently found to be pH sensitive and therefore unsuited for use in soils (Miller pers. comm.).

Problems with using ISEs for long term, in-situ DSM, are likely to include calibration drift and change in the Nernst slope (Adamchuk et al., 2005; De Marco et al., 2007), sensitivity to temperature (as specified in the Nernst Equation (Eqn.1.)), soil pH and moisture content, durability (Ito et al., 1996), and contamination of the selective and reference membranes (Hansen et al., 1977; Adamchuk et al., 2005; De Marco et al., 2007). These can be fairly easily controlled in the laboratory when making short term measurements where the ISE can be washed and recalibrated between measurements and soil types. To progress with the use of ISEs for real-time in-situ monitoring of soil NO_3^-, advances must be made in ISE technology so that long-term measurements in the field can be made without the need for regular and frequent recalibrations and changing of damaged/contaminated membranes.

Ion-selective field effect transistors

Ion-selective field effect transistors (ISFETs) are similar to ISEs in that they are electrochemical sensors and the output response is linearly related to the logarithm of the activity of the selected ion. They are also designed for use in measuring ions in solution and so will suffer the same problems as ISEs when faced with in-situ measurements in dry soil. The main difference between ISEs and ISFETs is that ISFETs have no internal solution and the selective electrode consists of two semi-conducting electrodes (source and drain) linked by a gate on to which an ion-sensitive membrane is directly affixed, as shown in figure 6.

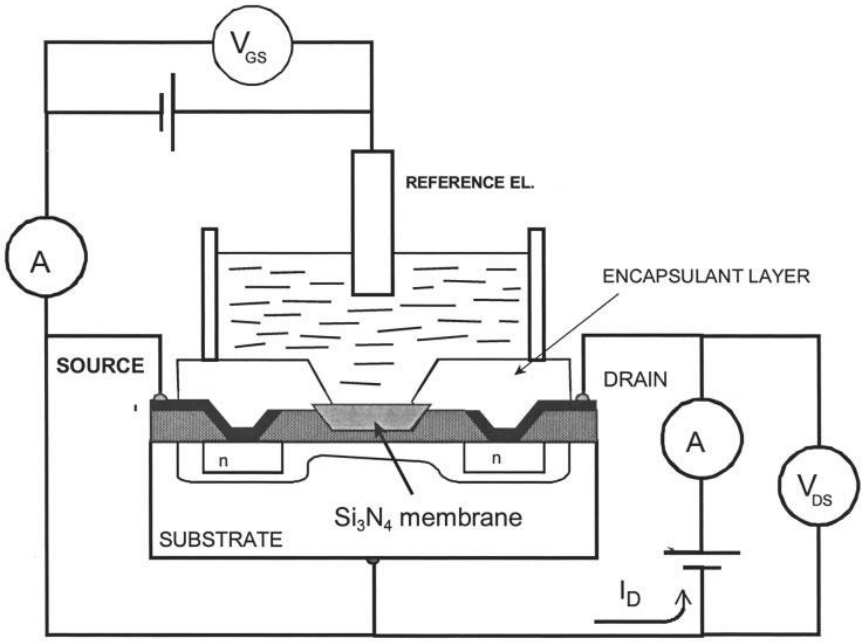

Figure. 6. Schematic of a NO_3^- Ion-selective field effect transistor (ISFET) (Artigas et al., 2001).

ISFETs have many properties that make them suitable for use as real-time sensors: they have a fast response time, low output impedance and signal to noise ratio, can be mass produced and have small dimensions (Birrell and Hummel, 2001; Kim et al., 2006). ISFETs, like ISEs but to a greater extent, typically suffer from long-term drift (Bergveld, 1991; Birrell and Hummel, 2001), hysteresis and durability issues (Sinfield et al., 2010). These issues have shown to be partially solved by using flow-injection technology (Birrell and Hummel, 2001; Price et al., 2003), which could be combined with an automatic soil sampling and extraction system (Price et al., 2003). Artigas et al. (2001) investigated the use of ISFETs for DSM and showed that they responded to the addition of a NO_3^- solution which was subsequently reversed by addition of H_2O. However, the NO_3^- ISFETs exhibited drift of 2-4 mV day^{-1}, so recalibration was performed each day and a significant loss of sensitivity occurred after a month of use. So ISFETs, like ISEs, require an advance in the technology to increase their durability and to minimise drift in electrode potential before they can be deployed in the field.

Other electrochemical sensors

There are a variety of amperometric/voltammetric sensors, including some biosensors (see section below), which can be used for the determination of NO_3^- in solution. These sensors work by enabling the reduction of NO_3^- at the electrode surface. This creates a measurable electrochemical response, which is related to the concentration of nitrate in the solution (Paixao et al., 2007; Atmeh and Alcock-Earley, 2011). There has been little research carried out using

this type of sensor for the analysis of soils but the potential for them to be used in this way has been demonstrated. A modified copper electrode, with a limit of detection for NO_3^- of 11 µM, has been used to analyse nitrate concentration in mineral water and sausage extracts with results comparable at the 95% confidence level to those obtained using the Griess reaction (Paixao et al., 2007).

The world of nano technology and materials is growing quickly and has led to the development of a variety of nano electrochemical sensors. It is beyond the scope of this review to detail the intricacies of these technologies, but it is likely that large advances in the sensing of environmentally important analytes will be achieved over the next few years. Atmeh and Alcock-Earley (2011) created a novel electrochemical sensor fabricated by electrodeposition of silver nanoparticles on pre-synthesized polypyrrole (PPy), to form a PPy/Ag composite matrix on a glassy carbon electrode. The electrochemical sensor exhibited strong catalytic activity with regards to NO_3^- reduction. The detection limit of NO_3^- was found to be 5 µM. Moreover, the sensor showed excellent sensitivity, selectivity, and stability. Of particular interest are nano wire electrodes. The cited advantage of these for analytic purpose include shorter response times, increased sensitivity and lower sample volumes (Arrigan, 2004). The application of discreet nano wire electrodes has been previously demonstrated, achieving low limits of detections across broad dynamic ranges for key target analytes (Dawson et al., 2011; Wahl et al., 2011; Dawson et al., 2012). Nano wire electrodes have yet to be demonstrated for the determination of NO_3^- and NH_4^+ in soil but the technology is developing rapidly and it is likely that we will see new sensors which can perform such analyses in the near future. One issue with nano sensors may be the small spatial scale at which they operate which may necessitate the deployment of large sensor arrays.

Biosensors

Biosensors allow measurement of NO_3^- in a range of diverse environments including soil slurry, food, waste water and sea water (Sinfield et al., 2010). The original concept behind NO_3^- biosensors involves the diffusion of NO_3^- across a membrane where it is reduced by immobilised bacteria, containing the nitrate reductase enzyme (NaR), to either N_2O or NH_3 which is then subsequently analysed (Larsen et al., 1997). More recently, NO_3^- biosensors have immobilised the NaR enzyme within a membrane, contained in an electrode, which has allowed direct amperometric analysis (Quan et al., 2005; Adeloju and Sohail, 2011). These sensors have been shown to have detection limits as low as 1 µM with a linear range of 0.04 to 2 mM (Adeloju and Sohail, 2011). As well as offering low detection limits these sensors can be

accurate and precise but suffer from very short lifetimes and a lack of robustness (Sinfield et al., 2010).

Lab-on-a-chip technology

Recent advances in microfluidic technologies have allowed the development of the miniaturization of chemical analytical techniques, which are normally undertaken in the laboratory. Such 'lab-on-a-chip' systems may have the potential to be used for in-situ environmental monitoring. Beaton et al. (2012) created a first generation 'lab-on-chip' sensor for the in-situ determination of NO_3^- and NO_2^- in natural waters based on the Griess reaction and subsequent colorimetric determination. The system detects NO_3^- in the range of 1.1×10^{-4} – 1.6 mM and is capable of performing 6 measurements per hour. Whilst this sensing system shows clear potential for NO_3^- monitoring in waters, using it for in-situ soil analysis would require coupling with an automated soil water sampling or soil extraction system. Such 'lab-on-a-chip' systems may also be useful for on-the-go soil NO_3^- sensing systems such as that used by Sibley et al. (2009). This technology could be also be modified for the determination of NH_4^+ using the colourimetric nitroprusside method (Mulvaney, 1996).

Soil solution sampling

As discussed above, one of the key limitations of most N sensor technology is that they are designed to measure the concentration of ions in solution and so may struggle to perform DSM, especially in dry or crack-prone soils. Automated, in-situ soil solution sampling, i.e. collecting only the soil water and not the soil solid phase, combined with on-line analysis may offer an alternative to DSM. There are many methods of soil solution sampling, the majority of which have been reviewed by Weihermueller (2007). These methods involve the sampling of the soil water at a specific point in time e.g. suction cups, or the gravimetric collection of soil water over a specified time period e.g. pan lysimeters, rather than the continuous sampling of soil solution. Furthermore, these methods require destructive sampling, leading to a perturbation of the system being evaluated and the temporal resolution of repeated sampling being dictated by the recovery of the local soil solution to pre-sampling levels. It is also likely that collection of soil water will prove problematic when soil moisture contents are low.

Attempts have been made to create systems capable of continuous monitoring based on the passive diffusion of soil solution solutes across porous membranes. Tuli et al. (2009) investigated the use of in-situ porous stainless steel cups, combined with NO_3^- ISEs and ultra violet (UV) spectrometry, and claimed proof of concept for in-situ monitoring of soil solution. The system relied on passive diffusion of solutes between the soil solution and the solution

inside the cup and so required no active suction. However, testing the system in a saturated sand matrix showed equilibration of internal and external solutions took 5 days. Modeling work has shown that the rate of diffusion into porous cups is a function of molecular diffusion (which has a particular relationship with soil moisture content for different soil mediums) and the radius of the porous cup (Riga and Charpentier, 1998). Consequently, in unsaturated soils, ionic equilibrium is likely to take much longer than 5 days and modeling work suggests that it could take in excess of a month, rendering it unsuitable for use in soil (Riga and Charpentier, 1998).

Microdialysis based sampling has been used extensively in neuroscience and pharmacokinetic research (Nesbitt et al., 2013) but more recently it has been applied to environmental studies for determining the bioavailability of trace metals (Miro and Hansen, 2006) and as a novel way of assessing soil N status (Inselsbacher et al., 2011; Inselsbacher and Näsholm, 2012). Figure 7 shows a diagram of a commonly used microdialysis probe. Microdialysis is a non-destructive sampling method based on the passive diffusion of solutes from within the soil solution, across a partially permeable membrane, into a flow of water – the perfusate. The resulting dialysate is collected over a specified sampling period and subsequently analysed. The rate of diffusion of solutes across the membrane is driven by the concentration gradient between the soil solution surrounding the membrane and the perfusate which is continuously being pumped through the probe. This creates a zone of depletion surrounding the probe, which will induce the diffusion of solutes through the soil down the resulting concentration gradient. As such, the concentration of solutes in the dialysate reflects not just the concentration in the soil solution, but also the rate at which these solutes can diffuse through the soil. In addition to this, each N pool is in flux, so differences in additive and consumptive processes may affect the concentration of each N-form over time.

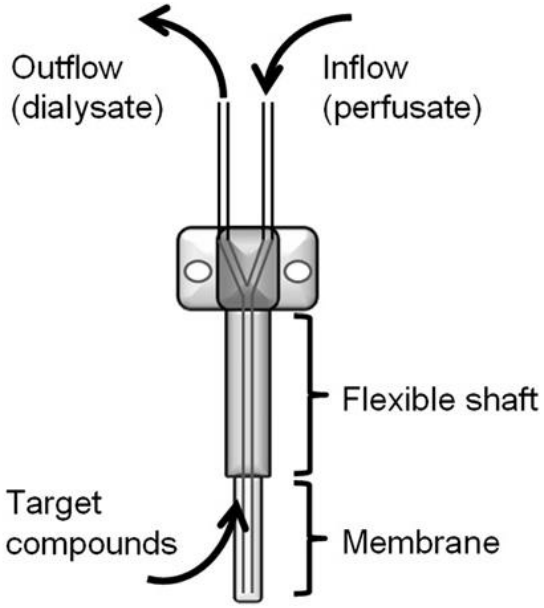

Figure 7. Diagram of a CMA 20 microdialysis probe. Image taken from Inselsbacher et al. (2011)

Because of these factors, it has been argued that microdialysis offers a method to assess soil N that better informs on the availability of N and the importance of different N-forms for plant nutrition (Inselsbacher and Näsholm, 2012). In addition, microdialysis probes, due to their small size and short sampling period, offer excellent spatial and temporal resolution.

Estimation of soil solution concentrations can be achieved by calculating the percentage recovery of the target solute from a standard solution at a specified perfusate flow rate (Inselsbacher et al., 2011). However, obvious differences between soil and the standard solution used may lead to errors when estimating intrinsic soil solution concentrations. An alternative method was used by Inselsbacher and Näsholm (2012), which involved measuring the absolute amounts of solutes in the dialysate to calculate the diffusive flux of solutes over the microdialysis membrane per unit area, per unit time.

Results from microdialysis sampling of boreal forest and agricultural soil, both in-situ and ex-situ following sieving and mixing, have suggested that the contribution of organic N, in the form of amino acids (AAs), to the total plant available N, in contrary to water and salt extractions, was higher than that of NH_4^+ and NO_3^- (Inselsbacher et al., 2011). This has led to the conclusion that for these soils, the pool size estimates determined by soil extractions are not representative of plant available N and that the contribution of organic N to plant nutrition has been underestimated.

In its current form, it is unlikely that microdialysis probes will prove sufficiently robust for long term, in-situ monitoring, although the principle of the technology could be improved

for agronomic purposes. Their small size and excellent spatial resolution are advantageous for laboratory research, and their unique method of diffusive based sampling may offer new insights in to soil N processing. The requirement for subsequent chemical analysis of the dialysate further complicates the use of microdialysis for in-situ monitoring. Although, coupling the probes with an on-line analyser, such as emerging lab-on-a-chip technology (Beaton et al., 2012) or ISEs, may provide a solution.

6. Spatial variance considerations for in-situ sensing

Optimising the spatial configuration of a sensor network is needed to ensure that a precise estimate of the mean N concentration across a field or management zone can be made whilst minimising economic costs. Many soil properties have been shown to exhibit spatial dependencies over a range of scales (Webster and Boag, 1992; Atteia et al., 1994; Fisher et al., 1998; Baxter et al., 2003; Corstanje et al., 2008). With specific reference to soil N, studies of the spatial variation at within-field scales in grazed pasture have shown considerable variation over small scales (< 5 m), which was mainly attributed to the uneven deposition of animal excreta (Bogaert et al., 2000; Van Meirvenne et al., 2003). This contrasts with results from arable fields where more variation was shown to occur at larger scales (Baxter et al., 2003; Haberle et al., 2004), which is likely due to the even input of fertiliser N and regular ploughing. Furthermore, issues with spatial variation are likely to be accentuated due to the likely small size (i.e. < 1 cm) of the sensing component.

It is, therefore important to make an assessment of the spatial variation of N across a range of scales to determine an optimum configuration prior to implementation of the sensor network. For example a field which has little large-scale variation may be served by a collection of sensors connected to one sensing hub, whereas a field with more variation at larger scales would require sensors to be located in multiple areas of the field. When determining an optimal configuration it is also important to consider the degree of uncertainty of the resulting estimations and the associated cost-benefit of reducing this uncertainty. Consideration also needs to be made as to whether the scale and magnitude of the observed in-field N variation is large enough to justify spatial variation in the optimum fertiliser input rate and, hence, the demarcation of within-field management zones. An effective way to collect such information is by spatially nested sampling and geostatistical analysis. This approach has been used to investigate the distribution of nematodes in soil at within-field scales (Webster and Boag, 1992), to examine the variation of ammonia volatilisation from soil at within-field to landscape

scales (Corstanje et al., 2008), to quantify regional-scale variation of metal concentrations in soil (Atteia et al., 1994) and to examine the interactions of soil and herbicide at within-field scale (Price et al., 2009).

7. Conclusions

Increasing NUE is a key objective of modern agriculture, in order to prevent the need for agricultural expansion into natural ecosystems and increasing levels of N pollution. In arable and grassland systems, improvements in fertiliser recommendations are needed to improve NUE. A move away from current methods, based on empirically derived relationships between N requirements over a growing season and predicted crop yields, to a more dynamic system that is better able to monitor and predict crop N requirements and soil N availability in real time, would have numerous benefits, viz. minimising surpluses of N in the soil and crop thus improving NUE and reducing losses to environment.

Sensing technology with the capability to assess crop N status is now well developed and its use is becoming more widespread. Improvements in soil N testing have so far been limited to on-farm rapid tests and on-the-go approaches. Whilst these represent an improvement on conventional sampling and laboratory analysis, farmer uptake of these methods is thought to be low and it is unlikely that they could provide data of a high enough spatial and temporal resolution to support PA. These issues may be resolved by the use of in-situ sensors which are capable of real-time monitoring of soil N, however, the development of such sensors has so far been limited.

Soil presents an extremely challenging environment for sensors to operate in, especially as most N sensors are designed to perform measurements in aqueous solutions. Of current sensing technologies, electrochemical methods, specifically ISEs, have the greatest potential to be used for in-situ monitoring as their use for DSM of NO_3^- has been demonstrated. However, further improvements in their accuracy, durability and stability of long-term in-situ soil measurements need to be made. Further innovation is required to overcome these challenges and there is a clear need for the development of viable NH_4^+ sensors. Sensing of plant available N in the immediate future is likely to be limited to NO_3^-. This may be sufficient in arable soils, which are typically dominated by NO_3^-, but may be limiting, for example, in acidic grassland soils, which have a higher NH_4^+ content. The emerging field of nano-technology may generate novel sensors, which are better able to deal with the challenges of soil sensing, although currently there has been little real development in this field.

Microdialysis sampling may offer an alternative to in-situ sensing, although the need for subsequent analysis of samples reduces its usefulness for real-time monitoring. This could be resolved by coupling microdialysis probes with an on-line system for sample analysis. Microdialysis has several advantages over current sensing technologies in that samples may be analysed for a range of N forms and other nutrients such as phosphate. Furthermore, it is likely that its unique diffusive-based sampling may better inform on plant N availability and offer new insights into soil N dynamics.

Given the likely small size of the sensing component of any given sensor and variation in soil properties over a range of spatial scales it is important that consideration is given to the optimum configuration of a sensor network. This may be achieved by using a nested sampling and geostatistical approach.

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Chapter 3

Article I

Assessing soil nitrogen availability using microdialysis-derived diffusive flux measurements

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Author contributions:

RS and DLJ conceived and designed the experiment. All the experimental work, data processing and statistical analysis was performed by RS. RS wrote the first draft of the manuscript with all authors contributing to the final version.

Nutrient Management & Soil & Plant Analysis Note

Assessing Soil Nitrogen Availability using Microdialysis-Derived Diffusive Flux Measurements

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Microdialysis-based soil sampling offers a potential alternative to traditional soil core extractions that better informs about the availability of nitrogen (N) for plant nutrition. This study compared soil N status, as estimated using 0.5 M K_2SO_4 and distilled water extractions, with microdialysis-derived diffusive flux measurements in eight grassland soils up an altitudinal gradient. Soil extracts and microdialysis samples were analyzed for plant-available N: total free amino acids, NH_4^+, and NO_3^-. In terms of the percentage contribution that amino acids, NH_4^+, and NO_3^- made to total plant-available N, the microdialysis-derived diffusive flux measurements were most similar to distilled water extractions. However, the relative magnitude of the diffusive flux measurements did not always reflect the pool sizes as estimated by the soil extractions, which suggests that the availability of N to plants, via diffusion, may be decoupled from concentration in these soils. The potential and limitations of microdialysis sampling and the implications of the results for soil N management are discussed.

Abbreviations: AAs, amino acids; DON, dissolved organic nitrogen; masl, meters above sea level; MW, molecular weight.

The ability to accurately quantify the nitrogen (N) status of a soil and determine its relevance for plant N nutrition is highly desirable, especially for agri-environments, where application of N fertilizers in excess of crop demand may lead to significant economic and environmental costs (Sutton et al., 2011). Traditionally, soil N status has been determined by using either concentrated salt (e.g., 2 M KCl or 0.5 M K_2SO_4) or distilled water batch extractions, which enable estimates of inorganic and organic N pool sizes at a specific time point to be made (Mulvaney, 1996; Jones and Willett, 2006). However, they do not inform on rates of pool turnover or the movement of solutes through the soil, both of which are important parameters when assessing soil N supply and potential plant uptake (Leadley et al., 1997; Darrah and Roose, 2001; Jones et al., 2005).

Microdialysis-based sampling of biological fluids in situ has been used extensively in neuroscience and pharmacokinetic research (Nesbitt et al., 2013). More recently, it has been applied to environmental studies for determining the bioavailability of trace metals (Miro and Hansen, 2006) and as a novel way of assessing soil N status, both in situ and ex situ (Inselsbacher et al., 2011; Inselsbacher and Näsholm, 2012a). Microdialysis is a nondestructive sampling method, based on the passive diffusion of solutes from within the soil solution, across a partially permeable membrane, into a flow of water—the perfusate. The resulting dialysate is collected over a specified sampling period and subsequently analyzed. The rate of diffusion of solutes across the membrane is driven by the concentration gradient, between the soil solution surrounding the membrane and the perfusate, which is

Soil Sci. Soc. Am. J. 78:1797–1803
doi:10.2136/sssaj2014.04.0128n
Received 4 Apr. 2014.
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continuously pumped through the probe. This creates a zone of depletion surrounding the probe, which induces the diffusive flux of solutes through the soil, down the resulting concentration gradient. As such, the concentration of solutes in the dialysate reflects not just the concentration in the soil solution, but also the rate at which these solutes can diffuse through the soil. In addition to this, each N pool is in flux, so differences in additive and consumptive processes may affect the concentration of each N-form over time. Because of these factors, it has been argued that microdialysis offers a superior method to assess soil N status that better informs us of the availability of N and the importance of different N-forms for plant nutrition (Inselsbacher and Näsholm, 2012a). However, using microdialysis to determine absolute concentrations of solutes in the soil solution is confounded by calibration complexities. Equilibrium between the solute concentrations of the dialysate and soil solution is never achieved and the relative recovery of solutes is dependent on a number of factors, including the flow rate of the perfusate, the molecular weight (MW) cut off for the membrane, the MW of the solute, temperature, solute species, and properties of the surrounding matrix (Menacherry et al., 1992). These problems are likely to be increased in heterogeneous soils where intrinsic properties such as moisture content, temperature, particle size, and composition may vary widely over short spatial scales. However, as the soil-related variables also affect the supply of N to plants, it has been suggested that microdialysis sampling better reflects the natural plant-soil systems they aim to evaluate. Consequently, simply measuring the rate of solute diffusion across the probe membrane over time may give a useful method for assessing soil N availability. This measurement has been termed the diffusive flux and is expressed in terms of the amount of solute diffusing across the membrane per unit area, per unit time (Inselsbacher and Näsholm, 2012b).

In this paper we directly compare estimates of soil N concentrations (total free amino acids [AAs], NH_4^+ and NO_3^-), as determined by traditional salt (0.5 M K_2SO_4) and distilled water extractions, with ex situ microdialysis diffusive flux measurements. Eight contrasting agricultural soils were used, to test the microdialysis technology in a range of soils with differing chemical, physical, and biological properties and management practices. The aim was to ascertain the usefulness of the microdialysis technique for assessing soil N availability and to evaluate the importance of different N-forms for plant nutrition. The potential for using microdialysis diffusive flux measurements as a research method or as tool for soil N management is also discussed.

MATERIALS AND METHODS

Soil Sampling

Soils were collected from eight contrasting agricultural grazed grasslands, along an altitudinal gradient, within an 8 km^2 region close to Abergwyngregyn, United Kingdom (53°14′ N, 4°01′ W). All the soils have developed since the last glacial period (10,000 yr before present) and are characterized by having a temperate-oceanic climate regime. Net primary productivity decreases with altitude; mean annual temperature ranges from 9.8°C at 5 m above sea level (masl) to 6.5°C at 700 m, with annual rainfall ranging from 800 mm at 15 masl to 2300 mm at 700 masl (Farrell et al., 2011). All the sites are permanent pastures, except Site 2, which had been plowed and reseeded 3 mo before sampling. Sites 1 to 3 are intensively grazed by sheep at a stocking rate of 8 to 10 ewe ha^{-1} and receive an inorganic nitrogen (NH_4NO_3) fertilizer input of 110 kg N ha^{-1} yr^{-1}. Sites 4 to 8 are extensively grazed by sheep at a stocking rate of <0.1 ewe ha^{-1} and receive no fertilizer inputs. Four replicate soil samples (n = 4) were collected from sites 1 to 3 and three (n = 3) from sites 4 to 8. Before sampling, the vegetation and litter layer were removed from an area 15 by 15 cm, and the top 15 cm of the soil collected for experimentation. The soil was stored in gas permeable polyethylene bags for immediate transport to the laboratory, where it was refrigerated at <5°C until needed. Immediately before use, the soil was sieved to 8 mm to remove large stones, roots, vegetation and earthworms, and thoroughly mixed. Soil was not sieved to 2 mm as this has been shown to stimulate microbial activity resulting in significant changes to both the organic and inorganic N pools in these soils (Jones and Willett, 2006). Site characteristics and soil properties are displayed in Table 1.

Background Soil Analysis

Soil pH and electrical conductivity were determined in a 1:2.5 (w/v) soil/distilled water suspension using standard electrodes. Moisture content was determined by drying for 24 h at 105°C. Total C and N were determined with a TruSpec CN analyzer (Leco Corp., St Joseph, MI). Exchangeable cations were extracted using 0.5 M ammonium acetate (Sparks, 1996), and the filtered extracts were analyzed by using flame emission spectroscopy (Sherwood 410 flame photometer, Sherwood Scientific, Cambridge, UK). Total dissolved phenolics, in the H_2O extracts, were assayed colorimetrically using the Folin–Ciocalteu reagent (F9252, Sigma-Aldrich, Gillingham, UK) according to Velioglu et al. (1998). Extractable phosphorus (P) was determined by extraction with 0.5 M acetic acid with subsequent colorimetric analysis by using the molybdate blue method of Murphy and Riley (1962). Microbial uptake of glucose-C was used as a proxy for soil microbial activity and was determined by using a method based on Jones et al. (2012). Briefly, 100 μL of uniformly labeled ^{14}C-glucose was added to 5 g of field-moist soil (8-mm sieved) and incubated at 20°C for 30 min. Evolved $^{14}CO_2$ was captured by using vials containing 1 mL of 1 M NaOH, to which 4 mL of scintillation fluid (Optiphase HiSafe 3 scintillation fluid [Wallac EG&G, Milton Keynes, UK]) was subsequently added. Following the incubation period, the soil was extracted with 25 mL of 0.5 M K_2SO_4. To prevent additional microbial activity, the K_2SO_4 was precooled to 1°C and soils were shaken for 10 min on ice. The mean temperature of the soil-extractant mix following shaking was 2.9°C ± 0.24 (mean ± SEM, n = 4). A 1.5-mL aliquot of the extract was removed and centrifuged (21,000 g, 5 min, 5°C) and 1 mL of the supernatant was added to 4 mL of scintillation fluid. The amount of ^{14}C-glucose in the soil extracts

and NaOH traps was determined by liquid scintillation counting by using a Wallac 1404 scintillation counter. Potentially mineralizable N was determined by using an anaerobic incubation method based on Keeney (1982). Briefly, 5 g field-moist soil (8-mm sieved) was placed in a 50-mL centrifuge tube, which was then filled to the top with deionized H_2O and the tubes sealed. Soils were subsequently incubated in the dark at 40°C for 7 d. The difference in NH_4^+ content between t = 0 and t = 7 d was attributed to N mineralization. Results are shown in Table 1.

Microdialysis Collection of Soil Solution

A syringe pump, holding two 20-mL polypropylene syringes (Terumo-Europe NV, Leuven, Belgium), filled with high-purity deionized water (the perfusate), were connected to two CMA 20 microdialysis probes (CMA Microdialysis AB, Kista, Sweeden), with a 100-kDa molecular weight cut off, polyethersulfone membrane (4 mm long, 500 μm external diameter). For each replicate sample, between 50 and 70 g of presieved (8 mm) soil was placed in a 100-cm^3 plastic beaker and packed to the bulk densities shown in Table 1, which were representative of field conditions. A microdialysis probe was inserted into the soil by using the needle and introducer supplied by the manufacturers. The flow rate of the pump was set to 5 μL min^{-1}. This was chosen as it has been shown to give a good compromise between volume of sample collected and the relative recovery of soil solutes (Inselsbacher et al., 2011). Dialysate was collected over a time period of 1 to 2 h in 1.5-mL microfuge tubes and stored at -18°C. Before sample collection, the probes were run for 20 min to ensure that the dead volume in the tubes had been flushed. The diffusive fluxes of AAs, NH_4^+, and NO_3^- were determined by calculating the amount of each N-form that diffused across the microdialysis probe membrane during each sampling period and are expressed in units of nmol cm^{-2} h^{-1}. The flow rate was monitored throughout the experiment to check that the performance of the pump and probe remained consistent throughout the sampling period.

Traditional Soil Nitrogen Extraction

All sieved soil samples were extracted following standard procedures (Jones and Willett, 2006). Briefly, field-moist 8-mm sieved soil (5 g) was extracted (175 rev min^{-1}, 1 h) using 0.5 M K_2SO_4 to determine exchangeable and free N, or distilled water, to determine free N, at a soil: extractant ratio of 1:5 (w/v), the extracts centrifuged (4000 *g*, 15 min), and the resulting supernatant collected and frozen (-18°C) to await chemical analysis.

Chemical Analysis of Soil Extractions and Microdialysis Samples

Amino acids were determined by the *o*-phthadialdehyde spectrofluorometric method of Jones et al. (2002). NH_4^+ was determined by the nitroprusside colorimetric method of Mulvaney (1996) and NO_3^- by the colorimetric Griess reaction of Miranda et al. (2001).

Table 1. Soil classification and properties of the eight soils used in this study.†

	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Site 7	Site 8
Soil	Typic endoaquepts	Dystric eutrudepts	Dystric eutrudepts	Typic endoaquepts	Dystric eutrudepts	Typic eutrocryepts	Typic eutrocryepts	Typic haplorthods
Altitude (masl)	5	10	15	35	45	110	200	370
Dominant vegetation	*L. perenne* L./*Trifolium repens* L.	*L. perenne* L.	*L. perenne* L./*Trifolium repens* L.	*L. perenne* L./*Trifolium repens* L./*Juncus effusus*	*L. perenne* L./*Trifolium repens* L.	*Agrostis canina* L./*Agrostis capillaris* L.	*F. ovina* L.	*F. ovina*/*T. cespitosum* (L.) Hartman
Texture	Silty loam	Sandy clay loam	Sandy clay loam	Clay loam	Sandy clay loam	Sandy loam	Sandy loam	Organic/sandy loam
Experimental bulk density	1.15 ± 0.01	1.04 ± 0.03	1.01 ± 0.01	0.49 ± 0.02	0.89 ± 0.04	0.90 ± 0.02	0.73 ± 0.04	0.45 ± 0.01
pH	6.81 ± 0.04	5.12 ± 0.11	6.43 ± 0.05	5.56 ± 0.04	5.94 ± 0.16	5.14 ± 0.02	4.94 ± 0.01	4.61 ± 0.02
EC, μS cm^{-1}	160.0 ± 19.5	227.8 ± 8.2	43.8 ± 0.8	31.8 ± 1.5	17.0 ± 3.9	15.2 ± 0.8	34.5 ± 0.7	36.4 ± 0.7
Moisture conten, g g^{-1} DW	0.22 ± 0.02	0.35 ± 0.05	0.42 ± 0.02	1.66 ± 0.06	0.39 ± 0.04	0.35 ± 0.04	0.51 ± 0.06	1.21 ± 0.01
Total C, g C kg^{-1}	23.5 ± 3.9	27.0 ± 1.8	29.5 ± 0.7	88.8 ± 4.2	36.7 ± 1.3	64.8 ± 11.2	66.8 ± 1.7	128.6 ± 2.7
Total N, g N kg^{-1}	2.8 ± 0.4	3.1 ± 0.1	3.4 ± 0.0	8.1 ± 0.2	4.5 ± 0.0	6.4 ± 0.7	6.3 ± 0.3	9.2 ± 0.2
Mineralizable N, mg N kg^{-1} d^{-1}	13.85 ± 4.25	15.53 ± 1.80	40.81 ± 8.28	121.89 ± 6.95	34.27 ± 2.64	35.98 ± 3.42	46.54 ± 2.91	47.36 ± 1.62
Microbial activity, μmol C kg^{-1} min^{-1}	37.64 ± 0.56	34.39 ± 0.86	46.20 ± 0.35	137.71 ± 1.03	44.96 ± 0.73	23.86 ± 9.34	58.65 ± 0.31	84.82 ± 0.52
Phenolic C, μg C kg^{-1}	5.80 ± 1.24	0.99 ± 0.28	2.95 ± 0.34	2.53 ± 0.13	0.82 ± 0.01	2.73 ± 0.64	2.99 ± 0.28	6.08 ± 1.54
Available P, mg P kg^{-1}	34.04 ± 4.68	4.67 ± 0.46	15.19 ± 0.87	5.94 ± 1.04	1.39 ± 0.78	2.52 ± 0.20	3.60 ± 0.97	6.84 ± 1.81
Exchangeable Na^+, mg kg^{-1}	273.2 ± 2.3	46.7 ± 2.6	51.7 ± 2.9	97.7 ± 2.9	52.1 ± 7.3	56.4 ± 13.7	129.2 ± 14.5	85.1 ± 5.4
Exchangeable K^+, mg kg^{-1}	172.8 ± 26.8	400.1 ± 5.9	168.4 ± 4.7	241.5 ± 65.0	214.5 ± 22.7	208.9 ± 21.5	300.3 ± 80.2	168.4 ± 4.7
Exchangeable Ca^{2+}, mg kg^{-1}	433.4 ± 11.4	952.2 ± 28.6	748.6 ± 11.4	617. ± 6.6	630.4 ± 78.8	164.2 ± 17.4	1438.1 ± 134.1	32.8 ± 13.1

† Values represent means ± standard errors (Soils 1–3, *n* = 4; Soils 4–8, *n* = 3). EC, electrical conductivity; masl, meters above sea level.

Calculations and Statistical Analysis

To determine the relative importance of each N pool for plant nutrition, the percentage contribution of each N-form to the total plant-available N (AAs, NH_4^+, and NO_3^-) was calculated for each of the three sampling methods (K_2SO_4 extraction, H_2O extraction, and microdialysis diffusive flux) for each soil type. Statistical analysis was performed by using one-way ANOVA followed by Fisher's least significant difference post hoc test using SPSS v.20 (IBM, Portsmouth, UK) with $P < 0.05$ used as the cutoff for statistical significance.

RESULTS

Table 1 shows that the eight soils along the altitudinal and productivity gradient differed greatly in their background characteristics. With the exception of Soil 4, the soils show a general increase in moisture content and total C and N, with altitude, reflecting the increasing annual rainfall and organic matter content of the soils. The characteristics of the soils used in this study correlates well with previous studies of this catena sequence (Farrell et al., 2011). The results of the glucose-C uptake assay suggests that Soil 4 has the most active microbial biomass and this is reflected in it having the largest pool of potentially mineralizable N. In general, the size of the potentially mineralizable N pool covaries with microbial activity and C and N content.

The percentage contribution of AAs, NH_4^+, and NO_3^- to total plant-available N for the soils, as estimated with the three different extraction techniques, is shown in Fig. 1. Overall, in Soils 2 to 8, the dominant N-form was the same for the three different extraction methods. For Soils 4 to 8, NH_4^+ was the dominant N-form, with percentage contributions, estimated by K_2SO_4 extractions, in the range of 51.6 to 78.7%. For Soils 2 and 3, NO_3^- was dominant, contributing, respectively, 94.3 and 76.0% of plant-available N, estimated by K_2SO_4 extractions. The results for Soil 1 shows a much more complicated picture, with the three methods giving very different results. Here, NH_4^+ and NO_3^- were the largest contributors to total plant-available N, as determined, respectively, by K_2SO_4 and H_2O extractions, whereas the contribution of each N-form, as determined by microdialysis, were not significantly different. For Soils 2 to 8, K_2SO_4 extractions gave the largest percentage contributions and absolute concentrations, shown in Fig. 2, of AAs, with an increase in AA_{K2SO4}, from 54 μmol AA kg^{-1} in Soil 3, to 526 μmol AA kg^{-1} in Soil 8. In contrast, the inverse was the case for microdialysis sampling, with the lowest AA_{DFLUX} of 0.07 nmol AAs cm^{-2} h^{-1} in Soil 8 and the highest of 0.68 nmol AAs cm^{-2} h^{-1} in Soil 1. Soil concentrations of AA_{H2O} were lower than AA_{K2SO4} and less varied across the eight soils, with the highest concentration of 116 μmol AA kg^{-1} in Soil 4. $NH_4^+{}_{K2SO4}$ soil concentrations were always higher than $NH_4^+{}_{H2O}$ soil concentrations, although the relative magnitude was similar across all the soils. The magnitude of the $NH_4^+{}_{K2SO4}$ and $NH_4^+{}_{H2O}$ soil concentrations were not always reflected in diffusive flux results. The highest $NH_4^+{}_{DFLUX}$ of 3.94 nmol NH_4^+ cm^{-2} h^{-1} was in Soil 6, which had the fifth lowest $NH_4^+{}_{K2SO4}$ and $NH_4^+{}_{H2O}$ concentration. However, there were no significant differences in $NH_4^+{}_{DFLUX}$ between Soils 2 and 4 to 8. NO_3^- was detected in all the H_2O extracts but not in the microdialysis samples for Soils 4 to 8 or in the K_2SO_4

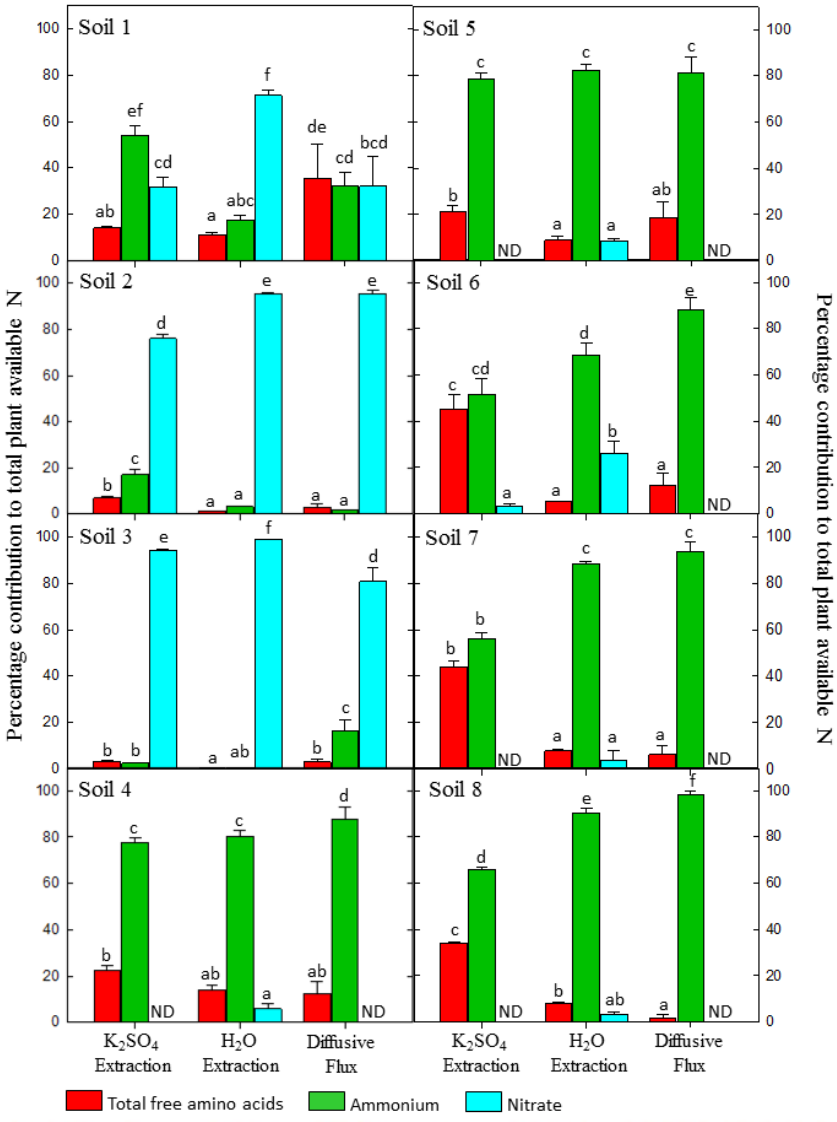

Fig. 1. Percentage contribution of total free amino acids, NH_4^+, and NO_3^- to total plant-available N in eight contrasting agricultural grassland soils, estimated by 0.5 M K_2SO_4 and H_2O soil extractions, and microdialysis-derived diffusive flux measurements. Values represent means ± standard errors (Soils 1–3, $n = 4$; Soils 4–8, $n = 3$). Letters show statistical differences between the percentage contribution made by each N-form, within each soil, for the three different methods of assessing soil N status.

extracts for Soils 4 and 5 and Soils 7 and 8. Soil 2 had significantly the largest $NO_3^-{}_{K2SO4}$ and $NO_3^-{}_{H2O}$ pool sizes but the second largest $NO_3^-{}_{DFLUX}$.

The average flow rate through the microdialysis probes over the experiment was 5.03 ± 0.2 µL min^{-1} (±SD), with the small variability attributed to slight changes in laboratory temperature.

DISCUSSION

This study compared traditional salt (0.5 M K_2SO_4) and H_2O soil extractions with microdialysis-derived diffusive flux measurements to determine the potential of microdialysis for assessing soil N status. The results clearly show that, in terms of percentage contribution of AAs, NH_4^+ and NO_3^- to plant-available N, the dominant N-form was the same, regardless of which method was used. The diffusive flux measurements were most similar to the H_2O extractions. Microdialysis will sample from the pool of N solutes that are free in soil solution and not bound to organic matter or mineral particles. This largely corresponds to the same pool that will be extracted using H_2O, so it is logical that the results from these two methods are similar. The management practices and environmental conditions are reflected in the N pools of each soil. The AA_{H2O} pool was small and varied little between the soils, with the exception of Soil 4. Previous studies on these soils has shown that the size of this pool is not due to a slow recharge time but a fast rate of microbial uptake, resulting in rapid pool turnover (Jones et al., 2004, Wilkinson et al., 2014). The AA_{K2SO4} pool showed an increasing trend up the altitudinal gradient and differences between the AA_{H2O} and AA_{K2SO4} pools are due mainly to the concurrent increase in soil organic matter. The $NH_4^+{}_{K2SO4}$ and $NH_4^+{}_{H2O}$ pools were much larger in Soils 4 to 8 but conversely had very low or undetectable pools of NO_3^-. These soils receive no NO_3^- input in the form of inorganic fertilizer, and rates of nitrification for this altitudinal gradient has been shown to decrease with increasing altitude (Jones et al., 2004). The largest pools of NO_3^- were found in Soils 1 to 3, which receive a fertilizer N input of 120 kg N ha^{-1} yr^{-1}. Of these three soils, Soil 2 has the largest NO_3^- pool, due most likely to the recent plowing and harrowing of the soil, releasing large quantities of labile organic matter, which would be rapidly mineralized and nitrified (Jones et al., 2004).

In terms of plant nutrition, the results of the soil extractions and the microdialysis diffusive flux suggests that the most important N source for Soils 2 and 3 was NO_3^- and for Soils 4 to 8 was NH_4^+. The results for Soil 1 were inconclusive. Figure 2 shows that the magnitude of the diffusive flux measurements, however, did not always reflect the pool sizes as estimated by the soil extractions. This was particularly evident when evaluating AA availability in our soils. This suggests that the availability of N to plants, via diffusion, may be decoupled from concentration in these soils. This supports evidence provided by mathematical modeling, which has shown that diffusion of solutes through the soil to the surface of the plant root not only depends on solute concentration but also the "tortuosity" of the diffusive pathway,

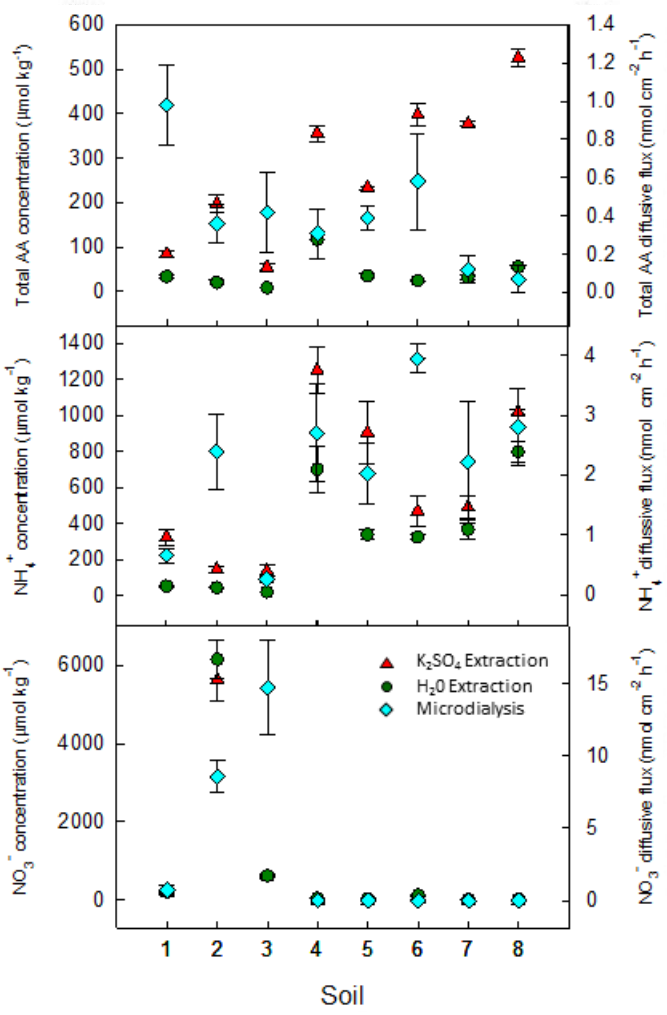

Fig. 2. Concentrations of total amino acids (AA), NH_4^+, and NO_3^- (µmol N kg^{-1}) in eight contrasting agricultural grassland soils, estimated by 0.5 M K_2SO_4 and H_2O soil extractions, and microdialysis-derived diffusive flux measurements (nmol N cm^{-2} h^{-1}), from eight temperate grassland soils. Values represent means ± standard errors (Soils 1–3, n = 4; Soils 4–8, n = 3).

the water content of the soil, and the buffering capacity of the soil (Leadley et al., 1997; Darrah and Roose, 2001). The lack of NO_3^- detection in the microdialysis samples from Soils 4 to 8 is due to their intrinsically low concentration in these soils, exacerbated by recovery dilution effects (typical relative recovery of AAs, NH_4^+, and NO_3 < 30%). Improving relative recoveries may be achieved by reducing microdialysis flow rates but this will also reduce absolute recoveries (Menacherry et al., 1992), requiring a much longer sampling time to collect sufficient sample for chemical analysis.

Based on our results and those of Inselsbacher and Näsholm (2012a), management of N, especially in agricultural soils, requires an assessment not just of absolute N pool sizes, but also moisture content and other physical soil properties that affect the movement of solutes through soil. Within the UK fertilizer practice guide, soil texture is included in the decision-making process, but other factors, such as structure and bulk density, are not (Defra, 2010). Microdialysis has the potential to be used as

a tool to assess the combined effects of pool size and soil physical properties for the determination of soil N availability, but there are some challenges to overcome. First, to obtain meaningful comparisons, a standardization of the method is needed. Second, and most importantly, is how best to interpret the diffusive flux data. There are currently several issues with this due to the fundamental functioning of microdialysis. Microdialysis sampling induces a diffusive flux through the soil as solutes diffusive across the probe membrane, creating a zone of depletion. Due to conservation of mass, the amount of solute extracted from the solution (that which is measured in the dialysate) reflects the diffusive flux of the solute through the soil immediately surrounding the probe. With increasing radial distance from the probe, the diffusive flux will decrease as the same mass is moving across a larger area of soil (Darrah and Roose, 2001). The rate of diffusion across the probe membrane is affected by the MW of the solute in accordance with Fick's law. Rates of solute diffusion, across the probe membrane, will increase with decreasing MW and the induced diffusive flux will subsequently increase. Therefore, the diffusive flux measurement is biased toward solutes with smaller MWs and low charge, invalidating direct comparison between solutes.

The length of time over which the diffusive flux can be maintained is also important. As sampling times increase and concentrations of the solutes are depleted in the immediate vicinity of the probe, the ability of the solute to be buffered by adsorbed solute and microbial generation become increasingly important. Different soils may initially have similar diffuses fluxes, but ability of the soils to maintain that flux may well differ.

Drawing conclusions for plant nutrition from the diffusive flux measurement is subject to further confounding factors. Although diffusion has been shown to be the key process by which plants obtain nutrients (Leadley et al., 1997; Darrah and Roose, 2001), mass flow delivery of nutrients to the root surface may be important in some contexts. Recent work, using microdialysis, also suggests that mass flow may indirectly increase N movement through soil (Oyewole et al., 2014). Having the ability to directly assess the availability of plant nutrients, via diffusion or mass flow, is therefore essential to the understanding of soil fertility and informing soil management. Another factor to consider is that solutes diffusing through the rhizosphere are likely to be exposed to different chemical, biological, and physical conditions compared with the bulk soil surrounding the microdialysis probes (Marschner, 1995; Jones et al., 2009). This rhizosphere effect, due mainly to the exudation and deposition of labile organic carbon from roots, may significantly change the availability of nutrients for plant uptake (Jones, 1998; Hinsinger, 2001; Kuzyakov, 2002). With respect to N, the increased activity and abundance of the microbial biomass may outcompete plants for uptake of organic N-forms (Jones et al., 2013), and increase inorganic N-forms due to faster mineralization of soil organic matter (Kuzyakov, 2002). Therefore, the diffusive flux of N at the probe surface may not accurately represent that which is available for plant uptake. Nutrient uptake from the soil is also an active and selective process, so the presence of N at the root surface does not necessarily result in its subsequent uptake.

The development of tools with the ability to monitor soil N availability is needed for the progression of precision agriculture, and the concept of microdialysis holds much potential. However, it is clearly evident that the methodology and the analysis of the results require significant development before microdialysis could be used to inform N management of agricultural land. Despite these issues, microdialysis is an exciting new research tool for investigating the complexities of soil N cycling and supply. The small size of the probes, combined with the potential for taking multiple samples over an extended period time without perturbing the system, may allow new insights into soil N dynamics at a fine temporal and spatial resolution. It is clear that standard salt and water batch extracts of soil provide a good estimate of plant-available inorganic N at the time of sampling; however, they provide little information on the rate of N supply. Further, recent studies have suggested that they are unsuitable for evaluating low molecular weight dissolved organic matter (DON) in soil due to microbial consumption of the DON during the extraction process (Rousk and Jones, 2010). These problems are overcome with a microdialysis-based approach; however, as with most methods, it is subject to uncertainty, is costly to perform, and the probes only sample small soil volumes. Microdialysis may, however, offer an alternative to standard methods for assessing N mineralization which can be both time-consuming and provide little information on DON (Khanna and Raison, 2013). Future research should therefore compare microdialysis with other techniques for evaluating soil N status (e.g., aerobic and anaerobic long-term incubations, ion exchange resins [Duran et al., 2013]).

ACKNOWLEDGMENTS

The authors would like to recognize the funding for this work provided by the English Beef and Lamb Executive (EBLEX), Hybu Cig Cymru (HCC), Quality Meat Scotland (QMS), and DairyCo. Thanks to Professor Tom de Luca for his help with USDA soil classification.

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Chapter 4

Article II

Nitrogen dynamics in the rhizosphere

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Unpublished manuscript

Author contributions:

RS and DLJ conceived and designed the experiment. All the experimental work, data processing and statistical analysis was performed by RS. PWH advised on the ^{14}C methodology and data analysis. RS wrote the first draft of the manuscript with all authors contributing to the final version.

Abstract

The rhizosphere is a zone of intense competition between plants and microbes for nutrients and these complex interactions may control the availability of nitrogen (N) for plant uptake. This study aimed to improve the spatial and temporal understanding of rhizosphere N dynamics using 3 different experimental approaches. In an initial experiment, net amino acid efflux from the roots of maize seedlings grown in a sterile nutrient solution was investigated. A significant exudate-derived concentration of amino acids, which peaked 1-2 cm from the root tip, was determined using a direct sampling method coupled with spectrofluorometric analysis. Determining rhizosphere N dynamics in-situ, has often been limited by a lack of non-destructive sampling methods. In a novel, in-situ approach, microdialysis probes were used to monitor N dynamics in the rhizosphere of maize seedlings. Microdialysis probes were inserted into microcosms filled with an agricultural Eutric Cambisol. Maize seedlings were grown, over a 68 hour period, in the rhizotubes, so that the growing root passed directly over the membrane of the microdialysis probe. Microdialysis samples were collected at 4 h intervals and chemically analysed for total amino acids, ammonium (NH_4^+) and nitrate (NO_3^-). In addition, the fate of amino acid inputs in the same soil was investigated using an isotopically-labelled amino acid mixture. Carbon dioxide traps were used to capture any $^{14}CO_2$ produced during mineralization while ^{14}C-labelled amino acids remaining in soil solution were collected using a centrifugation drainage technique. Whilst results from the microdialysis sampling showed that the concentration of NH_4^+ and NO_3^- decreased as the root grew past the probe, concentrations of amino acids remained relatively constant throughout the experiment. The lack of an amino acid spike from root exudation in the microdialysis samples, may be explained by the fact that the depletion of the isotopically labelled amino acid mixture from the soil solution was extremely rapid, with only 10% of the 10 µM treatment remained in the soil solution after 1 minute. This study demonstrates the feasibility of microdialysis sampling within the rhizosphere and suggests that LMW DON exuded by roots, is rapidly cycled by carbon-limited microorganisms.

1. Introduction

The rhizosphere is a zone of intense competition between plants and microbes for nutrients (Jones et al., 2013; Kuzyakov and Xu, 2013). Nitrogen (N) is often the limiting nutrient for plant growth and therefore, the complex processes which control the availability of N in the rhizosphere are key to ecosystem functioning (Vitousek et al., 1997). Gaining a better understanding of these processes is essential for improving predictions of rhizosphere N dynamics and their subsequent effects on plant N nutrition (Jones and Hinsinger, 2008). Nowhere is this more important than in agro-ecosystems, where the need to improve the efficiency of N fertiliser use and reduce polluting diffuse losses of N is set against increasing demand for food from a rapidly growing world population (Sutton et al., 2011; Tilman et al., 2011). The rhizosphere is extremely complex, exhibiting significant temporal and spatial variation in soil chemistry, microbial population and nutrient availability (Kuzyakov, 2002; Farrar et al., 2003). The rhizosphere effect is mainly attributed to root deposition of photosynthetically fixed carbon (C), which stimulates the growth of the microbial community (Jones et al., 2009). This, in turn, leads to increased microbial-plant competition for available nutrients. The growth of a root into undisturbed, root free soil will bring about significant changes to soil N dynamics. These changes can be attributed to 4 different but interrelated and competing processes: 1) Root uptake of both organic and inorganic N; 2) root exudation of organic C and N compounds; 3) priming effects on microbial N immobilisation and soil organic matter (SOM) decomposition rates facilitated by rhizodeposition of C; and 4) grazing of the microbial community by soil microfauna/mesofauna. These are discussed in more detail below: 1). Root uptake of both organic and inorganic N forms has been well evidenced (Marschner, 1995; Farrell et al., 2011; Hill et al., 2012). However, the ecological and nutritional importance of organic N plant uptake in-situ is still unclear (Näsholm et al., 2009) and is likely to depend upon a variety of environmental factors and local soil nutrient status (Jones et al., 2005). In agro-ecosystems, which receive large inputs of N, plant uptake of organic N forms is likely to be low (Owen and Jones, 2001; Schimel and Bennett, 2004; Jones et al., 2013). Conversely in low input, oligotrophic ecosystems the importance of organic N for plant nutrition may be significantly higher (Schimel and Bennett, 2004; Kielland et al., 2007; Inselsbacher and Näsholm, 2012b). Root uptake of organic N has shown to be spatially independent (Jones and Darrah, 1993; Jones and Darrah, 1994). Likewise, uptake of inorganic N has been shown to occur all along the root length; although some studies show that uptake in the root tip zone may be higher than older parts of the roots (Miller and Cramer, 2005).

2). It has been estimated that rhizodeposits may account for as much as 11 % of photosynthetically fixed C (Jones et al., 2009) or 15 to 25 % of C allocated below ground (Kuzyakov, 2002). Roots exude a variety of low molecular weight (LMW) organic compounds, of which amino acids are a significant component (Jones et al., 2009). Exudation of amino acids has been shown to be spatially dependent, with the largest efflux occurring in the zone behind the growing tip (Jones and Darrah, 1994; Jones et al., 2009). As such, there is a partial spatial decoupling of amino acid efflux and uptake. It has also been shown that active exudation of inorganic N may be initiated when an imbalance exists between uptake, growth and storage capacity, and passive leakage can occur through aquaporins and anion channels (Miller and Cramer, 2005).

3). The rhizosphere priming effect (RPE) is a change in the decomposition rate of soil organic matter (SOM) attributed to a change in the size and nutrition status of the rhizosphere microbial community (Kuzyakov, 2002). Large fluxes of available C from rhizodeposits at the root tip (Farrar et al., 2003) stimulates rapid growth of C-limited microbes, which may subsequently lead to N limitation. In this case, available N in the rhizosphere will be quickly immobilised leading to increased degradation of SOM in order to obtain limited N. This describes a positive RPE. In soils where N is abundantly available, such as agro-ecosystems, there may be no requirement for extra N to be obtained from SOM. In this case, there may be a decrease in SOM degradation as neither N nor C is limiting microbial growth. This is a negative RPE. However, as the rhizodeposition of C is spatial dependent, so is the nutritional status of the microbial community. As the amount of available C decreases away from the root tip zone, the large microbial community may become C limited again, leading to mineralisation of N from SOM and dead microorganisms (Kuzyakov, 2002).

4). The large growth in soil microbes within the rhizosphere is frequently accompanied by increased levels of predatory microfaunal grazing. This remineralises the N, which was previously immobilised by the microbial community, leading to increased N availability for plant uptake (Griffiths, 1994; Kuzyakov, 2002; Bonkowski et al., 2009).

Studies of N in the rhizosphere are particularly interesting as the importance of these processes are spatially dependent due to variations in the composition and amount of rhizodeposition along the longitudinal axis of a growing root. To further complicate matters, the nutrient status of the soil prior to root disturbance and other environmental conditions may have a considerable effect on these processes.

Over a range of spatial and temporal scales within the rhizosphere, there is intense competition for N between plants and microorganisms. Generally, microbes appear to be more

efficient at acquiring N when compared with roots (Jones et al., 2013), which may lead to plant N limitation (Kuzyakov and Xu, 2013). However, as the life cycle of the microbial pool is short and the pool size cannot be sustained due to increasing C limitation away from the root tip, the N within the microbial pool will be re-released for plant uptake. Hence, the long term flow of N is from the SOM to the plant and with plant C traded for microbial-derived N (Kuzyakov and Xu, 2013).

Investigating rhizospheric N dynamics is difficult to achieve due to the challenging sampling environment and the complex interactions between processes. Isolating individual processes, which may be achieved by growing plants in sterile nutrient solutions or investigating microbial dynamics in root free soil, is one approach that can be used to better characterise the dynamics of specific mechanisms. However, extrapolating these results to intact rhizosphere systems, where interactions between competing process and soil abiotic factors are not fully characterised, could lead to false conclusions. Measurement of the various soil N pools in-situ within an intact soil plant system may allow the net result of rhizospheric N processes to be assessed but inferring the importance of the individual processes and the rate of flux between the observed pools may not be possible. In-situ quantification of rhizosphere N dynamics are difficult to achieve without causing significant disturbance to the system being evaluated and has been limited by a lack of non-destructive sampling methods (Oburger et al., 2013). Recent studies have shown that microdialysis is an exciting new technique for the determination of soil N status, and may offer new insights into rhizosphere N dynamics (Inselsbacher et al., 2011; Inselsbacher and Näsholm, 2012a; Shaw et al., 2014). Microdialysis is a non-destructive sampling method, based on the passive diffusion of solutes from within the soil solution, across a partially permeable membrane, into a flow of water – the perfusate. The probes can be run continuously over a number of days, enabling continuous in-situ and near real-time monitoring of soil solution with minimal disturbance to the system being evaluated. The small size of the microdialysis probes used in this study and previous studies (diameter 0.5 mm, membrane length 4 – 10 mm), allows easy implantation into the soil and excellent spatial resolution, making them ideal for rhizosphere studies. The rate of diffusion of solutes across the probe membrane is driven by the concentration gradient between the soil solution surrounding the membrane and the perfusate, which is continuously pumped through the probe. This creates a zone of depletion surrounding the probe, which induces the diffusive flux of solutes through the soil, down the resulting concentration gradient. As such, the concentration of solutes in the dialysate reflects not just the concentration in the soil solution,

but also the rate at which these solutes can diffuse through the soil. In addition to this, each N pool is in flux, so differences in additive and consumptive processes may affect the concentration of each N-form over time (Shaw et al., 2014). Therefore, microdialysis sampling can be used to make a time-integrated assessment of in-situ concentrations that better reflects the importance of both biotic and abiotic processes.

The aim of this study was to investigate N dynamics in the rhizosphere using a variety of different approaches. In-situ determination of N dynamics in the rhizosphere of maize seedlings, grown in an agricultural soil, was performed using microdialysis sampling. To compliment these results, direct sampling of exudates from the root surface of maize seedlings, grown in sterile nutrient solution, was performed in order to determine the spatial variability in amino acid efflux. Furthermore, a ^{14}C isotopic labelling technique was used to assess the fate of exogenously applied amino acids to the same agricultural soil.

2. Materials and methods

2.1. Soil sampling and characterisation

Four replicate soil samples (n = 4) were collected from the Henfaes Research Station, Abergwyngregyn, Gwynedd, North Wales (53°14′N, 4°01′W). The site has a temperate, oceanic climate, receives an average annual rainfall of 1250 mm and has a mean annual soil temperature at 10 cm depth of 11 °C. Samples were collected from 0 – 10 cm from the Ahp horizon at the corners of a 2 m × 2 m quadrat. The soil was placed in gas-permeable plastic bags and transferred immediately to the laboratory, were it was refrigerated at 4 °C until use. These 4 replicates were used for the microdialysis experiment and 3 were used for the microbial uptake experiment. The soil is classified as a Eutric Cambisol which has a sandy clay loam texture and a fine crumb structure. The soil characteristics were determined using the methods described below.

Soil pH and electrical conductivity were determined in a 1:2.5 (w/v) soil:distilled water suspension using standard electrodes. Moisture content was determined by drying for 24 h at 105 °C. Total C and N of dry soil were determined with a TruSpec CN analyser (Leco Corp., St Joseph, MI, USA). Soil N pools were determined using a standard 0.5 M K_2SO_4 extraction (Jones and Willett, 2006). Field-moist, 2 mm sieved soil (5 g) was extracted (175 rev min^{-1}, 1 h) using 0.5 M K_2SO_4, to determine available N, at a soil: extractant ratio of 1:5 (w/v). The extracts were centrifuged (3200 *g*, 15 min), and the resulting supernatant was collected and frozen (-18°C) to await analysis. Soil solution was sampled using the centrifugal-drainage

technique of Giesler and Lundström (1993). Approximately 100 g of field-moist soil was placed in centrifuge tubes, the bottom of which are perforated to allow passage of the soil solution into a collection vessel, and centrifuged at 3200 *g* for 20 min. Soil solutions were subsequently frozen (-18°C) to await analysis. Soil extracts and solutions were analysed for dissolved organic carbon (DOC) and dissolved organic nitrogen (DON), total free amino acids (subsequently referred to as amino acids), ammonium (NH_4^+) and nitrate (NO_3^-) using the methods described in section 2.2 below. A chloroform fumigation-extraction (*t* = 7 days) using 2 g of fresh soil, was performed to determine microbial biomass C and N according to Voroney et al. (2008) (K_{EC} = 0.35 K_{EN} = 0.5). Exchangeable cations were extracted using 0.5 M acetic acid and the filtered extracts analyzed using flame emission spectroscopy (Sherwood 410 flame photometer: Sherwood Scientific, Cambridge, UK). Basal soil respiration was determined at 20 °C using an SR1 automated multichannel soil respirometer (PP Systems Ltd., Hitchin, UK) and steady state CO_2 production rates recorded after 24 h. Results are shown in Table 1.

Table 1. Characteristics of the Eutric Cambisol soil used in this study. Values represent means ± SEM (n = 4).

Soil property	Mean	SEM
pH	5.95	0.04
EC (µS cm^{-1})	36.90	2.18
Moisture Content (g g^{-1} DW)	0.33	0.01
Total C (g C kg^{-1})	22.75	0.79
Total N (g N kg^{-1})	2.91	0.09
C:N ratio	7.82	0.13
Microbial C (g C kg^{-1})	0.92	0.05
Microbial N (g N kg^{-1})	0.09	0.01
Basal soil respiration (µg CO_2 kg^{-1} h^{-1})	1233.1	94.5
Available Ca (mg kg^{-1})	930.8	9.43
Available K (mg kg^{-1})	63.27	1.84
Available Na (mg kg^{-1})	26.65	2.09
Available P (mg kg^{-1})	7.4	2.0
0.5 M K_2SO_4 extraction		
DOC (µmol C g^{-1})	13.49	0.93
DON (µmol N g^{-1})	1.27	0.13
Total free amino acids (µmol N g^{-1})	0.11	0.01
NH_4^+ (µmol N g^{-1})	0.57	0.08
NO_3^- (µmol N g^{-1})	0.16	0.04
Soil solution		
DOC (µmol C l^{-1})	2793	374
DON (µmol N l^{-1})	409	78
Total free amino acids (µmol N l^{-1})	9.29	1.97
NH_4^+ (µmol N l^{-1})	53.93	7.17
NO_3^- (µmol N l^{-1})	407.6	104.7

2.2. Chemical analysis

The following methods were used to analyse DOC, DON, amino acids, NH_4^+ and NO_3^- in soil extracts and solutions, microdialysis samples and nutrient solutions. DOC and DON were determined using an Analytik Jena Multi N/C 2100S (AnalytikJena, Jena, Germany). All samples were acidified using 2 M HCl (5 µl ml^{-1}) to remove dissolved inorganic C. Amino acids were determined by the *o*-phthaldialdehyde (OPA) spectrofluorometric method of Jones et al. (2002). NH_4^+ was determined by the nitroprusside colorimetric method of Mulvaney

(1996) and NO_3^- by the vanadate-catalyzed colorimetric Griess reaction of Miranda et al. (2001).

2.3. Root amino acid exudation

Root efflux of amino acids from sterile *Zea mays* L. plants was assessed using a direct sampling method combined with spectroflurometric analysis. Seeds of *Zea mays* L. were surface sterilised by shaking vigorously in 14% sodium hypochlorite followed by 90% ethanol. Sterile deionized (DI) water was used to rinse the seeds between and after these treatments. Individual seeds were then transferred to sterile Petri-dishes containing 25 ml of 10 % Long Ashton (LA) nutrient solution (Hewitt, 1952). The seeds were left to germinate and placed in a climate chamber with a temperature of 20 °C, 24 h photoperiod (*c*. 500 µmol photons m^{-2} s^{-1} PAR) and 70 % relative humidity. When the seedlings had reached the required size (see below) an individual plant was removed from its Petri-dish, rinsed well using sterile DI water and placed in a plastic dish partially filled with fresh sterile 10 % LA solution (Fig. 1.), so that the roots were submerged but the shoot remained out of the solution. The roots were arranged so exudates from the axial root would not be contaminated by those from secondary roots. The seedling was left undisturbed for 20 min to allow a dynamic equilibrium to be formed between the root boundary (unstirred) layer and the bulk solution. Samples of 1 µl were taken from the boundary layer along the length of the root using a Nano Drop®micro-pipette and analysed for amino acids using a Nano Drop® ND 3300 Fluorospectrometer (NanoDrop Products, Wilmington, USA) using a method based on Jones et al. (2002). Briefly 1 µl of sample was placed on the measuring window with 1 µl of the OPA reagent and fluorescence measured after 1 min. Standard solutions of leucine (0-50 µM), made up in 10 % LA solution, were used for the calibration and the resulting curve was found to be linear up to 50 µM, with a slope of 28.3 ± 1.0 and an r^2 of 1 ± 0 (n = 3, means ± SD).

Two sets of 3 replicate seedlings (n = 3) were analysed on 2 separate occasions. The first set had root lengths of between 6 to 7 cm and samples were taken at 0.5 cm intervals. The second set had root lengths of between 9 to 11 cm and samples were taken at 1 cm intervals. Statistical differences in the spatial dependence of amino acid exudation were determined using a one-way ANOVA. SPSS v.20 (IBM Ltd., Portsmouth, UK) was used for all statistical testing with $p < 0.05$ used as the cut-off for statistical significance.

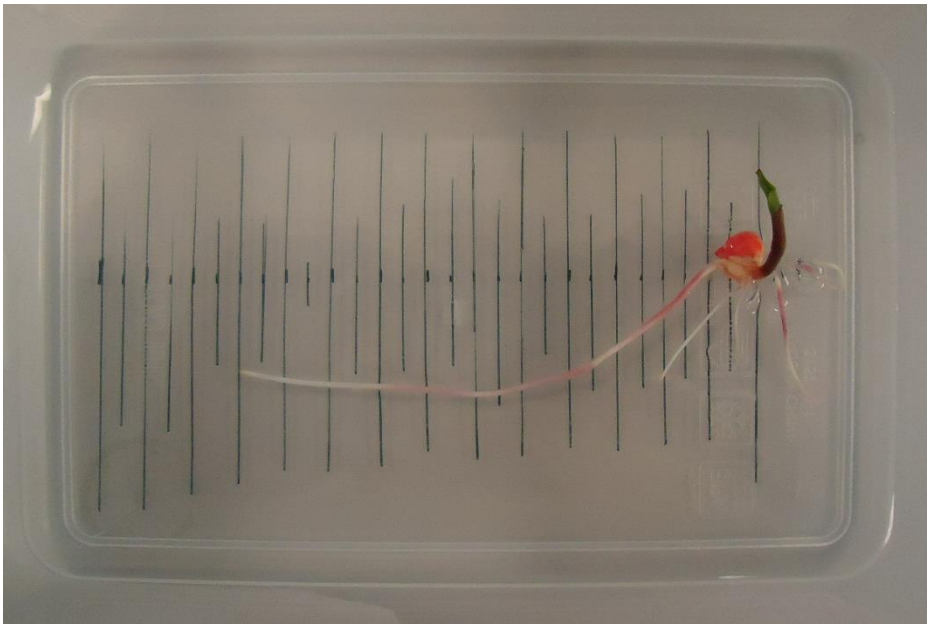

Figure 1. Experimental set up used for sampling root exudates from the rhizosphere of sterile Zea mays L. seedlings bathed in 10 % Long Ashton nutrient solution.

2.4. Microdialysis monitoring of rhizosphere N dynamics

Monitoring of rhizosphere soil N dynamics was achieved by growing the axial root of *Zea mays* L. seedlings within a soil filled rhizotube. A microdialysis probe was placed within the rhizotube. The primary root axis was then allowed to grow towards and then past the microdialysis probe enabling repeated and non-destructive sampling of soil solution chemistry to be made.

Microdialysis setup and calibration

A peristaltic pump (Watson Marlow 205u, Watson Marlow, Falmouth, UK) was used to perfuse high purity DI water through CMA 20 microdialysis probes (CMA Microdialysis AB, Kista, Sweeden), with a 20 kDa molecular weight cut off, polyethersulfone membrane (4 mm long, 500 µm external diameter). The membrane length of 4 mm was chosen to maximize the spatial resolution of the microdialysis sampling. These probes were chosen as they have been used previously, albeit with different membrane lengths and molecular weight cut-offs, to sample amino acids, NH_4^+ and NO_3^- from soils (Inselsbacher et al., 2011; Inselsbacher and Näsholm, 2012a; Inselsbacher and Näsholm, 2012b; Inselsbacher et al., 2014; Oyewole et al., 2014; Shaw et al., 2014) and it has been demonstrated that binding of N solutes to the membrane does not occur (Inselsbacher et al., 2011). The flow rate of the pump was set to 5 µl min^{-1} as this has been shown to give a good compromise between volume of sample collected

and the relative recovery (*RR*) of soil solutes (Inselsbacher et al., 2011). Prior to and following the rhizosphere sampling, the microdialysis probes were calibrated to determine the *RR* of amino acids, NH_4^+ and NO_3^- and to assess any changes in the performance of the probes during the experiment. Each probe was placed in a 100 µM standard solution of leucine, NH_4^+ and NO_3^- and perfused with DI H_2O at a rate of 5 µl min^{-1} and the dialysate was collected continuously at 2 h intervals for a total time period 6 h. Samples were frozen to await chemical analysis for amino acids, NH_4^+ and NO_3^- as described above. The *RR* of each probe was calculated as a percentage of the standard solution recovered in the dialysate as shown below.

$$RR\ (\%) = 100 \times ([N]_{md}/[N]_{std}) \quad \text{(Eqn. 1)}$$

Where $[N]_{md}$ is the concentration of the target N solute in the microdialysis dialysate and $[N]_{std}$ is the concentration of the target solute in the standard solution.

Microdialysis sampling of the rhizosphere

The rhizotube apparatus was assembled as shown in Figure 1. In detail, a rhizotube with a diameter of 9 mm and length of 30 cm was cut in half and a smaller plastic tube of 4 mm diameter and 2 cm length was secured in the bottom half. This was then filled with 1 mm sieved soil into which a pre-calibrated microdialysis probe was inserted. The top half of the tube was then connected to the lower half, secured and filled with soil to give a total soil mass of approximately 10 g with a bulk density of 0.75 g cm^{-3}. The microdialysis probes were connected to the peristaltic pump using marprene tubing (0.38 mm internal diameter).

Maize seedlings were pre-germinated for 3 d on damp tissue paper at 20 °C. Side roots were excised; leaving only the axial root which had a length of 2 cm. Excision of the side roots did not adversely affect the growth of the main root axis. Pre-germinated seedlings were then placed in the top of individual rhizotubes and covered with 10 mm of sieved soil. Controls were similarly assembled but without the addition of a pre-germinated seedling. Replicate microcosms were then transferred placed in a climate-controlled chamber as described above and samples recovered from the microdialysis probes every 4 h for a total of 68 h. Estimations of the intrinsic soil solution concentration ($[N]_{soil}$) of amino acids, NH_4^+ and NO_3^- for each 4 h sampling period were calculated as follows:

$$[N]_{soil} = [N]_{md} \times (100/RR) \quad \text{(Eqn. 2)}$$

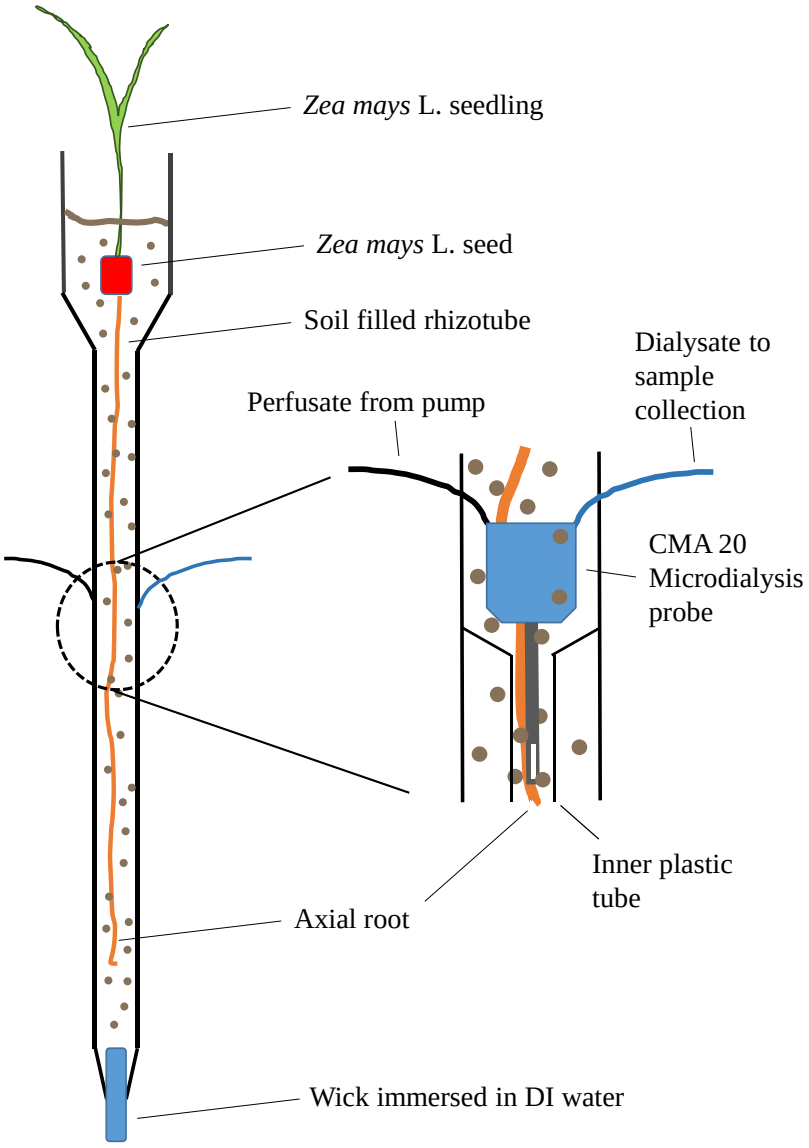

Figure 1. Equipment set-up for microdialysis monitoring of rhizosphere N dynamics in soil filled microcosms.

Statistical analysis

Differences in microdialysis derived soil N concentrations between specific time points or between the planted and unplanted treatments were assessed with two-sample t tests using SPSS v.20 (IBM Ltd., Portsmouth, UK) with $p < 0.05$ used as the cut-off for statistical significance.

2.5. Amino acid uptake and mineralisation by the soil microbial community

The fate of amino acids in the same soil was assessed using the methods of Hill et al. (2008) and Wilkinson et al. (2014). Briefly, a uniformly ^{14}C labelled equimolar mixture of 20

proteinaceous amino acids, at a range of concentrations representing that which could be found in the rhizosphere (1, 10, 100, 1000 µM), were used to determine rates of microbial uptake from the soil solution and subsequent mineralisation.

Microbial uptake of amino acids

1.6 g of field moist soil (2 mm sieved) was placed in 1.5 ml micro-centrifuge tubes, giving a bulk density of 0.81 ± 0.004 g cm^{-3} (mean ± SEM, n = 3). To facilitate collection of the soil solution via centrifugal-drainage, a small hole (0.5 mm) was made in the bottom of each 1.5 ml micro-centrifuge tube and the tube was placed inside an intact 1.5 ml micro-centrifuge tube (Hill et al., 2008; Wilkinson et al., 2014). A 300 µl aliquot of amino acid mixture, at each concentration, was applied to the soil and incubated, at 20°C, for 1, 5, 10, 30 and 60 min. At the end of each incubation period the soil solution was collected by centrifugation (4000 *g*, 1 min) and a 100 µl aliquot of the resulting solution added to 4 ml of Scintisafe 3 scintillant (Fisher Scientific, Loughborough, UK). The amount of ^{14}C remaining in solution after each incubation was measured by liquid scintillation counting using a Wallac 1404 scintillation counter (Wallac EG&G, Milton Keynes, UK). The same treatments were applied to sterilised soils (autoclaved at 120 °C, 20 min) and the results used as a control to determine the significance of abiotic (e.g. sorption, mineralisation) reactions.

Mineralisation of amino acid C

To determine mineralisation rates of the applied ^{14}C amino acid mixture, 1.6 g of field-moist soil was placed into a 10 cm^3 glass vessel. A 300 µl aliquot of the ^{14}C amino acid mixture at each concentration was applied to the soil. The vessel was sealed and moist air (20 ± 1 °C) passed over the soil surface at a rate of 100 ml min^{-1}. The outflow from the vessel was bubbled through 2 consecutive 3 ml 0.1 M NaOH vials to trap $^{14}CO_2$ evolved from the soil (capture efficiency > 95%; Hill et al., 2007). These traps were changed after 1, 5, 10, 20, 40 and 60 min, and ^{14}C in the traps determined by liquid scintillation counting as described above.

Data and statistical analysis

All experiments were undertaken in at least triplicate. Analysis of the microbial uptake data is complicated by the fact that the actual concentration of the added ^{14}C amino acids in the soil solution, at any given time point, is dependent upon the extent of mixing of the added solution with the intrinsic soil solution and adsorption of the added amino acids to soil particles. The theoretical maximum ^{14}C activity ($^{14}C_{max}$) in the recovered soil solution (^{14}C-activity per unit volume) can be calculated as follows:

$^{14}C_{max}$ = total ^{14}C added/(existing soil water + water added with substrate) (Eqn.3)

The effect of the abiotic factors on the ^{14}C activity in the recovered soil solution was assessed by comparing the recovered ^{14}C activity from the sterilised soils to calculate the theoretical maximum ^{14}C activity (Eqn. 4). These data are shown in figure 1 by plotting the recovered ^{14}C activity, as a percentage of the theoretical maximum ^{14}C activity, for each concentration, against time.

(Recovered ^{14}C activity/theoretical max ^{14}C activity) × 100 (Eqn. 4)

First-order, single exponential decay curves were fitted to these data:

$$R = y_0 + a \exp(-bt) \quad \text{(Eqn. 5)}$$

Where R is the amount of ^{14}C recoverable after mixing and sorption (as a percentage of the theoretical maximum ^{14}C activity), y_0 is the asymptote to which the actual recovered ^{14}C fell to after mixing with the intrinsic soil solution, t is the time after amino acid addition and a and b are the coefficients of the exponential decay. Figure 3 shows that the initial observed maximum ^{14}C activity, for all the concentrations, was greater than the theoretical maximum (>100%), which indicates that mixing with the intrinsic soil solution was incomplete. For concentrations 1 – 100 µM this value dropped below 100% between 10 – 20 min and for 1000 µM, between 30 – 40 min. At these time points it could be concluded that complete mixing had occurred, but this may not be case as it is not possible to disentangle the mixing and sorption processes at this time period. The inverse of the y_0 asymptote is likely to reflect the percentage of the added amino acid that is sorbed to the soil solid phase. The mean value of the inverse y_0 asymptote for the four added concentrations is 7.0 ± 1.6 % (mean ± SEM, n = 4). These curves were used to calculate the actual maximum soil solution ^{14}C activity, taking in to account mixing and sorption, for all sampling periods following substrate addition. In turn, this enabled the determination of the percentage of ^{14}C labelled amino acid remaining in the soil solution of the non-sterile soil, at each sampling time.

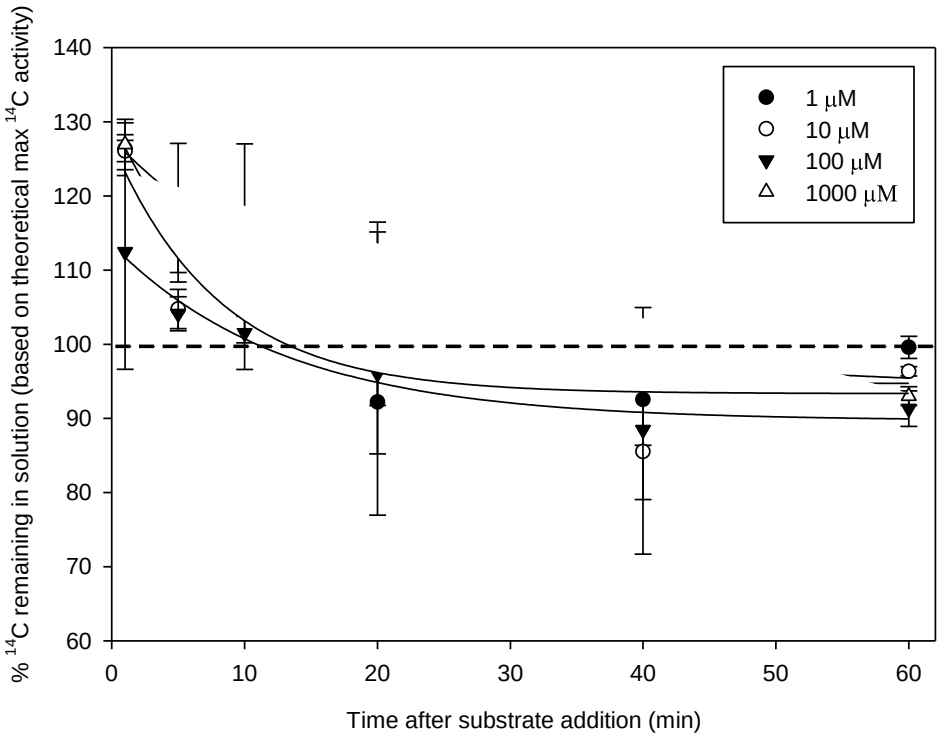

Figure 3. The effect of abiotic factors (mixing and sorption) on the percentage of added ^{14}C-label remaining in soil solution after the addition of a ^{14}C-labelled equimolar mix of 20 amino acids to a sterile (autoclaved) Eutric Cambisol agricultural soil. Percentages are based on the theoretical amount of ^{14}C available in solution assuming perfect mixing of the substrate with the soil solution. Deviation from 100% (dotted line) indicates incomplete mixing (values above 100%) or abiotic sorption (values below 100%). Lines represent fits of a single first order kinetic equation to the experimental data.

To determine the half-times of amino acids in the soil solution (for the 1, 10 and 100 µM treatments), a single first order exponential decay was fitted to the data describing the loss of amino acid from soil:

$$AA = a_1 \exp(-b_1 t) \quad \text{(Eqn. 6)}$$

where *AA* is the ^{14}C amino acid remaining in the soil solution, b_1 is the exponential coefficient describing depletion of soil solution ^{14}C by the microbial community, a_1 describes the pool size and *t* refers to time. The half-time of the amino acid soil solution pool can be defined as

$$t_{1/2} = \ln(2)/b_1 \quad \text{(Eqn. 7)}$$

For the 1000 µM amino acid treatment, depletion of ^{14}C from the soil solution was better described by a double first-order exponential decay equation:

$$AA = [a_1 \exp(-b_1 t)] + [a_2 \exp(-b_2 t)] \quad \text{(Eqn. 8)}$$

Half-times for the soil solution amino acid pools a_1 and a_2 were calculated as described above. The two exponential parts of the equation were assumed to represent uptake by independent carrier systems (Jennings, 1995).

Microbial uptake rates of applied amino acid C and N were calculated following the first minute of the incubation results, based on 1 mole of the ^{14}C amino acid mixture containing 5.15 moles of C and 1.5 moles of N.

The results from the mineralisation study were used to calculate the cumulative percentage of the total applied ^{14}C amino acid evolved as $^{14}CO_2$ for each time period. The inverse of this data is presented in Figure 5 and the half-times for mineralisation were calculated using a double first-order exponential decay curve on the premise of bi-phasic mineralisation of LMW C substrates in soil (Boddy et al., 2007).

$$AAs = [a_3 \exp(-b_3 t)] + [a_4 \exp(-b_4 t)] \quad \text{(Eqn. 9)}$$

Where *AAs* is the total amount of amino acid-derived ^{14}C remaining in the soil (i.e. $\sum$ soil solution + exchangeable + microbial), b_3 and b_4 are the exponential coefficients describing the primary and secondary phases of the mineralisation, a_3 and a_4 describes the pool sizes and *t* refers to time. Microbial mineralisation rates of applied amino acid C were calculated for the first minute of the incubation from the above results, based on 1 mole of the ^{14}C amino acid mixture containing 5.15 moles of C.

All exponential decay equations were fitted to the experimental data using a least squares iteration routine in SigmaPlot 12.3 (SPPS Inc. Chicago, USA). Statistical differences between pool half-times for the microbial uptake and mineralisation data were assessed using one-way ANOVA using SPSS v.20 (IBM Ltd., Portsmouth, UK).

3. Results

3.1. Root amino acid efflux

Root exudates were sampled from the roots of sterile maize seedlings using a 1 µl pipette, and analysed for total free amino acids using a nano-drop fluorimeter. It has been previously demonstrated that roots have the ability to recapture exuded amino acids (Jones and Darrah, 1994). Therefore, these results represent the equilibrium between gross efflux and re-uptake in the rhizosphere boundary layer. Figure 4 shows that both sets of seedlings exhibit a clear and statistically significant peak in the amino acid concentration of the exudates at 1 – 2 cm distance from the root tip. In the shorter roots (6-7 cm) this peak occurred at 1 cm with a concentration

of 11.2 ± 2 µM N (mean ± SEM; *n* = 3). In the longer roots (9 - 11 cm) the peak occurred at 2 cm and had a slightly lower concentration of 8.7 ± 0.5 µM N.

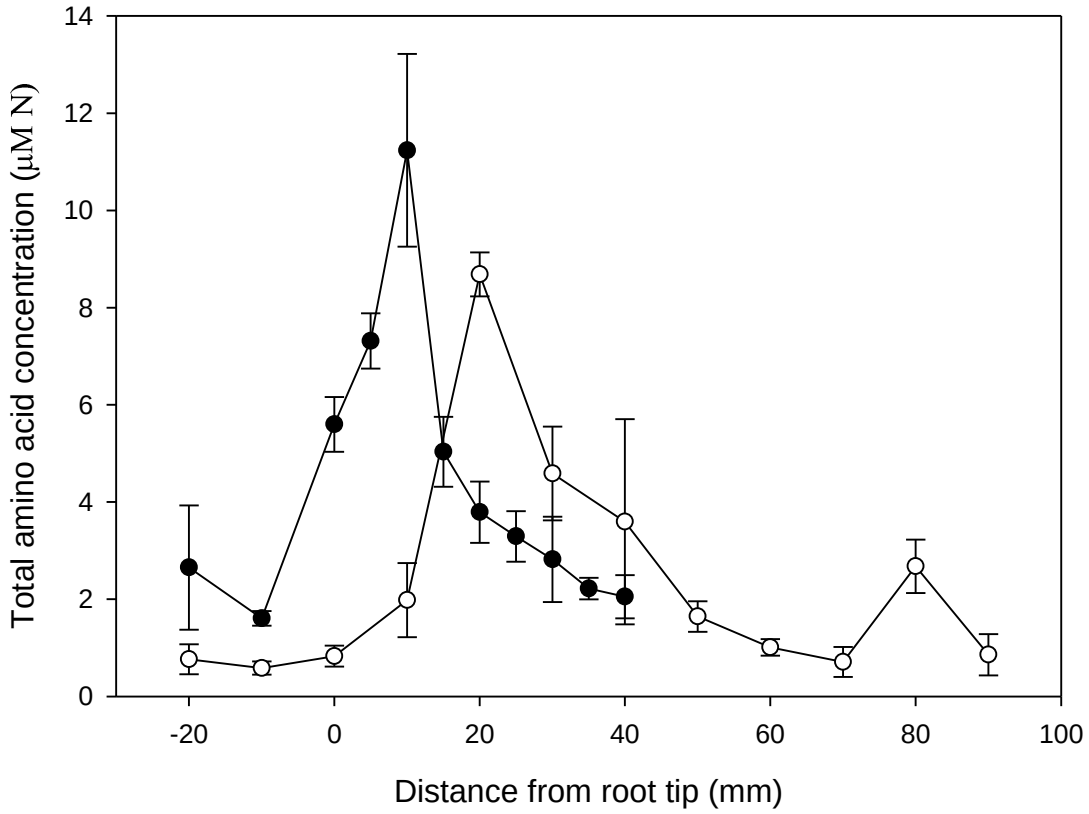

Figure 4. Spatial variation in amino acid concentration of root exudates sampled longitudinally along roots of sterile Zea mays L. seedlings grown in 10 % Long Ashton nutrient solution. Values represent means ± SEM (n = 3). Root length was 60 – 70 mm (closed circles) and 90 – 110 mm (open circles). Negative x values represent distance from root tip into bulk solution.

3.2. Microdialysis monitoring of rhizosphere N dynamics

Growth of maize seedlings in rhizotube

After 68 h, the plants were removed from the growth chamber and the rhizotubes split open longitudinally with a razor blade so that the roots could be inspected. The primary root length of the 3 replicates was 20.8 ± 0.8 cm (mean ± SD). The longitudinal distance from the root tip to the centre of the microdialysis probe membrane was 9.8 ± 0.8 cm. The average root growth rate was 0.31 ± 0.01 cm h^{-1}, from which it is estimated that the root tip reached the centre of the microdialysis probe membrane at 31.0 ± 1.2 h. Each replicate plant had also produced between 2 – 4 secondary roots although none of these had reached the microdialysis probe by

the time the experiment was terminated. Shoot length for all replicate microcosms was 9.0 ± 0.0 cm. In each of the planted microcosms, the primary root axis had grown directly over the surface of the microdialysis probe.

Performance of the microdialysis probes

The microdialysis probes were calibrated in a standard solution of 100 µM amino acid (leucine), NH_4^+ and NO_3^- prior to deployment in the microcosms. Based on the standard solutions, relative recoveries (RR) were calculated to be 4.8 ± 0.2 %, 14.5 ± 0.6 % and 10.8 ± 0.4 % (n = 7, ± SEM) for the three solutes respectively. At the end of the microcosm experiment, the probes were re-calibrated and the RR calculated to assess any change in the performance of the microdialysis probe. The RR decreased for all three N forms to 4.1 ± 0.3 %, 10.3 ± 0.4 % and 10.2 ± 0.7 % (n = 7, ± SEM) respectively, of which only the change for NH_4^+ proved statistically significant ($P < 0.001$).

The microdialysis derived estimates of soil solution concentration for the initial 4 h period in the control treatment were compared to the results achieved by centrifugal-drainage (see Table 1 and Figure 5). For amino acids and NO_3^- there were no significant differences between the two methods but the concentration of NH_4^+ was significantly higher when assessed by centrifugal-drainage ($P > 0.01$).

Microdialysis sampling

As shown in Figure 5, the concentration of amino acids in the soil solution from the control (unplanted) treatment decreased significantly ($p < 0.001$) over the course of the experiment from 10.6 ± 1.5 µM N at 4 h to 4.2 ± 0.2 µM N at 68 h. The concentration transiently peaked in the control treatment at 36 h with 14.1 ± 1.5 µM N and then decreased rapidly to 4.3 ± 0.4 µM N. The concentration of amino acids for the planted treatment also fell over the course of the experiment, but by a smaller amount, from 11.5 ± 1.2 µM N at 4 hr to 9.5 ± 4.4 µM N at 68 h, although this not statistically significant. The concentration peaked in the planted treatment at 48 h with 12.0 ± 4.6 µM N. Unlike the control, the concentration in the planted root treatment did not appear to stabilise, although none of the observed fluctuations proved statistically significant.

The concentration of NH_4^+ in the unplanted treatment varied throughout the experiment and showed no clear trend. Concentrations at 4 and 68 h were 22.4 ± 4.1 µM and 37.3 ± 12.4 µM respectively and were not significantly different from each other. Conversely, for the planted treatment, a statistically significant decreasing trend was observed with time as the NH_4^+ concentration dropped from 54.1 ± 10.9 µM at 4 h to 7.7 ± 0.3 µM by the end of the

experiment (P = 0.013). Between 28 and 40 h, the NH_4^+ concentration decreased by over 50 % from 29.3 ± 13.2 µM to 14.0 ± 4.9 µM, which corresponded to passage of the root tip past the microdialysis probe, although the difference here was not statistically significant. Furthermore the concentration of NH_4^+ prior to the passage of the root was variable and there also was a small peak at 36 h after the root had grown past the probe. As such, this noise makes attributing the observed decrease in NH_4^+ concentration to plant uptake difficult. However, the concentration does appear to flatline after 40 h.

The NO_3^- soil solution concentration in the control at 4 h was 301.0 ± 34.2 µM, which was statistically similar to the treatment of 203.8 ± 10.8 µM. However, between 30 and 36 h there was a large divergence in the two treatments as the unplanted soil increased by 67% to 505.7 ± 256.6 µM while the planted treatment decreased by 28% to 96.80 ± 45.4 µM. The mean concentration in the unplanted control after 36 h remained above 350 µM, although large inter-replicate was evident, resulting in no significant difference in NO_3^- concentration between the start and end of the experiment. In contrast, NO_3^- concentrations in the treatment containing roots continued to decline to a low point of 19.2 ± 3.9 µM at 52 h. This concentration was significantly different to the 32 h time point and coincides with growth of the root past the microdialysis probe (P = 0.003). The NO_3^- concentration then increased slightly to 32.4 ± 17.0 µM by the end of the experiment. No significant difference was found between the initial NO_3^- concentration at 4 h and the final concentration, however, a significant difference (P = 0.042) was found between the NO_3^- concentration at 8 h and 68 h despite the concentration at 8 h being lower than at 4 h. The difference between the control and root treatments at the end of the experiment was also significant.

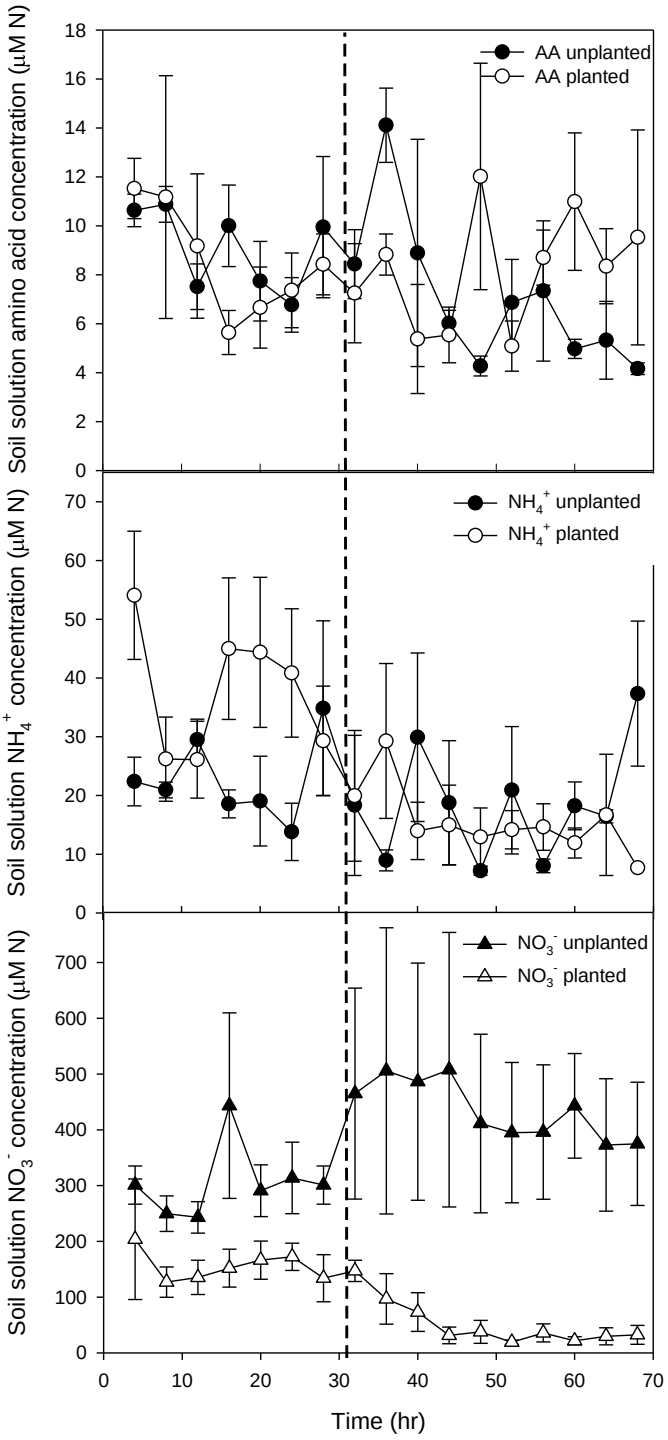

Figure 5. Soil solution amino acid, NH_4^+ and NO_3^- concentrations estimated by in-situ microdialysis sampling from root-free (unplanted control) and rhizospheric soil (planted). In the planted treatment the axial root of a maize seedling passed the membrane of the in-situ microdialysis probe at 31.0 ± 1.2 h (indicated with vertical dotted line). Microdialysis samples were collected over 4 h periods. Values represent means ± SEM (planted: n = 3, unplanted control: n = 4).

3.3. Microbial uptake and mineralisation of exogenous amino acids

To determine the rate of microbial amino acid uptake and subsequent mineralisation in soil, a ^{14}C labelled amino acid mixture was added to soil. Figure 6 shows microbial uptake of the applied amino acid solution was extremely rapid, with 92.5 ± 0.8 % (means ± SEM; *n* = 3) of the 1 µM solution removed from solution within 1 min. The amount remaining in soil after 1 min declined with increasing concentration to 54.8 ± 3.0 % in the 1000 µM treatment. The absolute microbial uptake of C and N however, increased with increasing amino acid concentration from 1.29 ± 0.01 µmol C kg^{-1} DW soil min^{-1} and 0.38 ± 0.001 µmol N kg^{-1} DW soil min^{-1} for the 1 µM treatment, to 777 ± 39 µmol C kg^{-1} DW soil min^{-1} and 225 ± 1 µmol N kg^{-1} DW soil min^{-1} for the 1000 µM treatment. These values of percentage and absolute microbial uptake are comparable to studies performed on similar soils using LMW C substrates (Hill et al., 2008; Wilkinson et al., 2014).

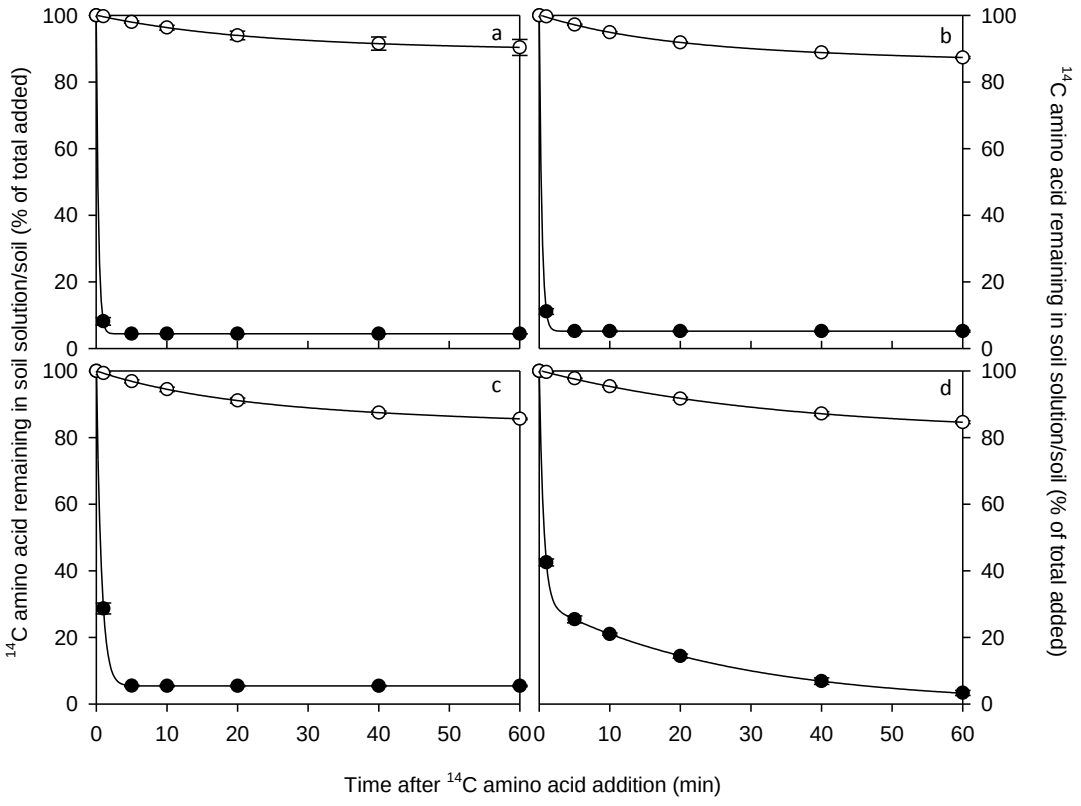

Figure 6. Relative amount of amino acid-derived ^{14}C in soil solution (solid circles), soil microbial community (open circles) and mineralised CO_2 (area above open circles) following the addition of a ^{14}C-labelled 1 µM (a), 10 µM (b), 100 µM (c) or 1000 µM (d) amino acid mixture to an agricultural Eutric Cambisol. Data points represent means ± SEM (n = 3) whilst

lines represent double or single exponential first order kinetic equations fitted to the experimental data

To describe the temporal dynamics of the observed microbial uptake, single first-order exponential decay curves (Eqn. 6; Fig. 6; $r^2 \geq 0.997$) were fitted to the experimental data for the 1, 10 and 100 µM treatments. For the 1000 µM treatment a double first-order exponential decay curve (Eqn. 7; Fig. 6; $r^2 \geq 0.995$) provided a better fit to the data. From these curves it was possible to calculate the half-times of amino acid-C in the soil solution (pool a_1; Eqn. 6 & 7; Table 2) for each concentration. The half-times of the a_1 amino acid pool were less than 1 min for all of the concentrations, with half-times for the 1 and 10 µM treatments significantly shorter than for the 100 and 1000 µM concentrations ($P < 0.001$). Although there was no significant difference in the 100 and 1000 µM pool a_1 half-times, it should be noted that a double first-order exponential decay curve was fitted to the 1000 µM and a large proportion of the added amino acid was allocated from the a_1 to the a_2 pool (35.1 ± 1.7 %), with a corresponding half-time of 18.9 ± 1.7 min.

Table 2. Half-times ($t_{½}$) for soil microbial uptake and mineralisation of a ^{14}C-labelled amino acid mixture added to an agricultural Eutric Cambisol. Half-times were calculated from the single or double exponential equations fitted to the experimental data. Values represent means ± SEM (n = 3). Superscript letters indicate values which are statistically similar (P<0.05).

Concentration of amino acid (µM)	Microbial uptake a_1 $t_{½}$ (min)	Mineralisation a_3 $t_{½}$ (min)
1	0.20 ± 0.02[a]	12.50 ± 1.46[a]
10	0.25 ± 0.01[a]	10.39 ± 1.00[a]
100	0.50 ± 0.02[b]	14.54 ± 2.91[a]
1000	0.40 ± 0.02[b]	16.91 ± 3.31[a]

Figure 6 shows that the mineralisation of the amino acid-derived C occurred at a much slower rate than the microbial uptake of amino acids from solution. The percentages of added amino acid that was mineralised in the first minute are shown in Table 3 was similar for all the concentrations and ranged between 0.3 – 0.8 %, with the 1000 µM addition having the lowest value of 0.30 ± 0.01 %. In absolute terms, the 1 µM addition, had the lowest rate of mineralisation of 6.0 ± 0.5 nmol C kg^{-1} DW soil min^{-1}. This value increased by 3 orders of magnitude to 3.5 µmol C kg^{-1} DW soil min^{-1} for the 1000 µM addition. After 60 min, 10.8 ± 1.2 % of the 1 µM addition had been mineralised compared to 15.5 ± 0.4 % in the 1000 µM treatment. As mineralisation of LMW C has been shown to be biphasic (Boddy et al., 2007),

double first-order exponential decay curves (Eqn. 9) were fitted to the experimental data to describe the temporal dynamics of the observed mineralisation (Hill et al., 2008; Wilkinson et al., 2014). These provided a good fit ($r^2 > 0.994$) to the data for all the concentrations, with the exception of the 1000 µM addition where a single first-order exponential decay curve (eq. 6) gave a better fit. The pool sizes and coefficients for a_3 and b_3 , representing the initial rapid mineralisation phase for the single were identical to the double first-order exponential decay curves, but the a_4 pool, representing the secondary slower phase, was attributed to the y_0 asymptote instead. This is likely to be an experimental artefact derived from the short time period (≤60 min) of the mineralisation study. Due to the uncertainty over connectivity between respiratory substrate pools (Boddy et al., 2007) we show only the half-times for the rapid initial phase of amino acid mineralisation (a_3 $t_{1/2}$, table 2). These ranged from 10.4 ± 1.0 to 16.9 ± 3.3 min for the four concentration treatments and were found to be statistically similar.

Table 3. Rates of C and N microbial uptake and subsequent C mineralisation of an added ^{14}C-labelled amino acid mixture for the first minute after addition to an agricultural Eutric Cambisol. Values represent means ± SEM (n = 3).

Initial amino acid concentration in solution (µM)	Rate of amino acid C uptake (µmol C kg^{-1} DW soil min^{-1})	Rate of amino acid N uptake (µmol N kg^{-1} DW soil min^{-1})	Rate of amino acid C mineralisation (µmol C kg^{-1} DW soil min^{-1})	Percentage of C taken up that is subsequently mineralised
1	1.29 ± 0.01	0.38 ± 0.002	0.006 ± 0.001	0.46 ± 0.04
10	12.41 ± 0.15	3.62 ± 0.04	0.05 ± 0.01	0.38 ± 0.04
100	101.24 ± 1.7	29.5 ± 0.5	1.01 ± 0.5	1.01 ± 0.51
1000	777 ± 39	225 ± 11	3.53 ± 0.1	0.46 ± 0.04

4. Discussion

4.1. N dynamics in the rhizosphere

This study investigated N dynamics in the rhizosphere using a multi methodological approach. The spatial dependence of amino acid root efflux was determined by direct sampling from the rhizosphere of intact sterile *Zea mays* L. seedlings. The likely fate of these exudates in soil was assessed using an exogenously applied ^{14}C-labelled amino acid mixture, which allowed determination of microbial uptake and mineralization rates. In order to look at the net effect of rhizospheric N process within an intact soil-plant-microbial system, microdialysis probes were used for in-situ quantification of N dynamics.

The spatial dependence of amino acid root efflux was clearly demonstrated, with a significant spike in rhizospheric amino acid concentration occurring in a 1-2 cm zone behind

the root tip. Similarly, a study performed on *Pissum sativa*, found the main site of amino acid exudation to be the root tip zone (van Egeraat, 1975). These results reflect the concentration in the rhizospheric boundary layer which would have been in dynamic equilibrium with the external nutrient solution. Several studies have demonstrated the ability of roots to recapture amino acids and other LMW carbon exudates from the rhizosphere (Jones and Darrah, 1994; Jones and Darrah, 1995), so the concentrations measured in this study represent the net efflux of amino acids. Uptake of amino acids by roots, has previously demonstrated to be non-spatially dependent (Jones and Darrah, 1994). Therefore, the spike in amino acid concentration shown in this study is most likely to be due to spatial differences in gross efflux rates. It is not possible though, to use these results to estimate rates of net or gross efflux and it is unlikely that these concentrations reflect actual soil concentrations. However, they do demonstrate that the input of N into the rhizosphere, via root exudation of amino acids, is spatially dependent, with the largest flux occurring immediately behind the root tip. As such, it may be expected that this peak in amino acid exudation would be apparent in the microdialysis sampling at the time the root tip grew past the probe. However, this was not the case, which suggests that (1) no exudation of amino acids occurred, (2) the exuded amino acids could not diffuse through the soil to the microdialysis probe, or (3) microbial uptake was extremely rapid. The linear diffusion distance (L) of amino acids in this soil can be estimated using the following equation (Jones et al., 2005):

$$L = (2D_e t)^{1/2} \qquad \text{(Eqn. 10)}$$

Where D_e is the effective diffusion coefficient, which for this soil can be assumed to be 0.01 $cm^2\ d^{-1}$ (Jones et al., 2005). As such, the distance over which amino acids from root exudates could diffuse in any 4 h sampling period is 2.5 mm, which would be more than sufficient to reach the surface of the microdialysis probe. As significant exudation of amino acids from the root tip zone has been demonstrated in this study this leaves rapid microbial uptake as the likely reason that no amino acid spike was observed in the microdialysis sampling. The likely fate of these amino acid exudates is depicted in Figure 5, which describes rates of microbial uptake from the soil solution and subsequent mineralization of exogenous amino acids. The extremely rapid uptake of amino acids reflects the C limitation of the microbial community that would exist prior to a root growing into a volume of soil. The rate of microbial C and N uptake of the applied amino acids over the first minute, and the half-times of the amino acids in the soil solution and C mineralisation, are similar to that of studies investigating LMW C dynamics conducted on the same soil (Hill et al., 2008; Wilkinson et al., 2014). Likewise, the decoupling

phenomenon between microbial uptake and C mineralisation of amino acids, as demonstrated previously for glucose, amino acids and peptides (Hill et al., 2008; Wilkinson et al., 2014), is evidenced here. The microdialysis sampling results are supported by a similar experiment conducted by Oburger et al. (2013), who used micro soil solution suction cups to sample amino acids and organic acids at increasing distances from the root surface. They found no significant gradient in either amino acid or organic acid concentration and no difference between rhizosphere and bulk soil, although there was a tendency for the concentration to decrease closer to the root surface, which was attributed to increased microbial activity. Re-uptake of amino acids and other LMW C exudates by roots has been demonstrated but it is likely that the soil microbes are much better competitors for DON and DOC than plants (Jones et al., 2013).

The soil solution concentration of both NH_4^+ and NO_3^- appears to be affected by root growth, with decreases observed around the estimated time that the root tip would have reached the microdialysis probe. Whilst it is difficult to definitively attribute the passage of the root to the decrease in the NH_4^+ pool due to "noise", the picture for NO_3^- is much clearer. The observed decrease could be due to uptake by the rapidly growing microbial community, plant uptake or a combination of these. It has been previously demonstrated that microbial uptake of NO_3^- from this soil is very low (Abaas et al., 2012), which would suggest that the observed decrease in soil solution NO_3^- concentration in our results is due to plant uptake. This is further supported by estimating the potential net NO_3^- influx into the maize root using the following equation (Barber, 1984):

$$I_{NO3-} = I_{max}(C_1 - C_{min})/(K_m + C_1 + C_{min}) \qquad \text{(Eqn. 11)}$$

Where I_{NO3-} is the net influx of NO_3^- into the root in nmol m^{-2} s^{-1}, I_{max} is the maximum influx rate (10), K_m is the Michaelis constant (10), C_1 is the initial NO_3^- concentration of the soil solution (147400 nM) and C_{min} is the minimum NO_3^- concentration at which net influx occurs (4 nM) (values taken from Barber, 1984). For the time period between 32 and 44 h, which corresponds to the passage of the root tip past the probe and a rapid reduction in the soil solution NO_3^- concentration, we estimate that a 1 cm root section could potentially uptake 13.5 nmols of NO_3^-. Given that the soil solution in the surrounding 1 cm section of soil (inside the 4 mm inner tube) would contain approximately 3.6 nmols of NO_3^-, the reason for the rapid depletion is clearly apparent. In the unplanted treatment, the NO_3^- concentration increased from 301 to 375 µM N over a 64 h period. A previous study on this soil reported nitrification rates in the soil solution of 4.1 µmol l^{-1} h^{-1} (Jones et al., 200), which is similar to the observed rate of 1.2 µmol l^{-1} h^{-1} in this study.

Microbial uptake of NH_4^+ is more likely to occur and rapid, and short term, immobilisation of NH_4^+ inputs into rhizosphere soil has been demonstrated using ^{15}N labelling combined with nano-scale secondary ion mass spectrometry (nanoSIMS) (Jones et al., 2013).

It has been suggested that N taken up by the rapidly growing microbial community during the initial period of LMW C availability will be remineralised when the supply of LMW C is exhausted or by micro-faunal grazing and subsequent excretion (Kuzyakov and Xu, 2013). The rate of mineralisation of the exuded amino acid-N cannot be determined from this study. Given the intrinsically high soil N concentrations, conventional thinking on C:N ratios would suggest that the microbial community would not have been N limited and much of the amino acid N would have been swiftly mineralised and released as NH_4^+, as evidenced by Jones et al. (2013). Despite this, accurate determination of microbial nutritional status is particularly difficult to infer at high spatial and temporal resolution, and growth may be limited by the availability of other nutrients, such as phosphorus as well as C or N (although this unlikely in this soil given the relatively high P concentration – see Table 1). Furthermore, it has also been suggested that the release of NH_4^+, following microbial uptake of amino acid, may depend more on the position of the amino acids along microbial biosynthetic pathways and the metabolic demand for individual amino acids rather than the C:N ratio of the substrate itself (Roberts et al., 2009). The microdialysis samples showed no recovery in NH_4^+ concentration but there was a small, non-significant increase in NO_3^- at the end of the experimental period.

4.2. Assessment of the microdialysis approach

Microdialysis sampling is an emerging technique for the in-situ assessment of soil N status and this study is the first to utilize this technique for rhizosphere research. It is therefore, important to consider the performance of the microdialysis probes used in this study and discuss how their unique method of diffusion-based sampling of soil solutes may be best used in future studies. The performance of the microdialysis probes was assessed by comparing the RR of amino acids, NH_4^+ and NO_3^-, before and after the in-situ monitoring period. Any change in the RR would suggest that the probe had been damaged or diffusion across the membrane affected by soil biotic or abiotic factors. The RR for all 3 N forms decreased slightly after the in-situ monitoring period, although the decrease was only significant for NH_4^+ ($p < .001$). If the probes had been damaged at all it would have affected the RR of all the N forms. There was a decreasing trend in the NH_4^+ concentration in the planted treatment over the 68 h experimental period. This could be explained by the observed reduction in RR, however this is unlikely to be the case as the unplanted treatment showed no such trend, which suggests that the

performance of the probes in-situ was maintained throughout the experiment. One possible explanation is that, despite cleaning and running the probes in DI H_2O after removal from the soil and prior to re-calibration, small soil particles remained on the membrane to which NH_4^+ could be adsorbed.

Inferring in-situ concentrations of N solutes in the soil solution using microdialysis has been the subject of some debate due to the obvious physical and chemical differences between the soil and the standard calibrating solution (Menacherry et al., 1992; Inselsbacher et al., 2011; Shaw et al., 2014). Previous work comparing microdialysis and lysimeter derived estimates of soil solution concentrations in an agricultural and forest soil found large differences in concentrations of NH_4^+ and NO_3^- and amino acids (Inselsbacher et al., 2011). However, in this study, the microdialysis estimations of soil solution N concentrations were statistically similar to those estimated by centrifugal drainage for amino acid-N and NO_3^-, but were significantly lower for NH_4^+, although this was only assessed for the first time point in the experiment. It was noticeable that there was large variation between replicates, especially for NO_3^- in the controls. Due to the small size of the membrane, the results may be affected by micro-heterogeneity of the soil's physical and chemical state. Such micro-heterogeneity in the availability of amino acids and NH_4^+ was found to occur in microdialysis samples from both a forest and agricultural soil despite sieving and mixing (Inselsbacher et al., 2011). This was not the case for NO_3^- in the same study, which was likely due to the ability of NO_3^- to diffuse freely through the soil, although the concentrations were much lower than for this study. The soil used in this study has particularly high turnover rates of amino acids (Wilkinson et al., 2014) and nitrification (Jones et al., 2004), so in root free soil, NO_3^- may rapidly accumulate in hotspots where there is an available supply of LMW DON (Kuzyakov and Blagodatskaya, 2015), which may explain the variability between the replicates in the unplanted treatment. Where roots are present, these micro-accumulations may not occur due to the rapid uptake of available NO_3^- by the roots, resulting in the lower heterogeneity observed in the planted treatment.

Continuous in-situ microdialysis sampling may enable estimation of rhizosphere N dynamics at high temporal and spatial resolution. However, the results give only a net picture of the many interacting processes occurring in the rhizosphere. Determination of the size and importance of individual fluxes is not possible with the methodology used in this study. In the future, combining the microdialysis approach with isotopic labelling techniques may allow continuous in-situ monitoring of specific C and N fluxes. For example, labelling of root

exudates by continuous exposure of plants to $^{14/13}C$ and/or a ^{15}N nutrient source may enable better tracking of the fate of plant derived C in the rhizosphere (Jones et al., 2009). Similarly, labelling of SOM may allow the importance of the RPE in soils with different nutrient status to be investigated (Murphy et al., 2015). Data generated by these approaches may also be used to parameterise and validate models of rhizosphere N fluxes (Darrah and Roose, 2001).

5. Conclusions

The complex nature of microbial C and N fluxes in the rhizosphere may control the amount and form of N available for plant uptake. This study in an agricultural soil clearly demonstrates that amino acids released in root exudates will be rapidly removed from the soil solution via microbial uptake. As such, it is likely that the importance of organic N for plant nutrition in this soil is limited, particularly given the size of the soil NO_3^- pool. Continuous in-situ microdialysis sampling from the intact rhizosphere of growing *Zea mays* L. allowed a unique insight into soil solution N dynamics at a fine temporal and spatial resolution. Depletion of soil solution NH_4^+ and NO_3^- was observed as the root grew past the microdialysis probe and for NO_3^- this was attributed to plant uptake. Microdialysis is likely to continue to offer new methodological approaches to enable the disentangling of complex rhizospheric processes, especially if combined with isotopic labelling techniques and mathematical modelling.

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Chapter 5

Article III

Assessing the potential for ion-selective electrodes and dual-wavelength UV spectroscopy as a rapid on-farm measurement of soil nitrate concentration

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Published: 2013. Agriculture, 3: 327 - 341

Author contributions:

RS and DLJ conceived and designed the experiment. All the experimental work, data processing and statistical analysis was performed by RS. RS wrote the first draft of the manuscript with all authors contributing to the final paper.

OPEN ACCESS
agriculture
ISSN 2077-0472
www.mdpi.com/journal/agriculture

Article

Assessing the Potential for Ion Selective Electrodes and Dual Wavelength UV Spectroscopy as a Rapid on-Farm Measurement of Soil Nitrate Concentration

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Received: 16 May 2013; in revised form: 21 June 2013 / Accepted: 24 June 2013 / Published: 2 July 2013

Abstract: Current fertiliser recommendations for nitrogen are limited in their accuracy and may be improved by the use of simple on-farm soil rapid tests. This paper investigates the potential for using nitrate (NO_3^-) ion selective electrodes (ISEs) and dual wavelength UV spectroscopy as part of a rapid soil NO_3^- diagnostic test. Three soil types, representing the major soil types for agriculture in the western UK, were tested. For the three soils, the ISE rapid test procedure gave a near 1:1 response (r^2 = 0.978, 0.968, 0.989) compared to the internationally-approved standard laboratory method. However, the accuracy of the ISE rapid test was reduced at low soil NO_3^- concentrations (<10 mg NO_3^- L^{-1}). We also show that NO_3^- analysis of H_2O soil extracts by dual wavelength UV spectroscopy was also highly correlated (r^2 = 0.978, 0.983, 0.991) to the standard laboratory method. We conclude that both ISE and dual wavelength UV spectroscopy have clear potential to be used for the rapid on-farm determination of soil NO_3^- concentration. Barriers to use of these field-based assessment tools include, farmer perception of cost-benefit, general attitude to new technologies and the ability to generate useful fertiliser use strategies from soil NO_3^- measurements.

Keywords: crop nutrients; fertiliser management; nitrogen use efficiency; soil analysis

1. Introduction

Improving nitrogen use efficiency (NUE) is a major goal within agricultural systems [1] and is key to the success of sustainable intensification [2]. Use of nitrogen (N) fertilisers represents the major N input in most farming systems and both under- and over-use of N fertilisers can represent an economic loss for the farmer, while over-use may cause significant environmental pollution [3–6]. In a purely economic sense, an optimum N fertiliser strategy can be defined as the point at which the cost of an additional unit of N is no longer covered by the resulting increase in crop yield. Defining an environmental optimum rate of N addition, however, is much more problematic. Calculating an optimum N fertilisation strategy is extremely desirable, but very difficult to achieve due to the inherent complexity of the soil-plant system, temporal and spatial variability and the importance of uncontrolled variables such as weather [5]. One theoretical method for improving NUE is to ensure synchronicity of supply and demand, both spatially and temporally, by maintaining the pool of plant available N in the soil at the minimum size required to meet crop demand [7,8]. NO_3^- is typically the most important crop-available form of N in most temperate climate, near neutral pH soils, although in some grassland soils ammonium (NH_4^+) may dominate. The mobility of NO_3^- within soil makes it easy for plants to uptake, but this property also makes it prone to being leached from the soil profile when field capacity is reached, with resulting water pollution issues. Regular testing of soil NO_3^- concentration, over the course of the growing season, may help farmers improve their nutrient management strategy by better matching supply and demand.

Current methods of calculating the N requirement of the crop over the growing season, and hence fertiliser N additions, require a prediction of the crop yield, based on soil type, climatic zone, topography and other variables. The amount of crop-available N that can be supplied by the soil in its pre-fertilised state over the growing season is then measured or estimated; this is known as the soil nitrogen supply (SNS). The difference between crop requirement and the SNS can then be made up for by addition of N fertiliser [5]. A variation of this method is widely used in the UK and is prescribed by The Fertiliser Manual RB209 [9]. A key component of the SNS is the concentration of soil mineral nitrogen (SMN), which consists of NH_4^+ and NO_3^-. RB209 provides tables to allow estimation of a field's SNS depending upon its soil type and previous management. This estimation can also be supplemented by measuring the SMN of the pre-fertilised soil.

Traditionally, laboratory analysis has been used for soil testing. However, it is expensive and time-consuming and therefore not suitable as a method of regular and frequent testing. There are also other problems with laboratory analysis. Unwanted mineralisation and nitrification/denitrification of the samples may occur during transport and storage prior to analysis. Significant changes to the intrinsic soil NO_3^- status may also occur, due to changes in the prevailing weather (e.g., during the delay between sampling and receiving the results). Farmer-operated rapid diagnostic testing potentially offer a cheap and instantaneous determination of soil NO_3^- status where the results can be used to directly inform nutrient management strategies, benefiting both the farmer and the environment. Previous work on rapid soil tests have largely been based on colorimetric strips combined with a handheld reflectometer [10–13] and ion selective electrodes (ISEs) [12,14], which have been described as semi-quantitative [15]. New, more quantitative methods are therefore required.

This study aimed to evaluate two contrasting rapid test methods for evaluating soil NO_3^- concentration. Firstly, we compared a rapid extraction method, which could be used in-field for the extraction of soil NO_3^-, coupled with NO_3^- determination using an ISE, to the standard laboratory determination of soil NO_3^-. Secondly, we evaluated the use of UV spectroscopy for NO_3^- determination in soil extracts in comparison to the ISE approach. The results were used to evaluate the potential of the two approaches for the on-farm measurement of soil NO_3^- status.

2. Materials and Methods

2.1. Soil Type and Sampling

Three contrasting soils were collected from Henfaes Research Station, Abergwyngregyn, UK (53°14′ N, 4°01′ W). Soil 1 is a lowland, clay loam textured Eutric Cambisol collected from an area of no vegetation cover, which had been used for potato production the previous season. Soil 2 is a lowland, silty loam textured Dystric Gleysol collected from a poorly draining area of an intensively sheep grazed field (ca. >10 ewe ha^{-1}) receiving regular fertiliser inputs (120 kg N ha^{-1} yr^{-1}) and dominated by *Lolium perenne* L. Soil 3 is a sandy loam textured Haplic Podzol collected from an upland, extensively grazed (<0.1 ewe ha^{-1}) unimproved acid grassland (*Pteridium aquilinum* L. Kuhn. and *Festuca ovina* L.). Prior to sampling, the overlying vegetation cover was removed and the soil sampled from a depth of 3–15 cm. After collection, the soil was stored in gas permeable polyethylene bags for immediate transport to the laboratory. The soil was refrigerated at <5 °C until it was needed for the experimental procedure. Immediately prior to use, the soil was sieved to 8 mm to remove large stones, roots, vegetation and earthworms and then thoroughly mixed.

2.2. Background Soil Analysis

Soil pH and electrical conductivity were determined in a 1:2 (w:v) soil:water mix using standard electrodes. Moisture content was determined by drying for 24 h at 105 °C. Organic matter content was determined by loss-on-ignition at 450 °C for 12 h. Total C and N were determined with a CHN2000 analyser (Leco Corp., St Joseph, MI, USA). Results for background soil analysis can be found in Table 1.

Table 1. Background characteristics of the three soils used in the experiments.

Soil	pH	EC (µS cm^{-1})	Moisture content (g g^{-1})	Total C (%)	Total N (%)
Eutric Cambisol	6.53	59.4	0.25	3.5	0.29
Dystric Gleysol	6.53	59.4	0.28	1.1	0.10
Haplic Podzol	5.34	12.9	0.70	6.1	0.57

2.3. Ion Selective Electrode (ISE)

A commercially available NO_3^- ISE (ELIT 8021) with a solid state PVC polymer matrix membrane was used in conjunction with a double junction lithium acetate reference electrode (RE) (ELIT 003n), supplied by Electro Analytical Instruments (EAI) (Wembley, UK). The NO_3^- ISE is reported to have an operational concentration range from 0.3–6300 mg NO_3^- L^{-1}, a response time of <10 s, working pH

range of pH 2–11, operational temperature range from 0–50 °C and an electrode slope of 54 ± 5 mV decade^{-1} at 25 °C. The ISE and RE were coupled with a multi-channel analyser (6+6-Channel Ion/pH/ORP/Tmp.Monitor MCC-SYSti-6+6b) and corresponding PC software (MCC-MON-6+6c, Version 2.1.1) supplied by Electro Analytical Instruments (EAI) (Wembley, UK). Prior to initial use, the ISE was pre-conditioned in a 1000 mg L^{-1} NO_3^- solution for 4 h. The calibration is calculated and stored by the software using a semi-logarithmic interpolation method.

2.4. NO_3^- Determination Using Ion Selective Electrode Rapid Test Method

Before each set of measurements, the ISE was calibrated using a range of NO_3^- solutions (1000, 100, 10, 1, 0.5 mg NO_3^- L^{-1}). The temperature of the calibrating solutions differed from the experimental measurements by a maximum of ±2 °C. 10 g soil (n = 3 for each soil type) was placed in a 50 cm^3 polypropylene tube and spiked with 1 mL of NO_3^- solution (2000, 1800, 1600, 1400, 1200, 1000, 800, 600, 400, 200, 100 or 0 mg L^{-1} (in addition the Eutric Cambisol was spiked with 20 and 10 mg L^{-1})) to achieve a range of intrinsic NO_3^- concentrations reflective of those that might occur in the field. Extraction was then performed by the addition of 20 mL of double distilled (DD) H_2O followed immediately by manual shaking by hand for 2 min. This extraction procedure is referred to as the rapid extraction method. The ISE was placed into the resulting soil slurry and a reading taken after 3 min. Between each measurement, the electrodes were rinsed with DD H_2O and dried with paper tissue. The soil slurry was subsequently centrifuged (20 min at 4000 rev min^{-1} followed by 20 min at 14,000 rev min^{-1}) and the supernatant decanted for NO_3^- analysis by the colorimetric Griess reaction method of Miranda *et al.* [16]—referred to as the standard laboratory method. This analysis reflects internationally accepted protocols for *ex situ* soil nitrate analysis [17].

It should be noted that ISEs respond not to the concentration but the activity of the specified ion. The activity of the ion depends upon both the concentration of the ion and the total ionic strength of the solution. There is little difference in concentration and activity at low ionic solution strength *i.e.* below 1 mM. However, above this they diverge leading to the potential for systematic error. In previous experiments using ISEs for determination of soil NO_3^- concentration, ionic strength adjustment buffers (ISAB) have been used to keep the ionic strength of the calibrating solutions equal and approximately matched to samples being tested [18]. This was not possible in this experiment as adding NO_3^- to the soil in varying amounts intrinsically meant that the ionic strength of the soil solution would be different between different treatments. The extracted solutions of soils amended with 1 mL of 2000 mg L^{-1} NO_3^-, would not exceed an ionic strength of 3 mM, which would result in a 6% difference between concentration and activity for monovalent ions. Whilst this has the potential to cause systematic error, this is partially offset by the fact that the ISEs were calibrated for concentration without ISAB. For the soils which were not spiked with NO_3^-, the ionic strengths of the measured extractions did not exceed 1 mM (equivalent to an electrical conductivity of 120 µs cm^{-1}), which would not cause a significant error.

2.5. Nitrate Extraction and Determination by the Standard Lab Method

NO_3^- was extracted from the soil using 1 M KCl or DD H_2O (10 g soil:20 mL) by mechanical shaking at 150 rev min^{-1} for 30 min. The resulting mixture was then centrifuged and analysed by the colorimetric Griess reaction method of Miranda *et al.* [16]. This is referred to as the standard lab method with KCl/H_2O extraction.

2.6. NO_3^- Determination by Dual Wavelength UV Spectroscopy

NO_3^- in the standard KCl and H_2O extracts were also analysed with dual wavelength UV spectroscopy at 205 nm and 300 nm using the method described in Edwards *et al.* [19]. NO_3^- absorbs strongly at 205 nm, however, dissolved organic matter (DOM) also absorbs strongly at this wavelength. To compensate for this, the DOM can also be measured at 300 nm, where no NO_3^- absorption occurs, and the relationship between the DOM absorbance at 205 and 300 nm can be incorporated into a traditional NO_3^- calibration curve to account for the DOM present as follows:

$$DOM_{205} = (2.841 \times DOM_{300}) - 0.0126 \quad (1)$$

where DOM_{205} = organic matter absorbance at 205 nm, DOM_{300} = organic matter absorbance at 300 nm. The DOM_{205} absorbance value is simply subtracted from the sample reading prior to calculating NO_3^- from the standard curve. This method was originally developed for testing natural waters and to our knowledge has not been used for NO_3^- determination of soil extracts.

2.7. Evaluation of the Methods across a Broad Range of Soils

A diverse range of different soils (n = 23) were sampled from within a 10 km^2 radius of the Henfaes Research Station. The samples were analysed using the ISE rapid test method (n = 23), the standard lab method with H_2O extraction (n = 23) and UV spectroscopy (n = 16) as described above. These soils were not spiked with NO_3^-. Background analysis of these soils can be found in Table 2.

Table 2. Background soil analysis for the broad range of soil sampled from within a 10 km^2 radius of the Henfaes Research Station. EC = Eutric Cambisol, DG = Dystric Gleysol, HP = Haplic Podzol.

Sample	Soil	pH	EC (µS cm^{-1})	Moisture content (g g^{-1})	Organic matter (%)
1	DG	6.04	19.3	0.33	5
2	EC	6.04	13.9	0.38	7
3	HP	5.85	6.4	0.51	10
4	HP	6.07	35.1	0.32	5
5	HP	4.85	43.2	0.52	18
6	HP	5.65	16.5	0.81	17
7	HP	5.89	84.6	0.36	9
8	EC	6.38	20.3	0.19	7
9	EC	6.19	13.8	0.17	5
10	EC	6.65	55.0	0.17	8

Table 2. *Cont.*

Sample	Soil	pH	EC (μS cm^{-1})	Moisture content (g g^{-1})	Organic matter (%)
11	EC	7.14	34.3	0.18	6
12	EC	6.48	43.5	0.29	7
13	EC	6.51	47.8	0.39	12
14	EC	6.61	18.1	0.25	4
15	EC	6.29	49.8	0.52	10
16	EC	6.23	45.7	0.30	8
17	DG	5.37	69.7	0.63	8
18	HP	4.53	32.6	0.37	5
19	EC	5.18	6.8	0.30	11
20	EC	6.28	45.7	0.24	6
21	EC	6.84	50.0	0.20	6
22	EC	5.39	38.6	0.39	5
23	EC	5.52	90.2	0.46	10

2.8. Statistical Analysis

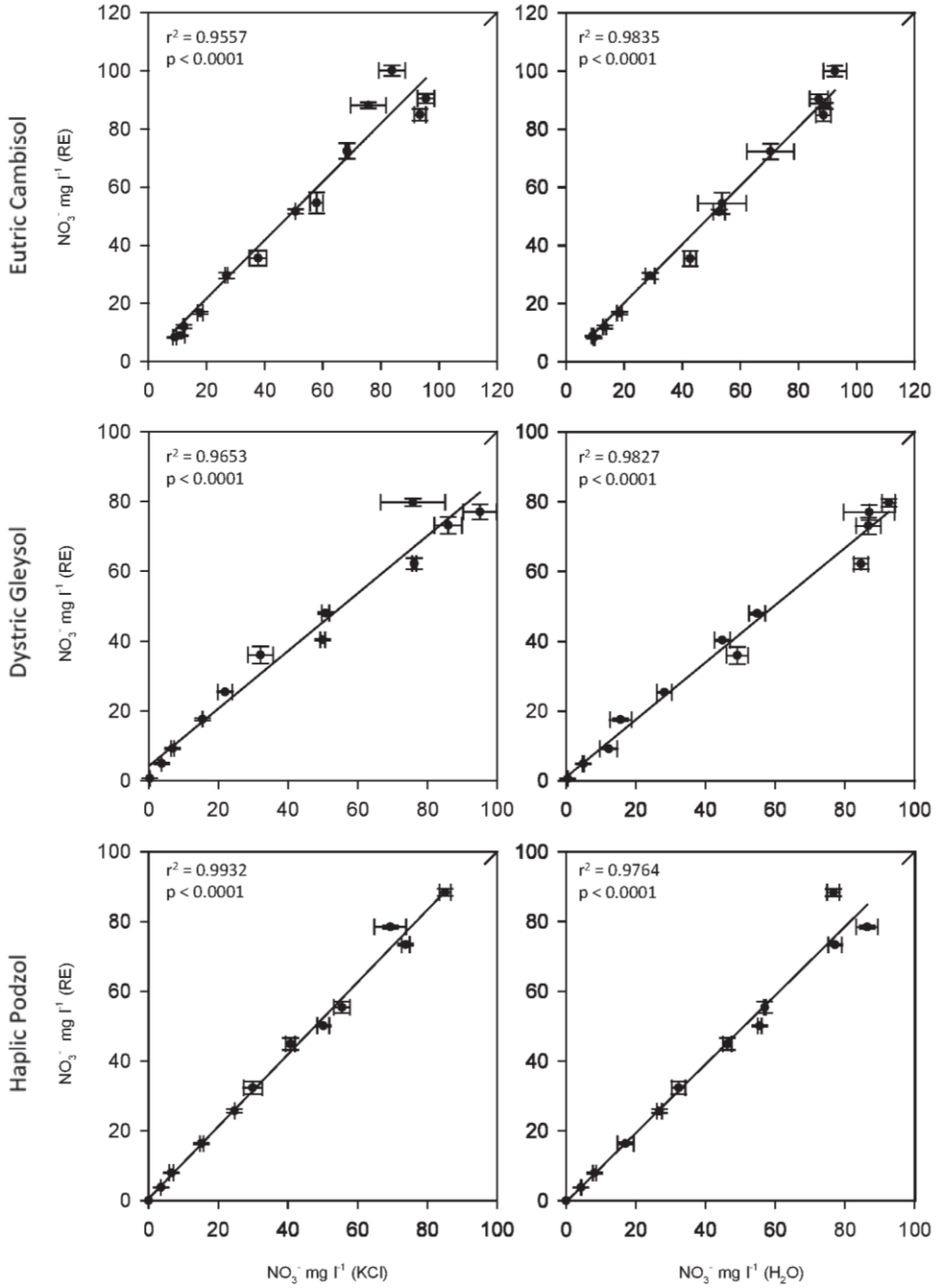
All concentrations given in this paper are reported in mg NO_3^- L^{-1}. Linear regression analysis was undertaken using SigmaPlot v12.3 (Systat Software Inc., Hounslow, UK) and paired *t*-tests were undertaken with SPSS v20 (IBM Ltd., Portsmouth, UK). $P < 0.05$ was used as the cut-off for statistical significance.

3. Results and Discussion

3.1. Efficiency of the Rapid Extraction Method

The efficiency of soil nitrate extraction from the three soil types using the rapid extraction method was tested by comparing it to standard soil extractions with 1 M KCl or H_2O. The standard lab method was used for the subsequent NO_3^- determination of all the extracts. Figure 1 shows an excellent correlation and a near 1:1 relationship between the rapid extraction method and the KCl or H_2O standard extraction method for the Eutric Cambisol and the Haplic Podzol, with no significant differences observed. In addition, there were no significant differences observed in NO_3^- extraction between the H_2O standard extraction and the 1 M KCl standard extraction for all three soil types, which shows that NO_3^- extraction using H_2O is acceptable. However, the efficiency of the rapid extraction method on the Dystric Gleysol was lower and was shown to be significantly different from the KCl standard extraction. The structure of the Dystric Gleysol was very poor so it is likely that shaking by hand for 2 min was not enough to allow complete dispersal of the soil particles. A similar problem may occur on heavy clay soils [15]. This may be rectified by increasing the shaking time of the extraction.

Figure 1. Comparison of the rapid extraction procedure (RE) with the standard 1 M KCl and H_2O extracts. All extracts analysed for NO_3^- using the standard lab method. The dotted line represents the theoretical 1:1 line for the two methods whilst the solid line represents the linear regression line describing the actual relationship between the two methods. Values represent means ± SEM (n = 3). The r^2 and p value from the regression analysis are shown for each graph.



3.2. Comparison of the ISE Rapid Test with the Standard Lab Method

The ISE rapid test method was compared to the standard lab method with KCl extraction. Figure 2 shows a good correlation between the ISE rapid test and the standard lab method with KCl extraction for the determination of soil NO_3^- in all three soil types. However, significant differences were found between the two methods when applied to the Eutric Cambisol and Dystric Gleysol. Analysis of the rapid extraction extracts with the standard lab method for these soils showed no significant differences when compared to the ISE rapid test method. This suggests that the significant difference between the ISE rapid test method and the standard lab method with KCl extraction was due to either differences in extraction efficiency or natural soil variation, but not the performance of the ISE. We have already shown above that the efficiency of the rapid extraction method on the Dystric Gleysol is lower than for the other soil types, which would explain the reduced accuracy of the ISE rapid test for this soil.

Figure 3 shows the results of the ISE rapid test compared to the standard lab method with H_2O extraction for the determination of NO_3^- on a range of soils, which were not spiked with NO_3^-. At these lower NO_3^- concentrations, the ISE rapid test tends to underestimate the NO_3^- concentration, the correlation between the two methods was not quite as strong and there was a significant difference between them. In particular, below 10 mg L^{-1} there is a poor response of the ISE to changing concentration. The efficiency of the rapid extraction method was not ascertained for the soils used here and the low values of NO_3^- would exacerbate any reduction in extraction efficiency, although this is only likely to be an issue for the two Dystric Gleysols. In addition, the response of the ISE below 10 mg L^{-1} was non-linear and so as NO_3^- concentration decreased below this the resolution of the ISE was reduced. The accuracy in the non-linear phase may also be reduced by the calibration method. The software used calculates the calibration from the standards using a semi-logarithmic interpolation method. Essentially, this works by joining the calibration points with a straight line, which has obvious implications for a non-linear curve. Accuracy in the non-linear phase may therefore be increased by using more calibration points. Alternatively, a curve could be fitted to the calibration data using a simplified version of the Nicolsky–Eisenman equation [20].

Figure 2. Comparison of the ion selective electrode (ISE) rapid test (ISE_{RE}) with the standard lab method—extractions in KCl (SLM_{KCl}) and rapid extraction procedure (SLM_{RE})—for NO_3^- determination in three soils amended with increasing amounts of NO_3^-. The dotted line represents the theoretical 1:1 line for the two methods whilst the solid line represents the linear regression line describing the actual relationship between the two methods. Values represent means ± SEM (n = 3). The r^2 and p value from the regression analysis are shown for each graph.

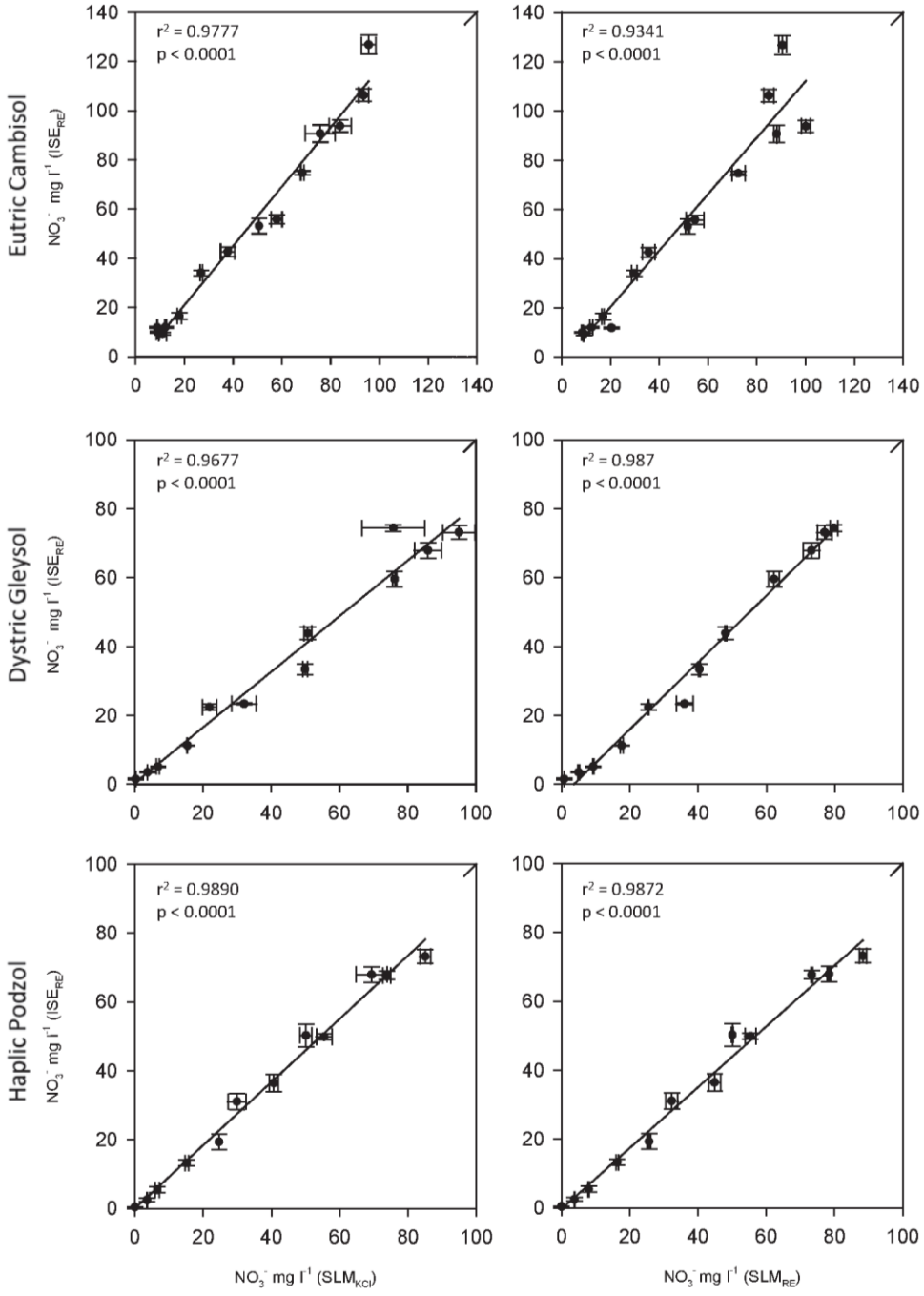
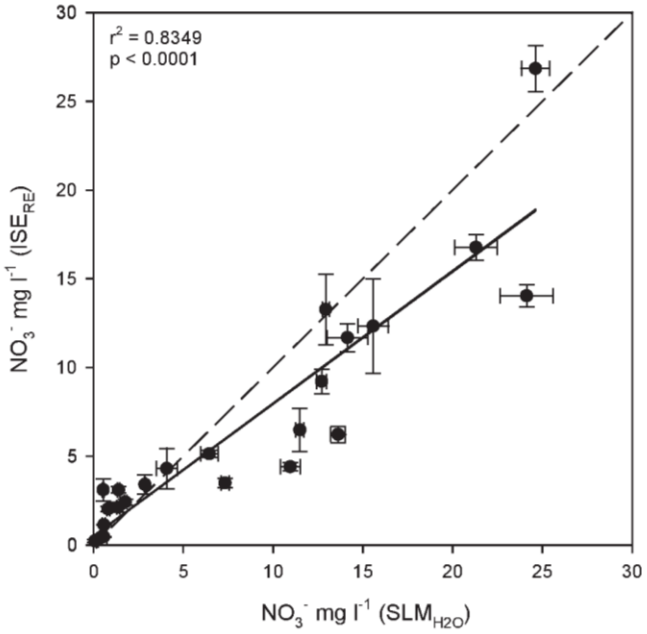


Figure 3. Comparison of the ISE rapid test with the standard lab method—extractions in H_2O (SLM_{H2O})—for NO_3^- determination across a broad range of agricultural soils (n = 23). The dotted line represents the theoretical 1:1 line for the two methods whilst the solid line represents the linear regression line describing the actual relationship between the two methods. Values represent means ± SEM (n = 3). The r^2 and p value from the regression analysis are shown.



3.3. Comparison of UV Spectroscopy with the Standard Lab Method

Figures 4 and 5 show an excellent correlation and a near 1:1 response between the standard lab method and UV spectroscopy method for the determination of NO_3^- in H_2O and KCl soil extracts. The response of UV spectroscopy to pure solutions of NO_3^- was linear from 0.05–12.5 mg L^{-1} (compared to 0.2–50 mg L^{-1} with the standard lab method). Consequently, most of the extracts required a 1:10 (v/v) dilution prior to NO_3^- determination. No significant differences were found between the methods using H_2O extraction for all three soil types and for KCl extraction with the Haplic Podzol. However, significant differences were found between the standard lab method and the UV spectroscopy for KCl extractions from the Eutric Cambisol and Dystric Gleysol. A closer look at Figure 4 shows that it is only the three lowest concentrations that appear to deviate significantly from the 1:1 regression line. These were the only samples, extracted in 1 M KCl which were not diluted prior to UV analysis, which suggests that the error is due to interference from the 1 M KCl. Edwards *et al.* [19], found no interference from saline constituents although they did not use solutions as strong as 1 M. Figure 5 shows that unlike the ISE rapid test there was no loss of accuracy at low concentrations. However, here only the analytical methods are being compared and both methods are using the same extracts, whereas with the ISE rapid test different extractions are used leading to variation in both the extraction efficiency and natural soil variation.

Figure 4. Comparison of UV spectroscopy—extractions in KCl (UV_{KCl}) and H_2O (UV_{H2O})—with the standard lab method—extractions in KCl (SLM_{KCl}) and H_2O (SLM_{H2O})—for NO_3^- determination in three soils amended with increasing amounts of NO_3^-. The dotted line represents the theoretical 1:1 line for the two methods whilst the solid line represents the linear regression line describing the actual relationship between the two methods. Values represent means ± SEM (n = 3). The r^2 and p value from the regression analysis is shown for each graph.

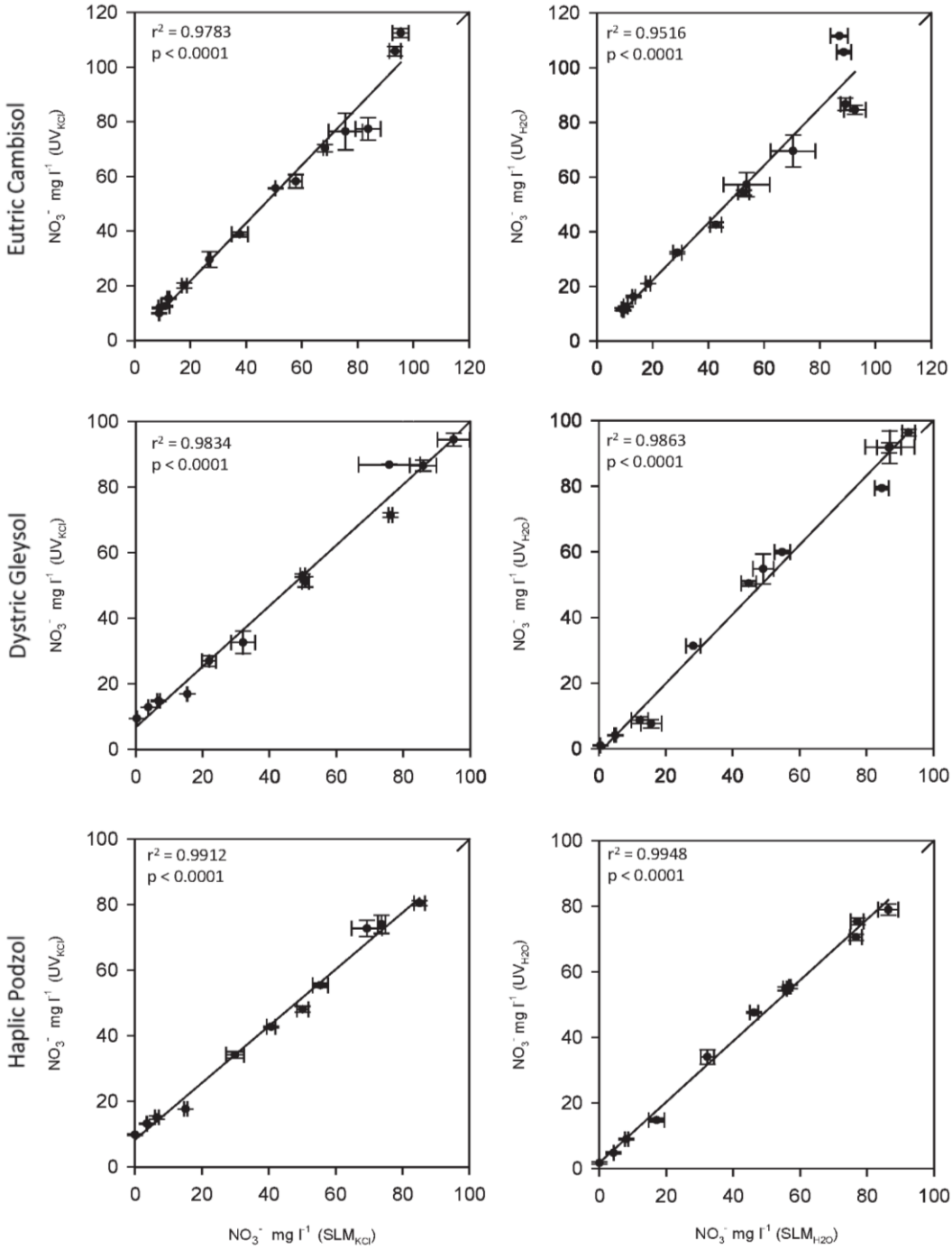
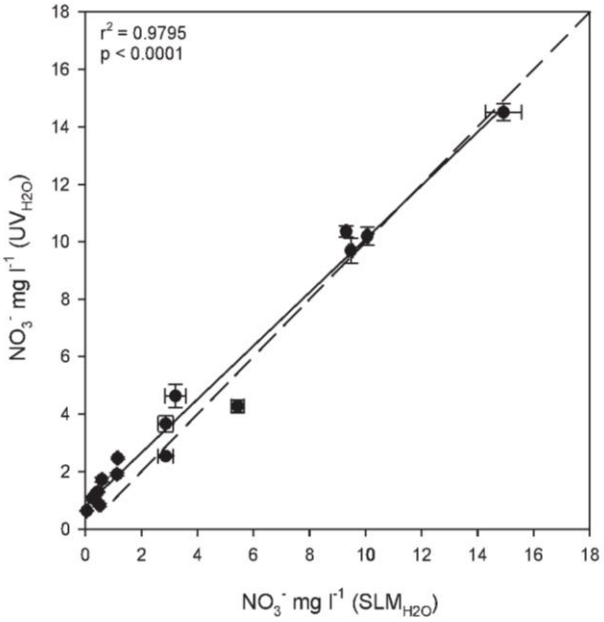


Figure 5. Comparison of UV spectroscopy—extractions in H_2O (UV_{H2O})—with the standard lab method—extractions in H_2O (SLM_{H2O})—for NO_3^- determination across a broad range of agricultural soils (n = 16). The dotted line represents the theoretical 1:1 line for the two methods whilst the solid line represents the linear regression line describing the actual relationship between the two methods. Values represent means ± SEM (n = 3). The r^2 and p value from the regression analysis is shown.



3.4. Evaluation of ISEs for Soil Nitrate Determination

The ISE rapid test procedure was conducted on three contrasting soil types (Eutric Cambisol, Dystric Gleysol and Haplic Podzol), which together represent the major agricultural soil types in the UK. In comparison to the internationally recognised standard laboratory method, the results clearly showed that current ISE technology combined with two min manual H_2O soil extractions has the potential to be used by farmers as an on-farm rapid-diagnostic test. However, the accuracy of the rapid test procedure decreased when testing a Dystric Gleysol and at low NO_3^- concentrations (*i.e.*, below 10 mg L^{-1}). There was also a problem with the electrode durability. During this experiment, three sets of ISEs were used. The second set was discarded when it began to show a large erratic response and a subsequent failure to stabilise. This type of malfunction is likely to be due to a failure of the electronics and can easily be spotted. The first and third set suffered from a loss of sensitivity at the lowest concentrations and the third set showed physical degradation manifest by a bulging membrane. The electrodes were discarded when they could not be calibrated correctly at the concentrations that were being determined. However, a subtle loss of sensitivity at lower concentrations or changes in the calibration parameters may not be spotted by a layman and could cause significant error if the calibration was not adjusted. A more rugged sensor housing design may improve this lack of durability alongside changes in the sensor chemistry

(e.g., inclusion of protective membranes). In addition, electrodes can also be constructed incorporating the latest improvements in ion sensor membrane design [21].

3.5. Evaluation of UV Spectroscopy for Soil Nitrate Determination

The results show clearly that dual wavelength UV spectroscopy can be used to accurately determine the concentration of NO_3^- in extracted soil solution. When combined with the rapid extraction method it has the potential to be used as an on-farm rapid test providing a hand-held UV spectrometer is available and the extracts are filtered or centrifuged. Dual wavelength UV spectroscopy is able to determine the NO_3^- concentration between 0.35 and 17.7 mg L^{-1} [19]. This means that extractions from soils with high NO_3^- input may need to be diluted before measurement. This was the case in our study for the majority of the samples. The results suggest that the 1 M KCl extractant causes some interference to the measurement. Diluting KCl extractions 1:10 (v/v) appears to prevent the interference that occurs due to 1 M KCl. Extracting with H_2O also solves the problem of the 1 M KCl interference and the results show there is no difference in NO_3^- extraction using 1 M KCl or H_2O. In addition, distilled water can be readily purchased by most farmers in comparison to KCl solutions. The advantage of using an ISE over UV spectroscopy for an on-farm rapid test is that ISE's can be used in soil slurry so no filtration or centrifugation needs to be carried out. However, UV spectroscopy offers better resolution at very low concentrations due to the linear nature of its response and is likely to be more durable. In addition, field portable UV spectrometers are now readily available. This approach, however, is readily suited to the evaluation of nitrate in agricultural drainage waters.

3.6. Implications for Fertiliser Application Guidelines

Extraction of NO_3^- from the soil and its subsequent determination does not provide all the information required to produce an agronomic relevant result. For the results to be meaningful for agricultural extension purposes, they must be up-scaled to units of kg ha^{-1}. This requires determination of the bulk density and moisture content of the samples. Schmidhalter [15] developed a method of determining both parameters using a simple in-field method requiring only standard bulk density cylinders, a graduated measuring cylinder and a solar powered balance.

For farmers to implement rapid soil NO_3^- testing, they must be convinced of the benefit as the process requires both time and money. When soil is sampled and sent for laboratory testing it can be analysed for a range of macro- and micro- nutrients. In the UK, fertiliser additions, as prescribed by RB209, require a calculation or estimation of SMN, which includes both NH_4^+ and NO_3^-. This rapid test would only determine soil NO_3^- concentration, and although nitrogen is fundamental to plant growth, it is not always the limiting nutrient. NO_3^- differs from other nutrients in that its concentration varies greatly both spatially and temporally, which is the main reason that farmer-operated NO_3^- rapid tests performed through the growing season may improve fertiliser management. This spatial and temporal variation does however pose a challenge as to determining the optimum sampling regime. Further, farmers need to have relevant and simple decision support systems so that collected data are interpreted correctly and can be implemented into a meaningful fertiliser strategy. Along with improving the technology, further work is therefore needed so that rapid soil NO_3^- can be adopted by industry as a way to optimise nutrient use efficiency.

4. Conclusions

This work shows that ISEs can offer a reasonably accurate and rapid way of determining NO_3^- concentration in soil slurries. This can be combined with a rapid extraction procedure using H_2O where the soil is shaken by hand for 2 min. For poorly structured or heavier clay soils a longer shaking time may be required. There is the potential for ISEs to be used by farmers for an on-farm rapid test; however, practicality issues and methods for integrating the data into a management plan may reduce its uptake. UV spectroscopy offers a similarly rapid and reagentless method of NO_3^- determination. Compared to ISE, it offers a lower detection limit and a greater resolution at low concentration—below 10 mg L^{-1}—but samples with a concentration greater than 18 mg L^{-1} will need to be diluted for accurate determination. The technology is likely to be more durable and less prone to error than ISEs. However, the cost of the technology is likely to be greater and samples will require filtering or centrifuging prior to measurement. For rapid tests to be used by the industry, farmers must be convinced of the cost-benefits and have a suitable decision support mechanism in place to turn the measurements into a fertiliser application plan.

Acknowledgments

Funding for the project was provided by the English Beef and Lamb Executive (EBLEX), Hybu Cig Cymru (HCC), Quality Meat Scotland (QMS) and DairyCo.

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Chapter 6

Article IV

In-situ monitoring of soil nitrate in real time using ion-selective electrodes

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Unpublished manuscript

Author contributions:

Prototype ISEs were developed at the John Innes Centre and adapted for in-situ soil measurements by RS. The experiments were conceived and designed by RS and DLJ. All the experimental work, data processing and statistical analysis were performed by RS. RS wrote the first draft of the manuscript with all authors contributing to the final version of the paper.

Abstract

The study of nutrient dynamics in soil is frequently limited by our inability to observe and measure processes in real time, without destroying or perturbing the system being evaluated. This is especially important in agroecosystems, where obtaining real time information from in-situ monitoring, could provide high resolution information on the spatial and temporal dynamics of plant-available nutrients such as nitrogen (N). Incorporation of such techniques with existing precision agriculture approaches may improve N management with subsequent reductions in environmental and economic costs. However, soil provides a very challenging sensing environment and currently there is a lack of sensors that have the capability for in-situ monitoring of soil N. Ion-selective electrodes (ISEs) are simple, cheap and accurate sensors and have previously been used for the direct ex-situ measurement of soil NO_3^-. In this study we demonstrate the use of a novel ISE for in-situ, real time monitoring of NO_3^- in a grassland agricultural soil over a 7 day period in both a laboratory and field trial. Results from the ISEs were found to be statistically similar to conventional laboratory analysis of contemporaneous soil samples on 16 out of 19 occasions. Results from the field trial showed a large variability in soil NO_3^- concentrations over small spatial scales reflecting the small diameter of the sensing membrane (0.8 mm) and the intrinsic heterogeneity of soil NO_3^-. In conclusion, NO_3^- ISEs provide a new opportunity for real-time measurement of soil N dynamics. Future development must focus on longer-term testing of the ISEs and determining how their results can be used to inform current agronomic management regimes.

1. Introduction

Soil science and the understanding of biogeochemical pathways are frequently limited by our inability to observe and measure processes in real time, without destroying or perturbing the system being evaluated. This is especially important in agroecosystems, where obtaining real time information from in-situ monitoring, could provide high resolution information on the spatial and temporal availability of nutrients for plant uptake. Of particular concern is the loss of nitrogen (N) from farmland where fertilisers have been applied in excess of crop demand resulting in significant environmental and economic costs (Sutton et al., 2011). One approach to tackling this issue is to ensure synchronicity of N soil supply with plant N requirement, which may be improved through the adoption of precision agriculture. Techniques involving GPS, crop canopy scanning and variable rate fertilisation are all in current use. However, precision agriculture is held back by a lack of suitable soil nutrient sensors (Sylvester-Bradley et al., 1999; Adamchuck et al., 2004; Kim et al., 2009). Current agronomic methods of soil N determination are limited to destructive sampling with subsequent laboratory analysis. This is time consuming, expensive and therefore, not suitable for generating high resolution temporal and spatial data needed to inform precision management of fertiliser applications (Kim et al., 2009). Furthermore, methods for predicting the supply of available N from the mineralisation of organic matter over the growing season are not very accurate or user-friendly. Because of this, farmers often rely on estimations based on previous cropping, soil type, growing conditions and fertiliser management (Defra, 2010), which have been shown to be seriously inaccurate (Sylvester-Bradley et al., 2008). To address this issue, sensors capable of real time, in-situ measurement of soil N, could be deployed in-field, throughout the growing season, to enable continuous monitoring of soil N status. However, soil presents an extremely challenging sensing medium due to large physical and chemical heterogeneity at micro-scales, variable environmental conditions and the presence of a large microbial population and potential sensor contaminants.

Ion-selective electrodes (ISEs) are able to directly measure a wide range of analytes and in general are cost-effective, relatively accurate, offer a short response time (<60 s) and from a practical perspective are suitable for field deployment (Kim et al., 2009; Sinfield et al., 2010). However, using ISEs for analysis in complex environments, such as lakes, seawater and soils, does present a challenge due to a range of potential problems including electrode fouling, drift, instability, dissolution and cross contamination (De Marco et al., 2007). ISEs have previously been assessed as an agricultural research tool for the rapid on-farm determination of soil nitrate

(NO_3^-) (Wetselaar et al., 1998; Shaw et al., 2013). The concept of the rapid-test has been extended by coupling NO_3^- ISEs with vehicle mounted, automated soil sampling and rapid extraction platforms (Adamchuk et al., 1999; Adamchuk et al., 2003). This approach has allowed geo-referenced, on-the-go determination of soil NO_3^- to be performed in real time (Sibley et al., 2009). The subsequent results were used to create soil NO_3^- maps which could be used to inform variable rate management of N fertiliser additions. These methods require the extraction of NO_3^- from the soil or the sampling of the soil water prior to measurement by the ISE. In contrast, in-situ monitoring, requires sensors to be capable of direct soil measurement (DSM), maintain calibration parameters over an extended period of time and be robust enough to deal with challenging and variable environmental conditions. At the time of writing, there has been little progress in developing in-situ NO_3^- monitoring in soils using ISEs or any other sensors. DSM of NO_3^-, using ISEs has been shown to work as a concept in the lab and in the field, albeit with varying results. NO_3^- ISEs were used during experiments on winter wheat at Rothamsted during the 1970's to determine NO_3^- concentration in soils (Nair and Talibudeen, 1973; Page and Talibudeen, 1977; Page et al., 1978). The method involved adding 1 ml of distilled water to an in-situ hole (maintained by polythene tubes) in the soil. This was left for 10 minutes to allow it to equilibrate with the soil and then the ISE was used to directly measure soil NO_3^- concentration (Nair and Talibudeen, 1973). Although this was not strictly DSM, it did show the potential for ISEs to be used in this way. More recently, Adamchuck et al. (2005), used a commercially available NO_3^- ISE with a poly-vinyl chloride (PVC) based membrane, to examine the possibility of DSM on a range of agricultural soils under laboratory conditions. The accuracy of the results compared to standard reference methods was fairly low (r^2, 0.41–0.51), although this was attributed to within sample variation rather than electrode performance (Adamchuk et al., 2005). Ito et al. (1996) developed a novel type of NO_3^- ISE, which utilised an Urushi matrix (a type of latex) membrane, rather than a more conventional PVC membrane. They claimed that this improved the hardness, mechanical strength, lifespan and response time of the membrane whilst retaining similar selectivity and detection range. These ISEs were tested on an agricultural Andosol, with a gravimetric water content of 80%. The soil-electrode contact was maintained for 1-3 minutes until the output stabilised. They found that NO_3^- determined with the Urushi membrane yielded a high level of agreement with the conventional colorimetric-based lab determination (Bremner method; r^2, 0.994).

As well as performing accurate DSM, any sensor used for in-situ monitoring must be able to maintain its performance over an extended period. This capability has been demonstrated for NO_3^- ISEs in agricultural drainage ditches and streams with high levels of accuracy achieved

(Le Goff et al., 2002; Le Goff et al., 2003). Problems with using ISEs for long term, in-situ DSM, include calibration drift and change in the Nernst slope (Adamchuk et al., 2005; De Marco et al., 2007), sensitivity to temperature, soil pH and moisture content, durability (Ito et al., 1996), and contamination of the selective and reference membranes (Hansen et al., 1977; Adamchuk et al., 2005; De Marco et al., 2007). To move forward with ISEs for real-time *in situ* monitoring of soil NO_3^-, advances must be made in ISE technology so that long term measurements in the field can be made without the need for regular and frequent recalibrations and changing of damaged/contaminated membranes.

In an attempt to overcome these challenges, the authors have developed a novel NO_3^- ISE, which is cost effective and simple to construct. The PVC-based ISE membrane contains the quaternary ammonium compound methyltridodecylammonium nitrate (MTDDA.NO_3) which acts as the NO_3^- selective ionophore. This study aims to demonstrate the concept of in-situ monitoring of soil NO_3^-, using the NO_3^- ISE, in both a laboratory and field context.

2. Materials and methods

2.1. Field site and soil characteristics

The field site used for this study is located within the Henfaes Research Station, Abergwyngregyn, UK (53°14′N 4°01′W). The site has a temperate oceanic climate, receives an average annual rainfall of 1250 mm and has a mean annual soil temperature at 10 cm depth of 11 °C. The field is roughly rectangular with a perimeter of 559 m and an area of 1.91 ha. It has an average altitude of 12.1 m and slope of 1.5 % with a northerly aspect. It is a semi-permanent sheep-grazed grassland, dominated by *Lolium perenne* L. The current ley was seeded by direct drill in April 2009 using a perennial and hybrid ryegrass mix. The field has been used for both all year round grazing and silage production since 2009, receiving an inorganic fertiliser input of between 100 – 130 kg N ha^{-1} in addition to potassium (K), phosphate (P) and sulphur (S) at recommended rates. Lime has also been applied when necessary to increase the pH. In 2014, inorganic fertiliser was applied on 12/5/14 and 11/7/14 at a rate of N:P:K 50:10:10 and 60:4:0 kg ha^{-1} respectively.

The soil at the field site is a free draining Eutric Cambisol with a sandy clay loam texture and a fine crumb structure. To assess the chemical characteristics of the soil, replicate samples (n = 4) were collected from 4 blocks (30 × 30 cm) at a depth of 0 – 10 cm, representing the Ahp horizon. The soil was placed in gas-permeable polyethylene bags and transported to the laboratory in a refrigerated box. All of the following procedures were performed on the same day as field sampling. Soil pH and electrical conductivity (EC) were determined in a 1:2.5

(w/v) soil:distilled water suspension using standard electrodes. Soil solution ionic strength was estimated from electrical conductivity (EC) measurements according to Griffin and Jurinak (1973). Moisture content was determined by drying for 24 h at 105 °C. Total C and N were determined with a TruSpec CN analyser (Leco Corp., St Joseph, MI, USA). Dissolved organic carbon (DOC) and dissolved organic nitrogen (DON) were measured in soil extracts (0.5 M K_2SO_4, 1:5 w:v) using an Analytik Jena Multi N/C 2100S (AnalytikJena, Jena, Germany) before and after chloroform fumigation incubation (t = 7 days) of 2 g (n = 4) of fresh soil, in order to determine microbial biomass C and N according to Voroney et al. (2008) (K_{EC} = 0.35, K_{EN} = 0.5). Exchangeable cations were extracted using 0.5 M acetic acid (Sparks, 1996) and the filtered extracts analyzed using flame emission spectroscopy (Sherwood 410 flame photometer; Sherwood Scientific, Cambridge, UK). Extractable phosphorus (P) was determined by extraction with 0.5 M acetic acid with subsequent colorimetric analysis using the molybdate blue method of Murphy and Riley (1962). Basal soil respiration was determined in the laboratory at 20 °C using an SR1 automated multichannel soil respirometer (PP Systems Ltd., Hitchin, UK) and steady state CO_2 production rates recorded after 24 h. Potentially mineralisable N was determined using an anaerobic incubation method based on Keeney (1982). Briefly, 5 g field moist soil was placed in a 50 ml polypropylene centrifuge tube, which was then filled to the top with de-ionized H_2O and the tubes sealed. Soils were subsequently incubated in the dark at 40 °C for 7 d. The difference in NH_4^+ content between t = 0 and t = 7 d was attributed to N mineralization. Above ground biomass was sampled on 26/6/2014. Replicate 1 × 1 m blocks (n = 4) were chosen at random from within the field. The vegetation was cut to ground level, stored in paper bags and subsequently oven-dried at 80 ^oC to determine dry matter content. A summary of the results are shown in Table 1.

Table 1. Characteristics of the grassland Eutric Cambisol used in this study (n = 4).

	Units	Mean	SEM
pH		6.57	0.05
EC	µS cm^{-1}	26.5	1.0
Soil solution ionic strength	mM	5.27	0.2
Basal soil respiration	mg CO_2 kg^{-1} h^{-1}	12.61	1.04
Total soil C	g C kg^{-1}	25.35	1.47
Total soil N	g N kg^{-1}	2.95	0.06
Soil C:N		8.62	0.64
DOC	mg C kg^{-1}	70.1	2.6
DON	mg N kg^{-1}	10.5	1.1
Ammonium-N	mg N kg^{-1}	1.60	0.01
Nitrate-N	mg N kg^{-1}	0.88	0.21
Mineralisable N	mg N d^{-1} kg^{-1}	3.92	0.54
Microbial C	g C kg^{-1}	1.03	0.10
Microbial N	g N kg^{-1}	0.16	0.01
Soil solution Cl^-	mg Cl^- l^{-1}	17.44	1.19
Exchangeable Ca^{2+}	mg Ca^{2+} kg^{-1}	501.0	121.8
Exchangeable K^+	mg K^+ kg^{-1}	46.0	12.6
Exchangeable Na^+	mg Na^+ kg^{-1}	25.4	5.1
Available P	mg P kg^{-1}	7.4	2.0
Above ground biomass	t DM ha^{-1}	1.56	0.14

2.2. Chemical analysis

The following methods were used to analyse DOC, DON, amino acids, NH_4^+ and NO_3^- in soil extracts and solutions, microdialysis samples and nutrient solutions. DOC and DON were determined using an Analytik Jena Multi N/C 2100S (AnalytikJena, Jena, Germany). All samples were pre-acidified using 2 M HCl (5 µl ml^{-1}) to drive off dissolved inorganic C. Amino acids were determined by the *o*-phthaldialdehyde (OPA) spectrofluorometric method of Jones et al. (2002). NH_4^+ was determined by the nitroprusside colorimetric method of Mulvaney (1996) and NO_3^- by the colorimetric Griess reaction of Miranda et al. (2001). Chloride (Cl^-) concentration in soil solutions was assessed using a Dionex DX120 chromatograph (Thermo Scientific, Waltham, MA, USA).

2.2. NO_3^- ISE construction

NO_3^- ISEs were constructed in our laboratory using a simple and reproducible protocol. A NO_3^- sensing membrane was constructed by dissolving the following reagents (all sourced from Sigma-Aldrich, St. Louis, MO, USA), mixed by weight (w/w), into 2 ml of tetrahydrofuran (THF): 23 % PVC (high-molecular-weight polymer); 1% methyltriphenyl phosphonium bromide (a lipophilic cation); 65 % 2-nitrophenyl octyl ether; 5 % nitrocellulose; 6% MTDDA.NO_3 (Miller and Zhen, 1991). High density polyethylene pipette tips (1250 µl, graduated; TipOne®, StarLab, Milton Keynes, UK) were dipped into the resulting membrane cocktail so that the tip filled via capillary action to a depth of 3 mm. The tips were then left in a fume hood for 24 h to allow the THF to evaporate and the membrane to harden. The tips were subsequently back filled with a 100 mM KNO_3/KCl solution into which an $Ag/AgCl_2$ wire was inserted. The pipette tip was then sealed and a standard electrical wire was attached. Throughout this study the NO_3^- ISEs were coupled with a commercially available double junction lithium acetate reference electrode (ELIT 003n; NICO2000 Ltd., Harrow, Middlesex, UK).

2.3. NO_3^- ISE calibration and interfering ions

Prior to performing in-situ measurements it was necessary to calibrate the NO_3^- ISE with standard NO_3^- solutions. ISEs respond to the activity of the target ion rather than the absolute concentration according to the Nernst equation:

$$E = E_0 + (2.303RT/z_iF)\log a_i \quad \text{(Eqn. 1)}$$

Where E is the potential, z_i and a_i are the charge and activity of the ion of interest, E_0 is a constant which includes all sample-independent potential contributions (influenced by the design of the ISE), R is the gas constant, T is temperature in Kelvin and F is the Faraday constant.

In order to calibrate for concentration, ionic strength adjustment buffers must be used to ensure consistent ionic strength across all the standards (Adamchuk et al., 2005). However, this will only produce reliable results if the ionic strength of the standards are similar to that of the samples being tested. Whilst it would be possible to determine the ionic strength of the sample soil solution, it may vary both spatially and temporally in-situ. Furthermore, from a plant physiological perspective, it is the activity of NO_3^- rather than the concentration which is of most agronomic relevance (Grattan and Grieve, 1992). The NO_3^- ISEs were, therefore, calibrated to NO_3^- activity rather than concentration. All NO_3^- ISE derived results are subsequently expressed as activity. NO_3^- ISEs were calibrated in the following concentrations:

100, 10, 1, 0.1, 0.01 and 0.001 mM NO_3^- which correspond to activities of 75.8, 9.1, 0.965, 0.0989, 0.01 and 0.001 mM respectively. A calibration curve was fitted to these data using a simplified version of the Nicolsky-Eisenman equation (Miller and Zhen, 1991):

$$E = E_0 - S \log ([NO_3^-] + K) \quad \text{(Eqn. 2)}$$

Where E is the measured output of the NO_3^- ISE in mV, E_0 is the constant reference potential, *S* is the gradient of the slope and *K* is a term that subsumes the concentrations of the interfering ions and the selectivity coefficients of the NO_3^- ISE for those ions. *K* is also used as the limit of detection (LoD). The calibration curve for the NO_3^- ISEs used in the field trial can be seen in Figure 1. They have a near-Nernstian slope of 60.3 ± 0.7 mv dec^{-1}, a linear range of 0.1 to 100 mM NO_3- and a limit of detection of 33 ± 1.2 µM NO_3^- (means ± SEM, *n* = 12).

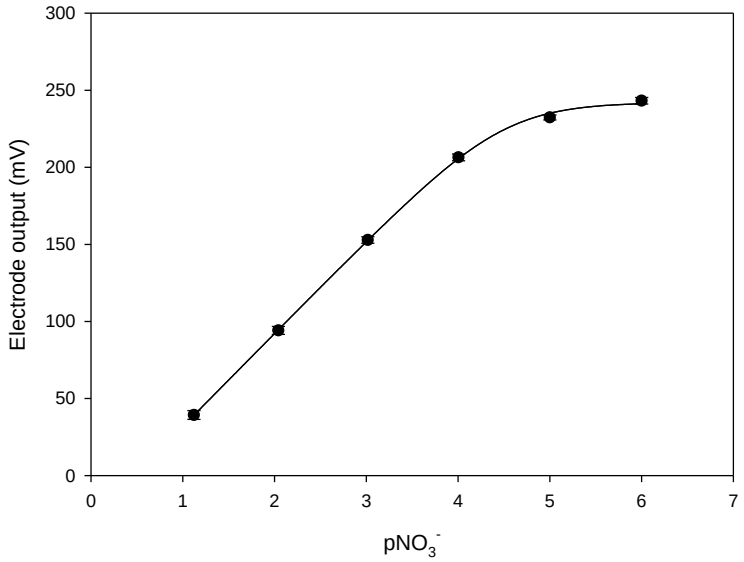

Figure 1. Calibration curve for the NO_3^- ISEs used for the in-situ soil monitoring field trial. Data points represent means ± SEM (n = 12). The line is a graphical representation of the simplified Nikolsky-Eisenman equation $E = E_0 - S \log ([NO_3^-] + K)$, where E is the measured output of the NO_3^- ISE in mV, E_0 is the constant reference potential, S is the gradient of the slope and K is a term that subsumes the concentrations of the interfering ions and the selectivity coefficients of the NO_3^- ISE for those ions. K is also used as the limit of detection. $pNO_3 = -\log_{10} [NO_3^-$ activity].

The selectivity of the NO_3^- sensing membrane to a range of anions has been previously determined by Miller and Zhen (1991) using the fixed interference method (Bakker et al., 2000). Of these interfering ions, only Cl^-, due to its relative abundance in soil and selectivity coefficient of 0.035, may have a significant effect on the NO_3^- ISE output when used for in-situ measurements in this soil. The Cl^- concentration of the soil solution was found to be 0.5

mM so the NO_3^- ISEs were recalibrated in standard solutions containing 0.5 mM Cl^-. No significant differences were found between the calibration parameters with and without 0.5 mM Cl^-.

2.4. Temperature effect on ISE output

The Nernst equation (Eqn. 1) contains a temperature term, therefore the output of the NO_3^- ISEs needs to be adjusted if the temperature of the sample varies from that of the calibration temperature. The temperature term makes up part of the slope (*S*) coefficient in the Nicolsky-Eisenman equation (Eqn. 2), which theoretically will respond by 0.1984 mV dec^{-1} $°C^{-1}$. This was tested experimentally for the NO_3^- ISE used in this study and the result (0.217 ± 0.04 mV dec^{-1} $°C^{-1}$) was found to be not significantly different to the theoretical temperature coefficient. As such, the results in this study have been calculated by adjusting the *S* calibration parameter by 0.1984 mV dec^{-1} $°C^{-1}$. This temperature coefficient is related only to the temperature of the sample being measured and not any direct temperature effects on the ISE itself or the data logging system, both of which will be subject to temperature variations when deployed in the field. To test for any such effects, a simple experiment was performed. Pre-calibrated NO_3^- ISEs (n = 5) were placed in individual 20 ml sample vials containing 10 ml of 1 mM NO_3^- and sealed with Parafilm®. The NO_3^- ISEs were connected, using a 1 m long piece of standard electrical wire, to a multi-channel data logger (DL2e, Delta-T Devices Ltd, Cambridge, UK). The data logger was programmed to make and record differential DC voltage measurements in the range 0 – 500 mV every 0.5 h. Initially, the data logger was placed in a temperature-controlled incubator which was programed to ramp the temperature from 9 to 45 and back to 9 °C, with the rate of change set to 3 °C h^{-1}. The NO_3^- ISEs were placed in an adjacent incubator, which was set to a constant 20 °C. NO_3^- ISE output was recorded over a 48 h period. Following this, the data logger and the NO_3^- ISEs were swapped so that the data logger was exposed to a constant temperature and the NO_3^- ISEs to the variable temperature. The NO_3^- ISE output was recorded for a further 62h.

2.5. Trial of NO_3^- ISE for in-situ soil monitoring under controlled environmental conditions

Replicate turfs (n = 5) of size 50 × 15 × 10 cm (length × width × depth) were cut from the experimental field and taken immediately to the laboratory. Here, each turf was cut into 3 sections. The first section was 10 × 15 × 10 cm and used for conventional NO_3^- determination. Briefly, the vegetation and the top 1 cm of the soil were removed from the turf and the remaining soil used for analysis. Soil was crumbled by hand, in order to prevent sieving

induced N mineralisation (Jones and Willett, 2006; Inselsbacher, 2014). Large stones, roots and vegetation were removed prior to gentle mixing of the sample. To further reduce mineralisation of organic N forms, sub-samples of field-moist soil (5 g) were extracted on ice (175 rev min^{-1}, 15 min) using cooled (5 °C) 0.5 M K_2SO_4 at a soil: extractant ratio of 1:5 (w:v) (Rousk and Jones, 2010). The extracts were centrifuged (4,000 *g*, 15 min), and the resulting supernatant collected and frozen (-18°C) to await chemical analysis. Soil solution was also obtained by the centrifugal-drainage method of Giesler and Lundström (1993). Briefly, the soil was prepared as described above and approximately 100 g of field-moist soil was placed in centrifuge tubes, the bottom of which are perforated to allow passage of the soil solution into a collection vessel, and centrifuged at 3,200 *g* for 20 min. Soil solutions were subsequently frozen (-18°C) to await analysis. Soil extracts and solutions were analysed for NO_3^- using the method previously described in section 2.2. In addition, approximately 3 g of soil was used for moisture content analysis.

The second and third sections of turf had equal dimensions of 20 × 15 × 10 cm, and were used for the experimental procedure. The turfs were placed on top of horticultural capillary matting in plastic containers, which had roughly the same dimensions as the turfs, and moved to a climate controlled chamber. The ends of the capillary matting were placed in a reservoir of distilled H_2O placed 4 cm below the base of the soil to ensure the soil stayed moist throughout the experimental period. In the second set of turfs, a hole of approximately 1 cm diameter was made to a depth of 6 cm in the center of each turf. A pre-calibrated NO_3^- ISE was placed into this hole. The hole was then backfilled and a gentle downward pressure was applied to the NO_3^- ISE to ensure good membrane-soil contact. The NO_3^- ISEs were then connected to the data logger using a 1 m length of standard electrical wire. The data logger was programmed to make and record differential DC voltage measurements in the range 0 – 500 mV every 1 h. The third set of turfs were used for destructive sampling and subsequent NO_3^- analysis throughout the experimental period in order to determine the performance of the NO_3^- ISE. This was performed in the middle of the day and night of the programmed diurnal cycle in order to try and identify any diurnal or temperature related variation in soil NO_3^- concentration. Soil cores were taken in triplicate from each turf between depths of 3 – 8 cm using a soil corer with a diameter of 5 mm. The triplicate cores were bulked prior to being hand crumbled and mixed. Soil extractions were performed using 2 g with 10 ml of 0.5 M K_2SO_4 as described above, with the resulting extracts frozen to await NO_3^- analysis. In addition, approximately 1 g of the bulked sample was used for moisture content analysis. The data from the soil extractions were used to calculate the NO_3^- concentration of the bulk soil, expressed on a dry weight basis (mg N kg^{-1}).

The extractions were also used to estimate the NO_3^- concentration in soil solution (mg N l^{-1}). The calculation assumes that all the NO_3^- in the soil extracts came from the soil solution pool so a simple soil solution dilution factor can be calculated using the soil moisture content. The climate chamber was programmed to run a 24 h diurnal cycle with conditions similar to that which may occur during a summer's day. The cycle had a 12 h day/night period with a temperature max of 25 °C after 6 h and a temperature minimum of 10 °C at 18 h. Photosynthetically active radiation (PAR) was set to 0 % for the 12 h night period. At the commencement of the day period the PAR was set to 50 % with a peak in the middle of the day of 100 % before returning to 50 % at the end of the day period. Relative humidity was set to 50 % for the day and 70 % for the night. This program was run for 3 whole cycles. Following this, the program was adjusted so that the temperature remained constant at 20 °C whilst the other variables remained unchanged. The program was then run for a further 4 diurnal cycles. At the end of the experimental period the NO_3^- ISEs were removed from the turfs. The vegetation and top 1 cm of the soil was removed and the soil extracted and soil solution sampled as described above. Extracts and soil solutions were subsequently frozen to await NO_3^- analysis. The NO_3^- ISEs were rinsed briefly in distilled H_2O and then soaked in 100 mM NO_3^- prior to being recalibrated to assess changes in calibration parameters.

2.6. Field trial of ISEs for in-situ monitoring of soil NO_3^-

A 2 × 2 m block was chosen at random from within the experimental field. Within this block, four 30 cm^2 sections of turf and topsoil were removed to a depth of 5 cm. 3 holes at a gradient of approximately 20° below the horizontal were made into the sides of these holes, into which the NO_3^- ISEs were inserted (see appendix 4, Fig. 5). Gentle pressure was applied to the NO_3^- ISEs to ensure good soil-membrane contact. The holes were back filled and the turfs replaced to ensure that the NO_3^- ISEs were completely buried with the exception of the electrical cable. In total, 12 NO_3^- ISEs were implanted into the soil (n = 12). The NO_3^- ISEs were connected, using a 1 m long piece of standard electrical cable, to the data logger, which was housed in a waterproof container. The data logger was programmed to make and record differential DC voltage measurements in the range 0 – 500 mV every 2 h. The NO_3^- ISEs were deployed during the afternoon of 6/8/2014 and logging commenced at 16:30h on the same day. Logging ceased at 08:30h on 12/8/14, giving a total logging time of 136 h. Failure of 1 NO_3^- ISE occurred immediately and it was assumed that this was caused by membrane damage during insertion into the soil. Results presented are means ± SEM (n = 11). At the end of the monitoring period the NO_3^- ISEs were removed from the soil and taken back to the laboratory for recalibration.

To make an assessment of the accuracy of the NO_3^- ISEs, soil samples were taken for conventional laboratory analysis. In an adjacent 2 × 2 m block, a soil corer with a diameter of 1 cm was used to take replicate soil samples (n = 4) from a depth of 5 – 10 cm. In a third block, larger volumes of soil (approx. 300 g field moist) were sampled (n = 4) from the same depth using a trowel. The soil was placed in gas-permeable plastic bags and transferred immediately to the laboratory, were they were refrigerated at 4 °C. Extractions were performed on the soil cores on the same day as sampling as previously described. The larger soil samples from the third block were used to obtain soil solution by centrifugal-drainage as previously described. Soil extracts and solutions were analysed for NO_3^- using the method described in section 2.2.

2.7. Statistical analysis of data

Significance testing was performed using one-sample t-tests, two-sample t tests and one-way ANOVA as appropriate. SPSS v.20 (IBM Ltd., Portsmouth, UK) was used for all statistical testing with $p < 0.05$ used as the cut-off for statistical significance.

3. Results

3.1. Temperature effect on NO_3^- ISE and data logger performance

The effect of varying the data logger temperature on the NO_3^- ISEs was investigated by placing the data logger in a variable temperature incubator whilst the attached NO_3^- ISEs were kept at a constant temperature. Figure 2 shows that there was a small temperature effect on the NO_3^- ISE results, with small peaks occurring concomitantly with the temperature maximum. However, closer inspection of these data revealed that the mV output of 2 of the NO_3^- ISEs covaried positively, 2 covaried negatively and 1 had little response to the increasing temperature of the data logger. Furthermore, the magnitude of the response, both positive and negative, was not consistent between the electrodes. This presented a challenge for developing an accurate temperature compensation calculation. It was necessary to calculate the maximum and minimum effect of temperature so that the real value would likely exist between the 2 extremes. As the output of one of the NO_3^- ISEs did not appear to be affected by the variable temperature, the minimum effect was assumed to be no effect. The maximum effect of temperature was determined by plotting the inverse of the NO_3^- ISE output (in mV), which showed the largest response, against temperature. A single first-order exponential increase curve (eqn. 3) was found to give the best fit to the data (r^2 = 0.992).

$$y = y0 + a \exp(b \times x) \qquad \text{(Eqn. 3)}$$

Where *y* is the electrode output in mV, *y*0 (0.0064) is an asymptote at which any further decrease in temperature has no effect on the logger output, *a* (1.07×10^{-5}) and *b* (0.0727) are the coefficients describing the exponential increase, and *x* is the logger temperature (°C). This equation was used to normalise the mV output of each electrode to 25 °C and these mV values subsequently used to calculate the NO_3^- activity of the substrate according equation 2.

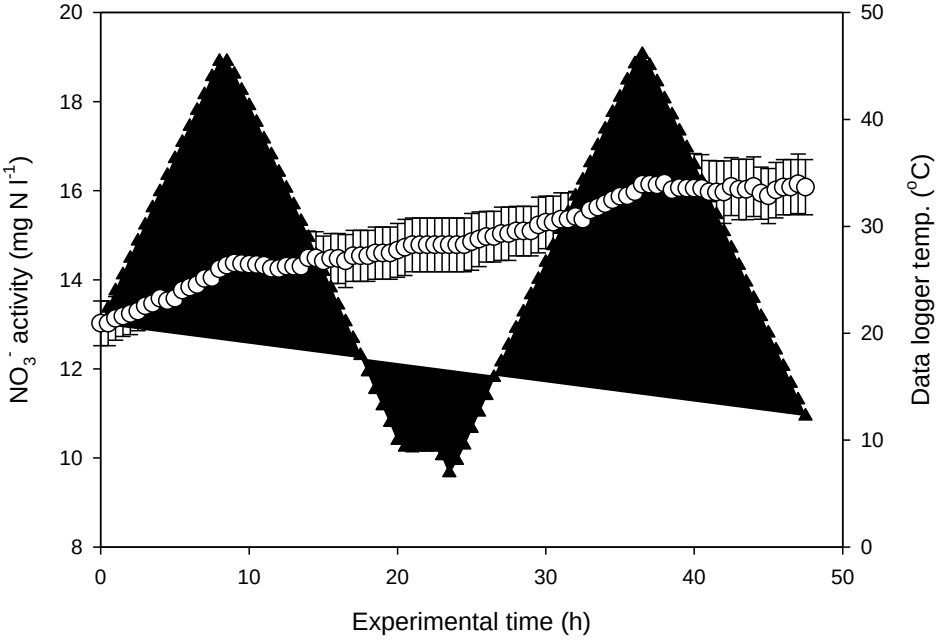

Figure 2. Effect of variable data logger temperature (triangles) on NO_3^- ISE estimation (open circles) of the NO_3^- activity of a standard NO_3^- solution (14 mg N l^{-1}). NO_3^- ISE data points represent means ± SEM (n = 5).

Figure 3 shows the effect of varying the temperature of the NO_3^- ISE and associated substrate on the NO_3^- ISE output. Changing the temperature from 10.5 °C to 45 °C decreased the output of the NO_3^- ISE from 23.3 ± 1.3 to 11.1 ± 0.5 mg N l^{-1}. Adjusting the *S* calibration parameter by 0.1984 mV dec^{-1} $°C^{-1}$, in accordance with Nernstian theory reduces the temperature effect. However, the theoretical temperature adjustment is not perfect in this case and there is still some apparent, albeit small co-variation in [NO_3^-] with temperature. This calibration adjustment was applied to all subsequent reported ISE results following any adjustments made to the electrode output (in mV) to compensate for variation in data logger temperature.

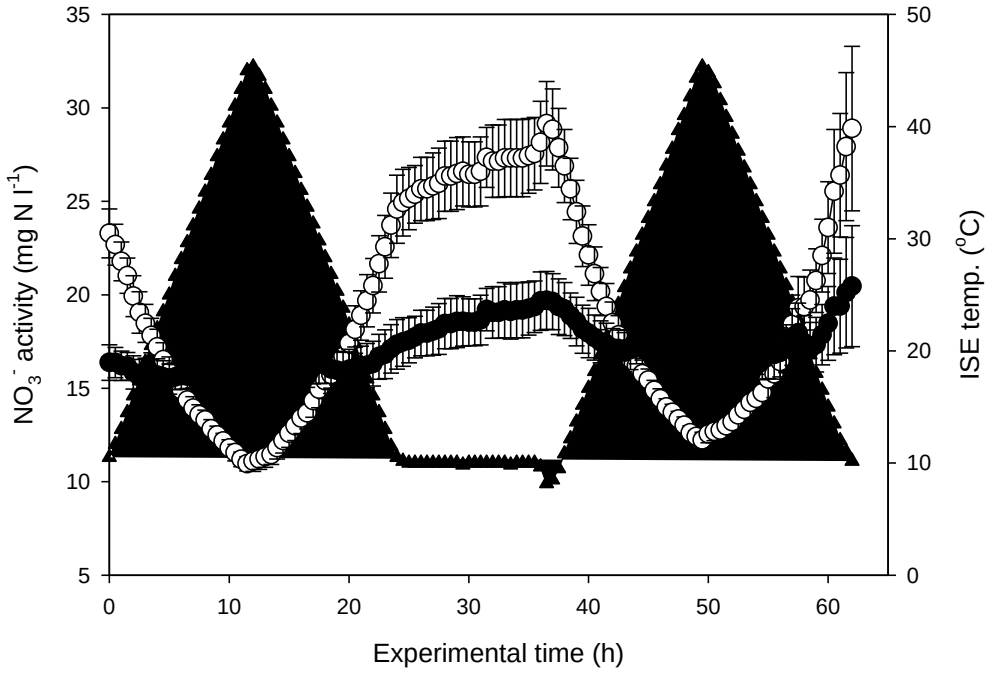

Figure 3. Effect of variable temperature (triangles) on NO_3^- ISE estimation (open circles) of the NO_3^- activity of a standard NO_3^- solution (14 mg N l^{-1}). Closed circle data points are the results after temperature adjustment to the NO_3^- ISE slope (S) calibration parameter by 0.1984 mV dec^{-1} $^oC^{-1}$. NO_3^- ISE data points represent means ± SEM (n = 4).

For both of the above experiments there was no significant difference between the initial NO_3^- ISE result and the concentration of the standard solution (14 mg N l^{-1}). However, the NO_3^- ISEs reported an increase in [NO_3^-] over the course of the experiments to 16.0 mg N l^{-1} ± 0.7 and 20.5 mg N l^{-1} ± 3.2, giving a rate of increase of 0.06 ± 0.01 mg N l^{-1} h^{-1} and 0.07 ± 0.04 mg N l^{-1} h^{-1} respectively. To determine whether this reported increase was due to evaporative loss or electrode drift a simple experiment was performed. This showed that evaporative loss at 20 °C resulted in an increase the activity of the standard solution at a rate of 0.05 mg N l^{-1} ± 0.01. As this rate of increase was statistically similar (p = 0.89) to results from the temperature experiments, the observed increase in the temperature experiments was attributed to evaporative losses rather than electrode drift.

3.2. In-situ soil NO_3^- monitoring under controlled environmental conditions

NO_3^- ISEs were used for the in-situ monitoring of soil solution NO_3^- activity in replicate turfs over a 160 h period under controlled environmental conditions in the laboratory and the results are presented in Figure 4. For the first 72 h, during which the temperature varied diurnally from 10 to 25 °C, the NO_3^- ISE estimates of soil solution NO_3^- activity showed a gradual decrease from 3.9 ± 1.4 to 1.8 ± 0.5 mg N l^{-1} (means ± SEM, n = 5). The NO_3^- ISE results also showed a cyclical variation during this time, which appeared to be positively correlated with both soil and data logger temperature and the diurnal cycle. For the remaining experimental time, during which the air temperature was set to a constant 20 °C, the NO_3^- ISE results increased slightly from 1.8 ± 0.5 to 2.6 ± 1.9 mg N l^{-1}, although this was largely due to the increase in 1 replicate from 2.9 to 10.2 mg N l^{-1}. There was still a small diurnal temperature variation (≈ 4 °C) apparent for the soil and the data logger, which was attributed to radiative heating. The NO_3^- ISE results exhibited a very small cyclical variation during this time period which corresponded to the above temperature variation and the day/night cycle. The soil solution NO_3^- concentration, estimated from 0.5 M K_2SO_4 extractions of soil cores, were statistically similar to the NO_3^- ISE estimations (p > 0.05) for all but 2 (132 & 156 h) of the 13 sampling time points. In general, the soil core derived estimates of soil solution NO_3^- were larger than the NO_3^- ISE results. There was no evidence of the temperature/diurnal related variation that was seen in the NO_3^- ISE results. The initial soil core derived estimates of soil solution NO_3^- were 6.2 ± 2.3 mg N l^{-1} (means ± SEM, n = 5). This increased to the experimental maximum of 16.9 ± 6.9 at 47 h. The concentration at the end of the experiment (156 h) was 9.9 ± 2.4 mg N l^{-1}. Once the ISE experiment had ended, soil solution was recovered from the NO_3^- ISE turfs using centrifugal-drainage. The resulting NO_3^- concentration was 2.2 ± 0.1 mg N l^{-1}, which was not significantly different from the NO_3^- ISE estimated activity (p > 0.05).

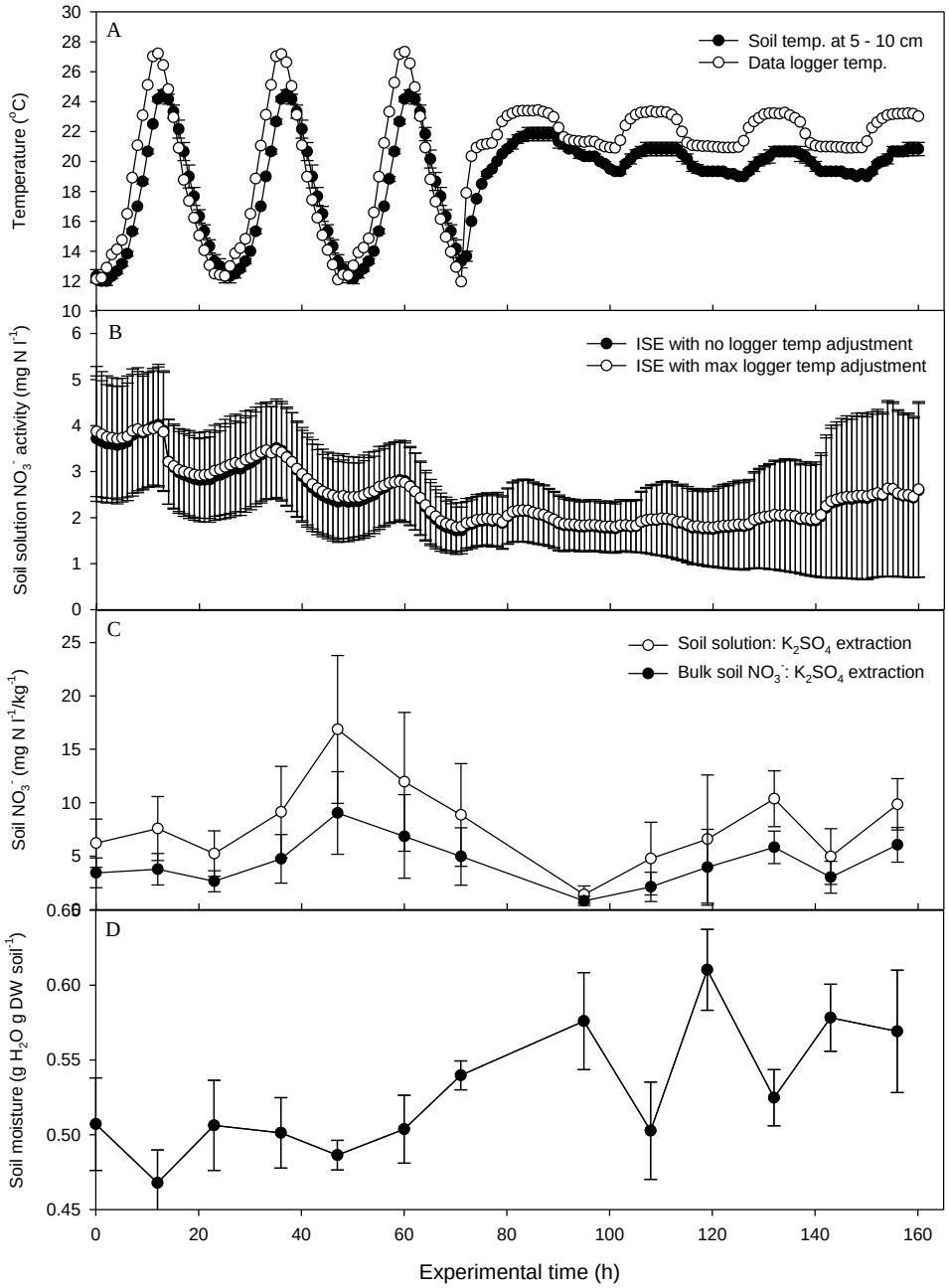

Figure 4. Results of in-situ soil NO_3^- monitoring under controlled environmental conditions over a 160 h period. Panel A shows the temperature (°C) of the data logger and soil at 5–10 cm depth (means ± SEM, n = 3). Panel B shows the estimation of soil solution NO_3^- activity by the in-situ NO_3^- ISE with (open circles) and without (closed circles) adjustment for data logger temperature (means ± SEM, n = 5). Panel C shows estimations of soil solution (mg N l^{-1}) and bulk soil NO_3^- concentration from soil core extractions and chemical analysis (mg kg^{-1}) (means ± SEM, n = 5). Panel D shows soil moisture content (means ± SEM, n = 5).

Adjusting the mV output of the NO_3^- ISE to compensate for the maximum observed temperature effect on the data logger had very little overall effect on the results. This was expected given the data logger temperature range and the exponential relationship between logger temperature and mV output described by equation 3. Recalibration of the NO_3^- ISEs was attempted at the end of the trial to assess changes in calibration parameters over the course of the monitoring periods. Despite rinsing with distilled H_2O and subsequent soaking in 100 mM NO_3^- it was not possible to remove all the soil from the membrane. Because of this, the ISE would not stabilise so calibration was not possible.

3.3. Field trial of ISEs for in-situ monitoring of soil NO_3^-

NO_3^- ISEs were deployed in-field over a 7 d period for in-situ monitoring of soil solution NO_3^- activity and the results are presented in Figure 4. During the monitoring period the environmental conditions were variable. A period of rain (8.8 mm) on the first day of the monitoring period meant that the soil moisture content was fairly high at the start of the experiment (0.28 ± 0.01 g H_2O g soil DW^{-1}, mean ± SEM, n = 4). During the monitoring period the mean daily temperature max and min was 19.4 °C and 12.9 °C respectively. On 10th August there was a further rainfall event (7.3 mm) and as a consequence, air temperature was much lower than previous days. There was also a resulting increase in the soil moisture content from 0.16 ± 0.01 g H_2O g soil DW^{-1} on 8th August to 0.23 ± 0.01 g H_2O g soil DW^{-1} on Sunday 10th. Due to the sunny and warm conditions on 4 of the days, the data logger temperature showed a large diurnal variation. For example, the data logger temperature decreased from a maximum of 44.2 °C at midday on August 10th to a minimum of 12.1 °C at midnight on August 11th. Soil temperature also showed a diurnal variation, although the variation was much lower than the data logger temperature and the air temperature. It remained between a maximum and a minimum of 22.8 °C and 17.7 °C for the duration of the monitoring period. 12 NO_3^- ISEs were deployed and of these one failed immediately on insertion into the soil. A noticeable feature of the NO_3^- ISE results are the peaks that occur during the day, with [NO_3^-] maximums occurring at 14:30h. These correspond to maximum daily soil and logger temperature. Ignoring these spikes, the NO_3^- ISE results showed a general increasing trend from 8.28 ± 2.25 mg N l^{-1} at 06:30h on August 7th to 13.09 ± 3.66 mg N l^{-1} at 06:30h on Sunday 10th. Following this, a gradual decline was observed. This decline occurred after the above-mentioned significant rainfall event and resulting soil moisture increase. The inter-replicate range of NO_3^- ISEs was large with differences of up to 57 mg N l^{-1} occurring between the lowest and highest replicate. Compared to the estimation of soil solution NO_3^- by conventional sampling and lab analysis,

the NO_3^- ISEs estimations were between 2 to 5 times greater, although this was only significantly different for the Sunday 10th sampling event. The conventional soil sampling was not performed at a fine enough temporal resolution to determine whether the diurnal variation observed by the NO_3^- ISEs was occurring. The adjustment made to the NO_3^- ISEs results for the temperature effect on the data logger caused a slight reduction in the maximum of each spike and a very small increase at data logger temperatures below 25 °C. Recalibration of the NO_3^- ISEs was attempted to assess changes in calibration parameters over the course of the monitoring periods. Despite rinsing with distilled H_2O and subsequent soaking in 100 mM NO_3^- it was not possible to remove all the soil from the membrane. Because of this, the ISE would not stabilise so calibration was not possible.

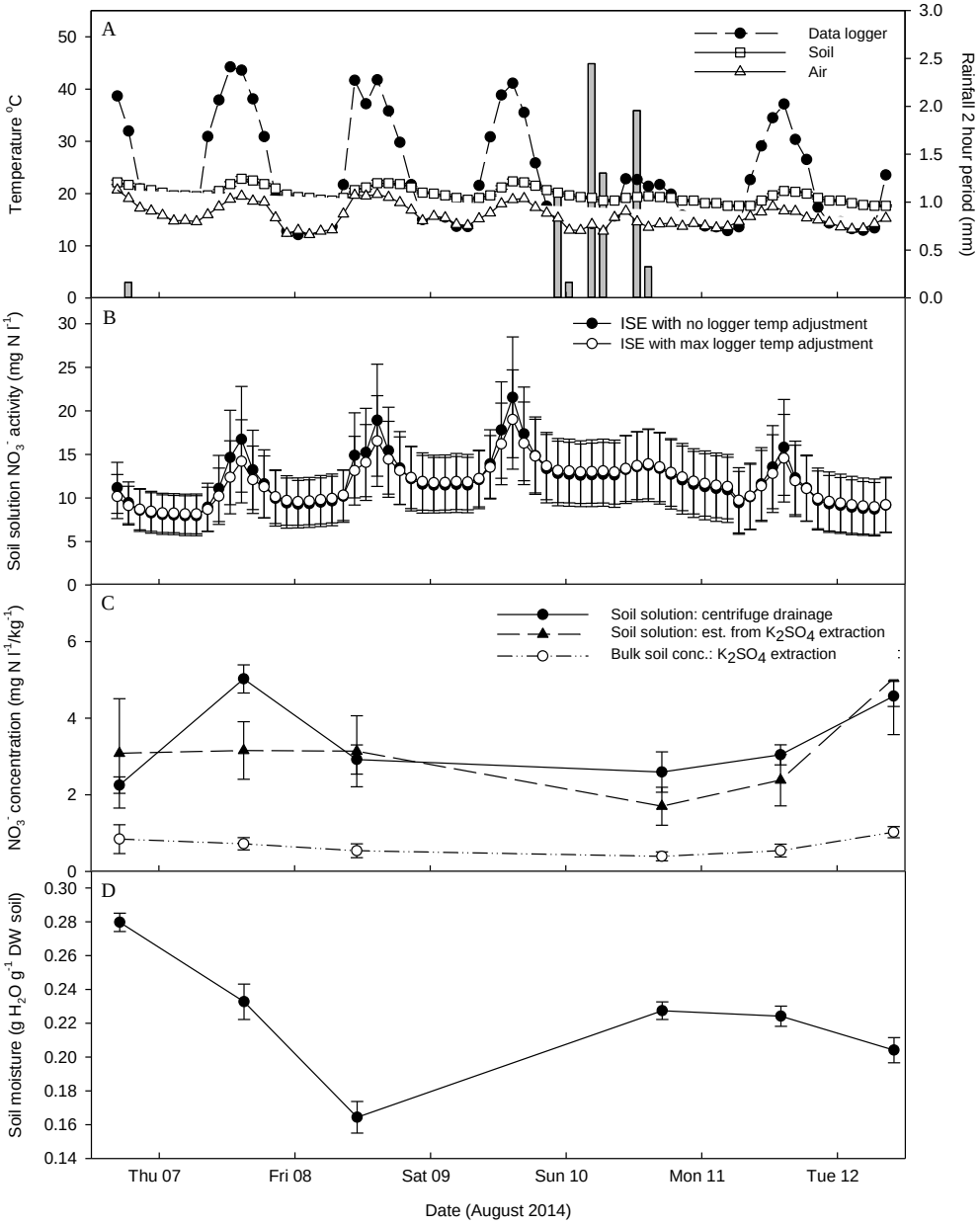

Figure 5. Results of in-situ soil NO_3^- monitoring in an agricultural grassland field over a 7 day period. Panel A shows the temperature (°C) of the air, data logger and soil at 5–10 cm depth (means ± SEM, n = 3). Rainfall totals (mm) are for 2 h time periods. Panel B shows the estimation of soil solution NO_3^- activity by the in-situ NO_3^- ISE with (open circles) and without (closed circles) adjustment for data logger temperature (means ± SEM, n = 11). Panel C shows estimations of soil solution and bulk soil NO_3^- concentration from soil core extractions and centrifugal-drainage followed by chemical analysis (means ± SEM, n = 4). Panel D shows soil moisture content (means ± SEM, n = 4).

4. Discussion

4.1. Overall ISE performance in soil

In this study, novel ISEs were used for in-situ, real time monitoring of NO_3^- in an agricultural soil. Experimental trials were performed in the laboratory, under controlled environmental conditions and in the field. During these trials, the NO_3^- ISEs estimation of soil solution NO_3^- activity was statistically similar to the conventional methods of sampling and analysis for 16 out of the 19 contemporaneous sampling events. Assuming that the conventional *ex-situ* NO_3^- measurement techniques are valid, the ISEs underestimated soil solution NO_3^- status in the laboratory experiment, but overestimated it during the field trial. It has been shown previously that different methods of soil N determination will access different fractions or pools of the soil solution and hence, results will depend to some extent on the method used (Geibe et al., 2006; Inselsbacher et al., 2011). The in-situ soil solution exists as an intrinsic part of the soil so it is highly likely that the NO_3^- ISEs will access a different fraction of the soil solution to the conventional centrifugal-drainage and soil extraction methods. During both monitoring periods only 1 of the 17 NO_3^- ISEs failed. The single failure occurred during the insertion of the electrode into the soil, which caused the membrane to be pushed up into the electrode barrel. This demonstrates that the NO_3^- ISEs are sufficiently robust for in-situ monitoring.

4.2. Influence of soil solution ionic strength on ISE monitoring

Assessing the absolute accuracy of the NO_3^- ISEs is confounded by a number of factors. The most obvious of these is that the NO_3^- ISEs sense the activity and not the concentration of the NO_3^- ions in soil solution. The NO_3^- activity will be lower than the absolute concentration, with the difference dependent upon the total ionic strength of the solution. For this soil, the ionic strength of the soil solution was estimated to be 5.3 ± 0.2 mM, resulting in a difference of approximately 8 % between the concentration and activity of any monovalent anion. The ionic composition and concentration of soil solution is dynamic and controlled by a number of factors, which means its ionic strength is likely to be both spatially and temporally variable.

4.3. Spatial consideration of soil NO_3^- and ISE performance

Some consideration must also be made as to the spatial variation of NO_3^- in the soil and the effect this may have on the interpretation of the NO_3^- results and comparisons with conventional methods of assessing soil NO_3^-. The sensing membrane of the NO_3^- ISE has a linear dimension of 0.8 mm, as such the resulting measurement is limited to a small volume of soil. Consequently, they are highly likely to be affected by micro heterogeneity of soil

properties. A separate study on this field using geo-statistical methods has shown that there is considerable variation in soil NO_3^- at scales below 1 cm (Shaw et al., unpublished, chapter 7 this thesis). It is therefore, unsurprising that the NO_3^- ISE results exhibited considerable inter-replicate variability. It is interesting to note that the inter-replicate variability was lower in the laboratory trial where the soil moisture was twice that of the field trial. Mobility of NO_3^- in the soil will be increased as the soil becomes wetter, which may reduce its heterogeneity. The conventional soil sampling may also be affected by small scale variance of soil properties given only 4 replicates, although this is offset by the larger sample support size used.

4.4. ISE drift and calibration

Changes in ISE calibration parameters over time, so called electrode drift, is one issue that must be minimised for accurate in-situ monitoring (De Marco et al., 2007). The NO_3^- ISEs used in the data logger temperature effect experiment reported an increase in concentration of the standard solution at a rate of 0.06 mg N l^{-1} h^{-1}. A subsequent experiment showed that this was due to evaporative loss rather than electrode drift. There was also no evidence of electrode drift occurring in-situ during either the laboratory or field experiments. Unfortunately, it was not possible to assess whether changes had occurred to the calibration parameters following the in-situ monitoring periods as the soil had formed an intimate contact with the ISE membrane preventing recalibration.

4.5. Influence of temperature on ISE performance

One obvious feature of the NO_3^- ISEs results from both the laboratory and field monitoring was the covariance with both logger and soil temperature and the diurnal cycle. In the laboratory monitoring experiment the temperature was variable for the first 3 diurnal cycles, then set to a constant 20 °C for the subsequent 4 diurnal cycles, although some small variation in data logger and soil temperature was still evident. The observed variation in the NO_3^- ISE results decreased markedly after the first 3 diurnal cycles. This suggests that temperature rather than the diurnal cycle was the cause of the observed variation. The fact that the results, which have been adjusted for temperature effects on both the data logger and the ISEs, still show co-variation with temperature could suggest that the measurements reflect a real soil phenomenon. However, a closer look at the results from the field trial would suggest otherwise. The diurnal variation in the NO_3^- ISE results in the field experiment were much larger than in the laboratory experiment despite variation in soil temperature being much lower. Temperature variation in the data logger conversely was much larger. The NO_3^- ISEs results increased dramatically as

the temperature of the data logger exceeded 25 °C in a manner that was consistent with an exponential relationship between temperature and the ISE output. As such, it is likely that the observed diurnal variation was mainly an experimental artifact caused predominantly by a temperature effect on the data logger. Although, due to the uncertainty over the size of the temperature effect, there may also be some temperature and diurnal related changes to the intrinsic soil NO_3^- concentration (Delhon et al., 1996; Marhan et al., 2015).

It is clear that precisely characterising the temperature effects on the logging-ISE system is crucial for truly accurate soil NO_3^- results to be achieved. Achieving this in our study was confounded by the fact that each ISE and data logger channel may have had a slightly different temperature response. In this study, the gradient of the Nernstian slope parameter, derived from the calibration of the NO_3^- ISEs, was adjusted based on the Nernst equation (Eqn. 1) by 0.1984 mV dec^{-1} $°C^{-1}$. This value was used as it was shown not to differ from the value which was derived experimentally. Applying this compensation largely, but not completely, negated the temperature effect on the ISEs. The theoretical temperature compensation is based on a theoretical Nernstian slope of 59.2 mV dec^{-1} at 25 °C, whereas the NO_3^- ISEs used for the field trial had a mean slope of 60.3 ± 0.7 mV dec^{-1}. As such, it is likely that each NO_3^- ISEs will have a slightly different response to temperature. Ideally, the temperature response of each NO_3^- ISE would be characterised individually although this would be very time consuming and there is no certainty that this would improve the accuracy of the in-situ monitoring. Adjusting the results for the temperature effect on the data logger proved much more difficult. The effect of temperature was shown to be inconsistent, in terms of both the size and direction of the effect. A maximum temperature effect was determined experimentally from the results of 5 replicate ISEs. However, the results from the field trial suggest that the temperature adjustment was not sufficient to negate the large variation in data logger temperature that occurred. To address this, further work needs to be carried out to better assess temperature related effects on the ISE – data logging system. Furthermore, it is recommended that for any future field work the data logger is stored in a thermally insulated container, out of direct sunlight, in order to maintain a more stable temperature.

5. Conclusions

This study has demonstrated the use of novel NO_3^- ISEs for in-situ, real-time monitoring of a grassland soil. Experiments were performed both in the laboratory, under controlled environmental conditions and in the field over 7 day periods. The NO_3^- ISE results were similar to those achieved by conventional soil sampling and laboratory analysis. There was no

evidence of electrode drift during any of the experiments although it was not possible to recalibrate the NO_3^- ISEs following the in-situ monitoring experiments so changes in calibration parameters could not be assessed. Improving the accuracy of the NO_3^- ISEs may be achieved by further investigations into the in-situ effects of temperature on the NO_3^- ISE – data logging system. Due to the small size of the sensing membrane, consideration must also be given to the spatial variance of NO_3^- within the experimental site, especially at small scales, in order to inform an appropriate sampling strategy. Alternatively, to reduce the effect of large small scale variance, the design of the NO_3^- ISE could be moderated to increase the size of the sensing membrane or incorporate multiple sensing membranes into one unit. It is likely that these NO_3^- ISEs will offer new insights into soil NO_3^- dynamics that has previously not been possible. Further development of the technology is required so that they can be deployed for the entirety of a growing season in order that research into their agronomic use can be fully evaluated.

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Chapter 7

Article V

Characterising the within-field scale spatial variation of different N forms in a grassland soil and the implications for in-situ N sensor technology and precision agriculture

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A version of this manuscript has been submitted to Agriculture, Ecosystems and Environment (July 2015) and is currently under review.

Author contributions:

RS, RML and DLJ conceived and designed the experiment. All the experimental work was performed by RS. RML provided all the geostatistical analysis and guidance on the results. RS and RML wrote the first draft of the manuscript with all authors contributing to the final version of the paper. RML's contribution appears with the permission of the Director of the British Geological Survey (NERC).

Abstract

The use of in-situ sensors capable of real-time monitoring of soil nitrogen (N) may facilitate improvements in agricultural N-use efficiency through better fertiliser management. Optimising the deployment of in-situ sensors for both accuracy and cost requires consideration of the spatial variation of N forms at within-field scales. In this study, a geo-statistical nested sampling approach was used to characterise the spatial variability of amino acids, ammonium (NH_4^+) and nitrate (NO_3^-) in the soil of a grazed grassland field (1.9 ha). Within the growing season, two nested sampling campaigns were undertaken both before and after the application of fertiliser N (60 kg N ha^{-1}) and the removal of grazing sheep. The field was stratified into four quarters with four mainstations located at random within each quarter (stratum). Within each mainstation, sampling at the following spatial scales: 1 cm, 10 cm, 50 cm and 2 m, was performed using a soil corer with a 1 cm diameter. Further investigation into small-scale spatial variance was investigated using smaller soil samples (approx. 70 mg) that represented the "aggregate" scale. Short-range variation was found to be dominant, with at least 61%, 86% and 61% of the total accumulated variance of amino acid-N, NH_4-N and NO_3-N, respectively, occurring at scales < 2 m. Variation at larger scales (> 2 m) was not as significant but was still considered an important spatial component for all N forms. Fertiliser N application and removal of sheep had a small effect on the spatial variance of N forms. In the case of NO_3-N, the total accumulated variance was lower, with more of the observed variance attributed to scales > 2 m. The aggregate-scale sampling revealed further large variation at the sub 1-cm scale. Calculations based on the sampling showed that given a budget of £5000, the NO_3^-N field mean could be estimated with a 95% confidence interval of 1.69 µg N g^{-1} using 2 randomly positioned data loggers each with 5 sensors (based on units costs of data logger and sensor being £2000 and £200, respectively). These calculations assume that the sensor used would sense a similar volume of soil to that sampled by the 1 cm soil corer. Sensors with sensing elements < 1 cm will be subject to further spatial variability and local replication at scales < 1 cm would be needed to maintain accuracy. Therefore, achieving accurate estimates of the field mean is likely to incur significant costs. Whilst the cost of technology is likely to decrease, investigation of how best to integrate this approach within a PA framework to improve NUE is still required.

1. Introduction

Improving nitrogen (N) use efficiency (NUE) remains one of the key challenges for global agriculture (Cassman et al., 2002; Robertson and Vitousek, 2009) and is essential for the success of sustainable intensification (Tilman et al., 2011). The deleterious environmental effects and economic costs of diffuse N pollution from farmland in Europe, where N has been applied in excess of crop requirement, are well documented (Sutton et al., 2011).

One often cited approach to reduce N losses and improve NUE, is to ensure synchronicity of N supply with crop demand (Shanahan et al., 2008; Robertson and Vitousek, 2009), although, achieving this in practice is challenging due to the complex nature of the soil-plant system. Precision agriculture (PA) attempts to address this issue by reducing uncertainties surrounding the measurement of key variables to determine optimum N fertiliser management (Pierce and Nowak, 1999; Dobermann et al., 2004). Key to the success of PA is the accurate assessment of within-field soil N status at a high spatial and temporal resolution to enable the variable rate application of N fertiliser. This approach allows areas of N deficiency and surplus to be addressed as well as in-season adjustment of fertiliser rates in accordance with current and predicted growing conditions. However, conventional soil sampling techniques, coupled with laboratory analysis are expensive, labour-intensive, and time-consuming and cannot provide real-time data of sufficient resolution to accurately inform PA management (Sylvester-Bradley et al., 1999; Kim et al., 2009).

A number of different approaches have been used to address this issue. Crop canopy sensing techniques, for determination of plant N status, are now in commercial use and can be used to inform variable rate fertiliser application (e.g. wheat, maize; Raun and Johnson, 1999; Diacono et al., 2013). Whilst the advantages of this approach in some situations have been evidenced (Diacono et al., 2013), plant N status and yield is the product of many variables and may not always correlate with soil mineral N status. On-the-go soil sampling for nitrate, using electrochemical sensor platforms attached to agricultural vehicles have been developed (Adsett et al., 1999) and, for the case of pH, commercialised (Adamchuk et al., 1999). The results have been used to develop field nitrate maps (Sibley et al., 2009) which could be used to define within-field management zones and to calculate variable fertiliser application rates. On-the-go sampling is generally more spatially intensive than manual field sampling, allowing better spatial resolution, although key information on how soil mineral N varies over small spatial scales may not be obtained. This can lead to increased uncertainties of interpolative predictions, especially if the sample volume is small (Schirrmann and Domsch, 2011). Furthermore,

increasing the temporal resolution of this approach requires additional economic costs and as both these approaches rely on reactive management, crucial changes in soil mineral N status may be missed.

One approach, which has yet to be explored, is the use of in-situ sensors capable of monitoring soil mineral N in real time. This approach may enable a step away from predetermined fertiliser N recommendations (Defra, 2010) to a more dynamic system that responds in real-time to changes in growing conditions. It potentially has many benefits compared to on-the-go soil sampling and crop canopy sensing. The data provided by in-situ sensors will be of significantly higher temporal resolution, negating the need for repeated sampling surveys throughout the year, which represent an economic cost to the farmer. Furthermore, this may enable more accurate timing of fertiliser application, reducing the risk of yield penalties caused by N-nutrition deficiencies, and reducing the risk of N transfers to water and air as a result of excessive fertiliser N applications. It is also likely that the high resolution data generated by an in-situ sensor network will increase knowledge of the controls of soil N processes and thus enable development of models which allow for a proactive approach to fertiliser N management.

At the time of writing, there are no such sensors in use commercially. However, potential for the development and deployment of such sensors exists (Shaw et al., 2014). Ion-selective electrodes have many characteristics suitable for soil sensing networks. They are relatively cheap, simple to use, require no mains electrical power supply and the concentration of the target ion can be easily calculated via a pre-calibration. Nitrate (NO_3^-) selective electrodes have previously been successfully deployed for monitoring streams and agricultural drainage ditches (Le Goff et al., 2002; Le Goff et al., 2003) as well as for on-the-go soil sampling of agricultural soils (Sinfield et al., 2010) and on-farm rapid tests for soil NO_3^- (Shaw et al., 2013). Direct soil measurement, which is essential for the success of in-situ monitoring, has been shown to be possible (Ito et al., 1996; Adamchuk et al., 2005), although improvements in accuracy and robustness of the sensing membrane are required. Increasing use of nano technologies for the construction of electrochemical sensors may result in significant advances in sensor performance (Arrigan, 2004; Atmeh and Alcock-Earley, 2011).

Optimising the spatial configuration of a sensor network is needed to ensure that precise estimate of the mean across a field or management zone can made whilst minimising economic costs. It is, therefore, important to make an assessment of the spatial variation of soil mineral N across a range of scales to determine an optimum configuration prior to implementation of the sensor network. This is particularly important in grazed grasslands where N returns from

livestock occur unevenly (Bogaert et al., 2000). Since sensors must be organized in clusters around a hub with a logger, sensor networks can be regarded as multi-scale sampling schemes with hubs, the primary units, and sensors (secondary units) randomly placed in an area around each hub. As shown by de Gruijter et al. (2006), the optimum configuration of such a sampling scheme depends on the relative costs of additional primary and secondary units and the within- and between-primary unit variability. For example a field which has little large-scale variation may be served by a collection of sensors connected to one sensing hub, whereas a field with more variation at larger scales would require sensors to be located in multiple areas of the field. When determining an optimal configuration it is also important to consider the degree of uncertainty of the resulting estimations and the associated cost-benefit of reducing this uncertainty. Consideration also needs to be made as to whether the scale and magnitude of the observed in-field N variation is large enough to justify spatial variation in the optimum fertiliser input rate and, hence, the demarcation of within-field management zones.

As seen in the discussion above, the feasibility and optimal design of sensor networks depends on the variability of the target properties at different within-field scales. An effective way to collect such information is by spatially nested sampling. This was first used in soil science by Youden and Mehlich (1937) and rediscovered as a technique for investigation of multiscale soil variation by Webster and Butler (1976). In nested spatial sampling, sample sites are arranged in a nested hierarchical design which allows the partition of the variance of the measured variable into components associated with a set of pre-determined scales. At the highest level of the hierarchy sample, points are arranged in clusters associated with "main stations" which may be at randomly-located sites or on nodes of a grid or transect. Within a mainstation, sample points may be divided between two or three stations at level 2 which are separated from each other by some fixed distance. For example, the stations at level 2 may be on the vertices of an equilateral triangle with sides length d_2 m, or at two locations d_2 m apart. While these distances are fixed, the orientation of the level-2 stations relative to each other is randomized to ensure lack of bias. Within each level-2 station, sample points may be ordered at further nested spatial scales.

This approach has been used to investigate the distribution of nematodes in soil at within-field scales (Webster and Boag, 1992), to examine the variation of ammonia volatilization from urea amended soil at within-field to landscape scales (Corstanje et al., 2008), to quantify regional-scale variation of metal concentrations in soil (Atteia et al., 1994), and to examine the interactions of soil and herbicide at within-field scale (Price et al., 2009).

Recently, Lark (2011) showed how setting nested sampling in the linear mixed model framework allows the nested sampling scheme to be optimized in different circumstances.

The aim of this study was to investigate the spatial variation of plant-available N (amino acids, ammonium (NH_4^+) and NO_3^-) concentrations in soil within a grassland field over a 2 month period. A geo-spatial statistical approach was used to quantify the observed variation and the results were used to explore how an in-situ soil N sensor network may be optimally designed and deployed. The potential and challenges of integrating this approach within a PA framework are discussed.

2. Materials and methods

2.1. Field site and soil characteristics

The field used for this study is located within the Henfaes Research Station Abergwyngregyn, Wales, UK (53°14′N 4°01′W). The site has a temperate, oceanic climate, receives an average annual rainfall of 1250 mm and has a mean annual soil temperature at 10 cm depth of 11 °C. The field is roughly rectangular with a perimeter of 559 m and an area of 1.91 ha. It has an average altitude of 12.1 m asl with a slope of 1.5 % in a northerly aspect. It is a semi-permanent sheep-grazed grassland, dominated by *L. perenne* L. The current ley was established by direct drill in April 2009 using a perennial and hybrid ryegrass mix. The field has been used for both all year round grazing and silage production since 2009, receiving an annual inorganic fertiliser input of between 100 – 130 kg N ha^{-1} in addition to potassium (K), phosphate (P) and sulphur (S) at recommended rates. Lime has also been applied when necessary to restore the pH to a target value of 6.5. In 2014, inorganic fertiliser was applied on 12/5/14 and 11/7/14 at a rate of N:P:K 50:10:10 and 60:4:0 kg ha^{-1}, respectively. The field was grazed until 9/6/14 and the field remained sheep free until the 2/9/14. The soil is a free draining Eutric Cambisol with a sandy clay loam texture and a fine crumb structure.

To assess the chemical characteristics of the soil, replicate samples (n = 4) were collected from 4 blocks (30 × 30 cm) at a depth of 0 – 10 cm, representing the Ahp horizon. The soil was placed in gas-permeable polyethylene bags and transported to the laboratory in a refrigerated box. All of the following procedures were performed on the same day as field sampling. Soil pH and electrical conductivity were determined in a 1:2.5 (w/v) soil:distilled water suspension using standard electrodes. Moisture content was determined by drying for 24 h at 105 °C. Total C and N were determined with a TruSpec CN analyser (Leco Corp., St Joseph, MI, USA). Dissolved organic carbon (DOC) and dissolved organic nitrogen (DON)

were measured in soil extracts (0.5 M K_2SO_4, 1:5 w:v) using an Analytik Jena Multi N/C 2100S (AnalytikJena, Jena, Germany). Chloroform fumigation and incubation (t = 7 days) of 2 g (n = 4) of fresh soil was performed to determine microbial biomass C and N according to Voroney et al. (2008) (K_{EC} = 0.35 K_{EN} = 0.5). Exchangeable cations were extracted using 0.5 M acetic acid (Sparks, 1996) and the filtered extracts analyzed using flame emission spectroscopy (Sherwood 410 flame photometer; Sherwood Scientific, Cambridge, UK). Extractable phosphorus (P) was determined by extraction with 0.5 M acetic acid with subsequent colorimetric analysis using the molybdate blue method of Murphy and Riley (1962). Basal soil respiration was determined in the laboratory at 20 °C using an SR1 automated multichannel soil respirometer (PP Systems Ltd., Hitchin, UK) and steady state CO_2 production rates recorded after 24 h. Potentially mineralisable N was determined using an anaerobic incubation method based on Keeney (1982). Briefly, 5 g field moist soil was place in a 50 ml centrifuge tube, which was then filled to the top with de-ionized H_2O and the tubes sealed. Soils were subsequently incubated in the dark at 40 °C for 7 d. The difference in NH_4^+ content between t = 0 and t = 7 d was attributed to N mineralization. Above ground biomass was sampled on 26/6/2014. Replicate 1 × 1 m blocks (n = 4) were chosen at random from within the field. The vegetation was cut to ground level, stored in paper bags and subsequently oven-dried at 80 °C to determine dry matter content. A summary of the results are shown below in Table 1.

Table 1. Background properties of the agricultural grassland Eutric Cambisol used in the study. Values represent means ± SEM (n = 4). All values are expressed on a dry weight soil basis.

Soil property	Mean ± SEM
pH	6.57 ± 0.05
EC (µS cm^{-1})	26.5 ± 1.0
Basal soil respiration (mg CO_2 kg^{-1} h^{-1})	12.61 ± 1.04
Total soil C (g C kg^{-1})	25.35 ± 1.47
Total soil N (g N kg^{-1})	2.95 ± 0.06
Soil C:N	8.62 ± 0.64
DOC (mg C kg^{-1})	70.08 ± 2.57
DON (mg N kg^{-1})	10.48 ± 1.07
Mineralisable N (mg N d^{-1} kg^{-1})	3.92 ± 0.54
Microbial C (g C kg^{-1})	1.03 ± 0.10
Microbial N (g N kg^{-1})	0.16 ± 0.01
Exchangeable Ca (mg Ca kg^{-1})	501 ± 122
Exchangeable K (mg K kg^{-1})	46.05 ± 12.61
Exchangeable Na (mg Na kg^{-1})	25.43 ± 5.13
Available P (mg P kg^{-1})	7.38 ± 2.02
Above ground biomass (t DM ha^{-1})	1.56 ± 0.14

2.2. Sampling design and protocol

Nested sampling for spatial variability

The sampling was designed in the light of the following considerations. The aim was to characterize the variability of forms of soil N at a range of spatial scales relevant to planning the design of an in-situ sensor network. In particular, it was necessary to examine the relative importance of variance between and within local regions each of which might be represented by a cluster of NO_3^- electrodes or similar sensors deployed around a single data logger such that the maximum distance between any two sensors is about 2 m. In a grassland environment it was expected that one source of variation would be urine patches of linear dimensions about 40 cm (Selbie et al., 2015). Otherwise we had no prior information on the likely distribution of variance between the scales of interest.

Given these considerations we planned a nested sampling design with length scales within each main station of 1 cm, 10 cm (intermediate between the fine scale and urine patch scale), 50 cm (urine patch scale) and 2 m (upper bound on the "within-region served by a sensor cluster" scale). We distributed mainstations by stratified random sampling with the target field

divided into four quarters (strata) of equal area. Four mainstations were established at independently and randomly-selected locations within each quarter (stratum), giving a total of 16 mainstations. The design of the sampling scheme within each mainstation was obtained by the optimization procedure of Lark (2011) on the assumption of a fractal or quasi-fractal process in which the variance is proportional to the log of the spatial scale. The objective function was the mean estimation variance of the variance components. Figure 1 shows the optimized sample design. With 12 samples per mainstation the total sample size was 192. The sample sites were then selected at each mainstation by randomizing the direction of the vectors between the substations at each level of the design shown in Figure 1, while keeping the lengths of the vectors fixed. An initial nested sampling campaign was performed over 2 days on the 4th & 5th June 2014. This was repeated on the 31st July and 1st August 2014, 3 weeks after the field received a N fertiliser input of 60 kg N ha^{-1}. Sample site locations were set up the day before sampling took place. At each sampling location a soil corer, of diameter 1 cm, was used to sample soil. A 5 cm soil core from between depths of 5 -10 cm was sampled and placed in gas-permeable plastic bags, and stored in a refrigerated box. This depth was chosen as it represents the middle of the rooting zone and would make installation of any in-situ sensor a straight forward process. Following the sampling event the samples were transferred immediately to the laboratory where they were refrigerated at 4 °C. Extraction of soluble N from soil was performed on the soil cores on the same day as sampling as described below. During the second nested sampling event, duplicate sub-sampling and chemical analysis were performed on 4 out of the 12 samples from each mainstation in order to make an assessment of measurement error.

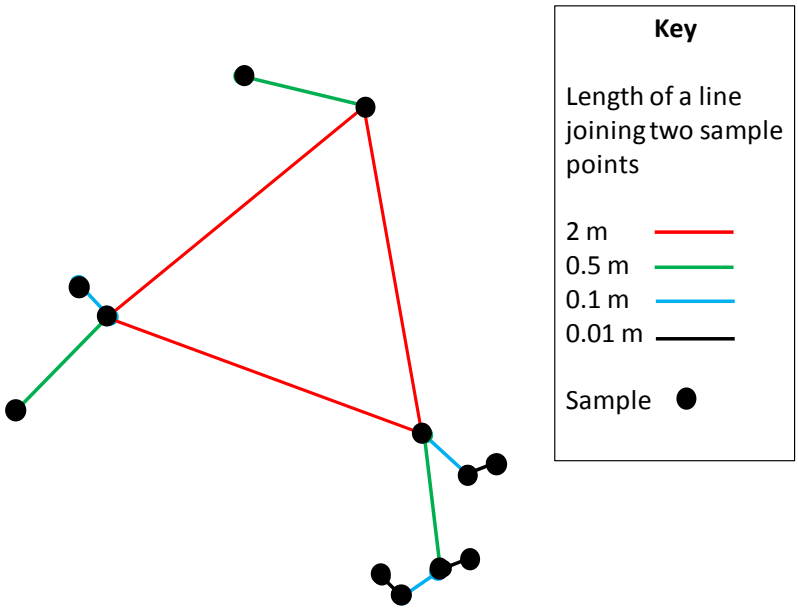

Figure 1. The optimised sampling design of a mainstation. Distances between sampling points were fixed but angles were randomized, with the exception of the 2 m vectors.

To investigate spatial variability of N forms at the sub-core scale a further sampling design and protocol was developed and performed on the 25th June 2014. Two sampling locations were chosen at random within each of the 4 strata. At each location, a pair of samples were taken, using the protocol described above, with a distance of 1 cm between each sample. This resulted in a total of 16 core samples. In the laboratory the cores where broken apart and 4 "aggregates" of weight 60 – 80 mg were collected. These aggregates were then soluble N extracted and analysed using the protocol described below.

Investigating depth effects

To investigate the variability of forms of N with depth an equilateral triangle, with sides 50 cm, was randomly located within each strata. The 50 cm scale was chosen as the initial nested sampling showed that it encompassed most of the variance for all N forms. At the vertices of the triangle, a core was taken to 25 cm depth and was subsequently split into 5 cm sections, giving a total of 60 samples. This sampling was performed on the 27th June 2014.

2.3. Extraction and chemical analysis of soil samples

All soil extractions were performed on the same day as sample collection, according to the following protocol. Samples were crumbled by hand, in order to prevent sieving induced N mineralisation (Jones and Willett, 2006; Inselsbacher, 2014). Large stones, roots and vegetation

were removed prior to gentle mixing of the sample. To further reduce mineralisation of organic N forms, sub-samples of field-moist soil (2 g) were extracted on ice (175 rev min^{-1}, 15 min) using cooled (5 °C) 0.5 M K_2SO_4 at a soil: extractant ratio of 1:5 (w:v) (Rousk and Jones, 2010). The extracts were centrifuged (4,000 *g*, 15 min), and the resulting supernatant collected and frozen (-18°C) to await chemical analysis. The protocol differed slightly for the soil aggregate samples. Each aggregate, of weight 60 – 80 mg, was placed in a 1.5 ml Eppendorf micro-centrifuge tube and crumbled gently using a metal spatula. The soil was then extracted in 500 µl of 0.5 M K_2SO_4 as described above. Total free amino acid-N was determined by the *o*-phthaldialdehyde spectrofluorometric method of Jones et al. (2002). NH_4-N was determined by the nitroprusside colorimetric method of Mulvaney (1996) and NO_3-N by the colorimetric Griess reaction of Miranda et al. (2001).

2.4. Statistical analysis

Nested Sampling

The *n* data from the nested sampling may be analysed according to the following statistical model. An $n \times 1$ vector of observations, **y**, is regarded as a realization of a random variate, Y, where

$$Y = X\beta + U_s\eta_s + U_m\eta_m + U_2\eta_2 + U_{0.5}\eta_{0.5} + U_{0.1}\eta_{0.1} + U_{0.01}\eta_{0.01} + U_r\eta_r. \quad (1)$$

X is a $n \times p$ design matrix which represents fixed effects in the model (e.g. p levels of a categorical factor, or p continuous covariates) and β is a length-p vector of fixed effects coefficients. There are 4 strata in the sampling design, and U_S is a $n \times 4$ design matrix for the strata. If the ith observation is in stratum j then $U_S[i, j] = 1$ and all other elements in the ith row are zero. The design matrix associates each observation with one of 4 random values in the random variate η_s. These values are assumed to be independent and identically distributed Gaussian random variables with a mean of zero and a variance σ^2_s which is the between-stratum variance component. Similarly U_m is a $n \times 16$ design matrix for the mainstations, and the variance of η_m is the between-mainstation variance component. The terms with subscripts 2, 0.5. 0.1 and 0.01 represent the design matrices and random effects for the components of variation associated with the 2-m, 0.5-m, 0.1-m and 0.01-m scales respectively. If duplicate material from some or all of the soil specimens is analysed then the random effect η_r which represents the variation due to subsampling and analytical variation can be estimated, otherwise it is a component of the variation estimated for the finest spatial scale.

Under the linear mixed model Y has covariance matrix H where

$$\mathbf{H} = \sigma^2_s \mathbf{U}_s^T\mathbf{U}_s + \sigma^2_m \mathbf{U}_m^T\mathbf{U}_m + \sigma^2_2 \mathbf{U}_2^T\mathbf{U}_2 + \sigma^2_{0.5} \mathbf{U}_{0.5}^T\mathbf{U}_{0.5} + \sigma^2_{0.1} \mathbf{U}_{0.1}^T\mathbf{U}_{0.1} + \sigma^2_{0.01} \mathbf{U}_{0.01}^T\mathbf{U}_{0.01} + \sigma^2_r \mathbf{U}_r^T\mathbf{U}_r, \quad (2)$$

and the superscript T denotes the transpose of a matrix. The parameters of this matrix are therefore the variance components, and these can be estimated by residual maximum likelihood (REML), see Webster & Lark (2013). Once this has been done then the fixed effects coefficients in the model can be estimated by generalized least squares (see Lark & Cullis, 2004). Note that there is an explicit assumption that the data are a realization of a Gaussian random variable. For this reason the values were transformed if exploratory analysis suggested that this is not a plausible assumption.

Because all sampling could not be done in one day the sampling day was randomized within strata, so as not to be confounded with the spatial variance components of interest. For this reason it is regarded as a fixed effect in the model. The significance of the between-day effect was tested with the Wald statistic — Lark & Cullis (2004) discuss this.

The significance of a random effect in the model can be tested by comparing the residual log-likelihood for a model with the term dropped (L^-) with the residual log-likelihood for the full model (all random effects, L). Any variance accounted for by a term which is dropped will contribute to variance at lower levels in the hierarchy (finer spatial scales) for the dropped model. For this reason the ultimate component of the model (η_r when there are duplicate analyses and $\eta_{0.01}$ otherwise) cannot be dropped. Dropping a term from the model will usually reduce the log-likelihood (and will not increase it). Whether the reduction in likelihood is strong enough evidence that the inclusion of the term in the full model is justified can be assessed by computing Akaike's information criterion *AIC* for each model:

$$AIC = -2L + 2P \quad (3)$$

where P is the number of parameters in the model. The *AIC* penalizes model complexity, by selecting the model with smaller AIC, one minimises the expected information loss through the selection decision (Verbeke & Molenberghs, 2000).

Aggregate scale sampling

After any necessary transformations the n data collected to investigate variation within cores were analysed according to the following statistical model. An $n \times 1$ vector of observations, y, is regarded as a realization of a random variate, Y, where

$$\mathbf{Y} = \mathbf{X}\beta + \mathbf{U}_s\eta_s + \mathbf{U}_p\eta_p + \mathbf{U}_c\eta_c + \mathbf{U}_a\eta_a, \quad (4)$$

As in Equation (1), X is a design matrix for fixed effects and β is a vector of fixed effects coefficients (here just a constant mean). Again, as in Equation (1), U_s is a $n \times 4$ design matrix for the strata and η_s is assumed to be an independent and identically distributed Gaussian random variate with a mean of zero and a variance σ^2_s. In the same way U_p and η_p are the design matrix and the random variate for the between-pair within stratum effect, with variance σ^2_p; U_c and η_c are the design matrix and the random variate for the between-core within-pair component, with variance σ^2_c and U_a and η_a are the design matrix and the random variate for the between-aggregate within-core component, with variance σ^2_a. This latter component is effectively the residual as there are no duplicate measurements on any aggregate. The same method based on the AIC was used to assess the evidence for including each term in the model above the between-aggregate effect.

Depth sampling

After any necessary transformation the data were analysed by a nested linear mixed model of the form

$$Y = X\beta + U_s\eta_s + U_c\eta_c + \varepsilon. \quad (5)$$

As in previous models X is a design matrix for fixed effects, here associating each observation with one of the five depth increments. The fixed effects coefficients in β are therefore mean values of the target variable for each increment. As before U_s and U_c are design matrices for between-stratum and between-core-within-stratum random effects. The term ε is an identically and independently distributed random variable of mean zero, the residual. This model was fitted by REML, and Wald tests were used to test the null hypothesis of equal mean values for the depth increments

2.5. Design of sensor arrays

The variance components derived from the nested sampling were used to examine the theoretical performance of different configurations of an in-situ sensor array, where a cluster of n_e sensors are randomly located within a region of 2 m diameter around each of n_l data logging hubs, which are located by simple random sampling. The between-sensor within-logger component of variance can be approximated by

$$\sigma^2_{sens} = \sigma^2_2 + \sigma^2_{0.5} + \sigma^2_{0.1} + \sigma^2_{0.01}, \quad (6)$$

and the between-logger variance by

$$\sigma^2_{log} = \sigma^2_s + \sigma^2_m. \quad (7)$$

As such, the standard error of the mean can be estimated as follows:

$$\sigma_{mean} = \{(\sigma^2_{log} / n_l) + (\sigma^2_{sens} / n_l n_e)\}^{½} . \quad (8)$$

The 95% confidence interval of the mean could therefore be calculated given variance components and particular values of n_l and n_e and the limits were back-transformed to the original units of measurement.

3. Results

3.1 Nested sampling to evaluate the spatial distribution of soluble N in soil prior to application of N fertiliser

Table 2 shows summary statistics for amino acid, NH_4^+ and NO_3^- concentrations in soil for the nested sampling undertaken over 2 days in June. Note that in all cases a Box-Cox transformation was chosen, conditional on sampling day as a fixed effect. Variance components for the different spatial scales are shown in Table 3 and the accumulated variance components, from the smallest to largest scale, are plotted in Figure 2. In total, 192 samples were collected and processed over 2 days.

Table 2. Summary statistics describing the spatial variability of soluble N derived from the nested sampling of a grassland soil prior to the application of N fertiliser. Alongside the raw data, an estimate of the Box-Cox transformation parameter (λ) is also provided.

Variable (μg N g^{-1})	Mean	Median	Skewness	Minimum	Maximum	λ
Nitrate-N	1.71	1.10	5.41	0.29	22.51	-0.426
Ammonium-N	1.87	1.27	12.82	0.29	80.49	-0.541
Amino acid-N	1.44	1.39	3.37	0.65	5.20	-0.492

Table 3. Variance components for the (Box-Cox transformed) variables and associated Wald tests describing the spatial variability of soluble N derived from the nested sampling of a grassland soil prior to the application of N fertiliser. The Wald statistic and associated p-value describe differences between the two sampling days. Those variance components marked with an asterisk are ones which caused an increase in AIC if they were dropped from the model (finest scale cannot be dropped).

Variable	Variance component						Wald statistic	*p*-value
	σ^2_s	σ^2_m	σ^2_2	$\sigma^2_{0.5}$	$\sigma^2_{0.1}$	$\sigma^2_{0.01}$		
Nitrate-N	0.0629*	0.0362*	0.0	0.0795*	0.0937*	0.0628	0.001	0.974
Ammonium-N	0.0087	0.0121*	0.0078	0.00008	0.0153	0.0751	6.8	0.009
Amino acid-N	0.0058	0.0035*	0.0	0.0	0.0124*	0.0307	1.89	0.17

The results show that there was a significant difference (as assessed by the Wald statistic) between the 2 sampling days for NH_4-N concentration only. The different forms of N showed slightly different scale-dependencies, although in general, short-range variance dominated. For amino acid-N, the 1-cm scale had the largest variance component, constituting 58.6% of the total accumulated variance. The 10-cm and the between-mainstations within strata term were important spatial components as they were judged by the AIC to improve the likelihood of the model sufficiently to justify their inclusion. Similarly, for NH_4-N the 1-cm scale had the largest variance component, constituting 63.0% of the total accumulated variance. However, for spatial scales greater than 1 cm, only the between-mainstations within strata term was important as judged by the AIC. For NO_3-N, there was more variance at larger scales compared to the other forms of N, with the 10-cm scale having the largest variance component, constituting 28.0% of the total accumulated variance. Furthermore, all the spatial scales, with the exception of the 2-m scale, exhibit variance that was considered important by the AIC. Short-range scale variation still dominated though, with 70.4% of the variance occurring at spatial scales up to 50 cm. It should be noted that the 1-cm scale component will also include any measurement error.

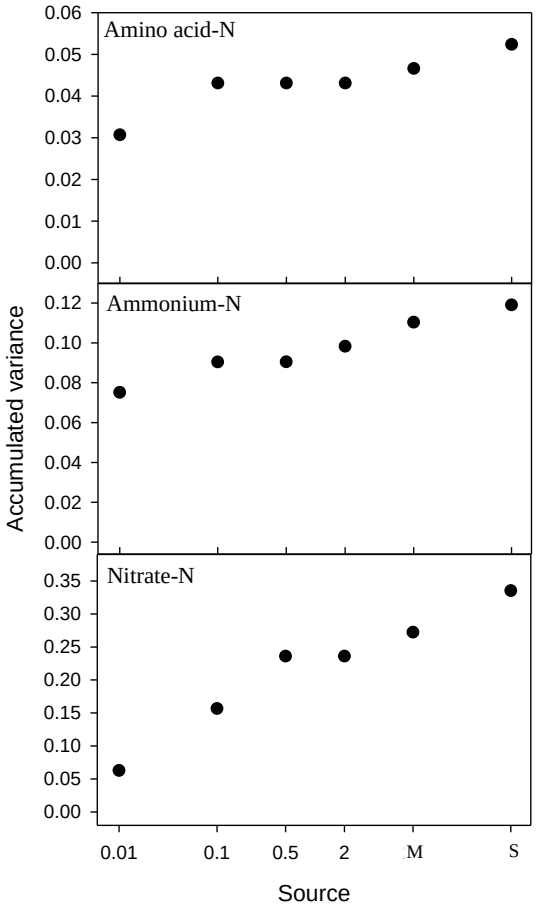

Figure 2. Accumulated variance components from the finest to coarsest spatial scale, derived from 5th and 6th June nested sampling results (before fertiliser addition). Source is the spatial-component in meters, with M and S representing the between-mainstation and between-strata components respectively.

3.2. Nested sampling to evaluate the spatial distribution of soluble N in soil after application of N fertiliser

To assess the influence of nutrient management regime, a second nested sampling was undertaken in July, 3 weeks after the field had been fertilised with 60 kg N ha^{-1}. Summary statistics for soluble N in soil are shown in Table 4 and variance components for the different spatial scales are shown in Table 5.

Table 4. Summary statistics describing the spatial variability of soluble N derived from the nested sampling of a grassland soil after the application of N fertiliser. Alongside the raw data, an estimate of the Box-Cox transformation parameter (λ) is also provided.

Variable ($\mu g\ N\ g^{-1}$)	Mean	Median	Skewness	Minimum	Maximum	λ
Nitrate-N	1.36	1.25	0.89	0.26	3.45	0.103
Ammonium-N	1.96	1.71	3.28	0.26	9.88	-0.424
Amino acid-N	1.25	1.18	2.58	0.56	4.40	-0.481

Table 5. Variance components for the (Box-Cox transformed) variables and associated Wald tests describing the spatial variability of soluble N derived from the nested sampling of a grassland soil after the application of N fertiliser. The Wald statistic and associated p-value describe differences between the two sampling days. Those variance components marked with an asterisk are ones which caused an increase in AIC if they were dropped from the model (finest scale cannot be dropped).

Variable	Variance component							Wald statistic	*p*-value
	σ^2_s	σ^2_m	σ^2_2	$\sigma^2_{0.5}$	$\sigma^2_{0.1}$	$\sigma^2_{0.01}$	σ^2_ε		
Nitrate-N	0.0	0.0575*	0.0	0.0052	0.0472*	0.0263*	0.0121	8.09	0.0045
Ammonium-N	0.0039	0.0069*	0.0	0.0	0.015	0.045*	0.0109	15.43	8.60×10^{-15}
Amino acid-N	0.002	0.0241*	0.0025	0.0	0.0086*	0.0199*	0.0103	0.708	0.4

The accumulated variance components, from the smallest to largest scale, are plotted in Figure 3. The same protocol was used as for the first nested sampling with the addition of duplicate measurements on 4 samples from each mainstation. This allowed the 1-cm spatial variance component to be resolved from the subsampling and measurement error, which is the residual variance in this analysis. As this forms the ultimate term in the model, it allows an assessment of the importance of the 1-cm spatial component by the AIC. For all of the N forms, the 1-cm scale was considered important by the AIC, and was larger than the residual variance. However, the residual variance, which was similar for all N forms, constitutes a substantial component of the accumulated variance and was, for all N forms, larger than the variance at 50 cm and 2 m. The different forms of N showed slightly different scale-dependencies, although in general short-range variance dominated. For amino acid-N the between mainstations within strata had the largest variance component, constituting 35.8% of the total accumulated variance, although 57.7% of the total accumulated variance occurred at scales up to 10 cm. The 1-cm, 10-cm and the between-mainstations within-strata term were important as they are judged by the AIC to

improve the likelihood of the model sufficiently to justify their inclusion. For NH_4-N the 1-cm scale had the largest variance component, constituting 55.1% of the total accumulated variance. Spatial scales greater than 10 cm accounted for only 13.3% of the total accumulated variance. Only the 1-cm and the between-mainstations within-strata terms were important as judged by the AIC. For NO_3-N, the variance at larger scales was similar to that of amino acids, with the the between-mainstations within strata scale having the largest variance component, constituting 38.7% of the total accumulated variance. As for amino acid-N, the 1-cm, 10-cm and the between-mainstations within-strata term were important as they are judged by the AIC. Short-range scale variation still dominated though, with 61.2% of the variance occurring at spatial scales up to 50 cm.

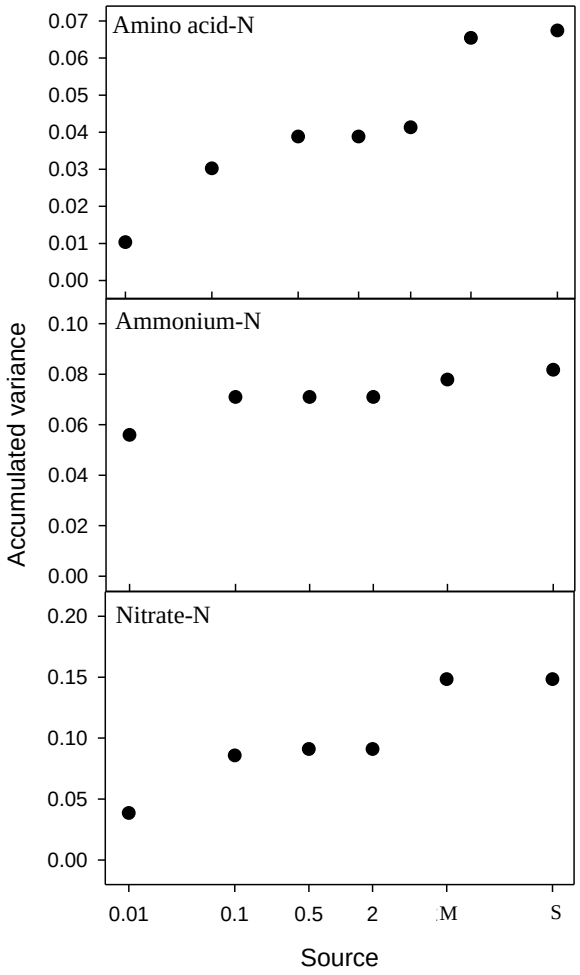

Figure 3. Accumulated variance components from the finest to coarsest scale, derived from 31st July and 1st August nested sampling results (after fertiliser addition). Source is the spatial-component in meters, with M and S representing the between-mainstation and between-strata components respectively.

3.3. Aggregate-scale variability of soluble N in soil

The grassland soil is characterized by small aggregates (ca. 1-2 mm diameter) relative to the size of the bulk soil cores used in the nested samplings (1 cm diameter). Table 6 shows the summary statistics for the within-core, aggregate scale variability while the variance components are shown in Table 7.

Table 6. Summary statistics describing the aggregate-scale variability of soluble N within a grassland soil. An estimate of the Box-Cox transformation parameter (λ) is also provided.

Variable ($\mu g\ N\ g^{-1}$)	Mean	Median	Skewness	Minimum	Maximum	λ
Nitrate-N	1.20	1.04	0.80	0.19	3.13	0.282
Ammonium-N	2.00	1.78	1.24	0.30	5.85	0.200
Amino acid-N	1.56	1.50	0.49	0.77	2.69	0.508

Table 7. Variance components for the (Box-Cox transformed) variables describing the aggregates-scale spatial variability of soluble N in a grassland soil. Those variance components marked with an asterisk are ones which caused an increase in AIC if they were dropped from the model (finest scale cannot be dropped).

Variable	Variance component			
	σ^2_s	σ^2_p	σ^2_c	σ^2_a
Nitrate-N	0.0	0.0	0.072*	0.289
Ammonium-N	0.0	0.0311	0.005	0.3766
Amino acid-N	0.0	0.0055	0.0167*	0.0499

In all cases, the largest variance component was the between-aggregate within-core scale. For NH_4-N and NO_3-N, 91.3% and 80.1% respectively of the total accumulated variance occurred at this scale, which was an order of magnitude higher than the variance at the between core scale. The variance at the aggregate scale for amino acid-N was slightly lower at 69.2%. It should be noted that any analytical error that occurred will also appear in this variance component. The between-core component, which represents the 1-cm spatial scale, was important for amino acid-N and NO_3-N, but not NH_4-N, as judged by the AIC. Neither the between-pair component, which is similar to the between-mainstations scale, nor the between-strata component were found to be important as judged by the AIC. Due to the limited replication (2 pairs of cores per strata), the results at stratum and mainstation scale should be interpreted in light of the above nested sampling results. The main interest is in the relative

magnitude of the between-core within-pair and between-aggregate within-core components of variance.

3.4. Influence of depth of the variability of soluble N in soil

Table 8 shows summary statistics for residuals of the Box-Cox transformed data collected from different soil depths. All forms of N showed a decreasing trend down the soil profile (Figure 4). Table 9 presents the estimated mean values for all N forms (Box-Cox transformed) for each depth increment. There is a clear reduction in the concentration of each N form with depth. The variation with depth was significant for NH_4-N and NO_3-N ($p < 0.001$) but not for amino acid-N ($p = 0.55$).

Table 8. Summary statistics for the residuals of the Box-Cox transformed data describing the influence of soil depth on the vertical variability of soluble N within a grassland soil. An estimate of the Box-Cox transformation parameter (λ) is also provided.

Variable	Mean	Median	Skewness	Minimum	Maximum	λ
Nitrate-N	0	0.08	-1.29	-1.29	1.27	-0.343
Ammonium-N	0	0.05	-0.11	-0.83	0.84	-0.365
Amino acid-N	0	0.02	-0.04	-0.36	0.36	0.203

Table 9. Estimated mean values for all forms of soluble N (Box-Cox transformed units) for each depth increment, and the results of a Wald test of the null hypothesis of equality of the means at all depths.

Variable	Mean (transformed units) by depth interval (cm)					Wald statistic	*p*-value
	0-5	5-10	10-15	15-20	20-25		
Nitrate-N	0.103	-0.124	-0.397	-0.533	-0.766	45.9	2.6 × 10-9
Ammonium-N	-0.196	-0.364	-0.540	-0.624	-0.776	44.8	4.2 × 10-9
Amino acid-N	0.335	0.294	0.271	0.263	0.230	3.02	0.55

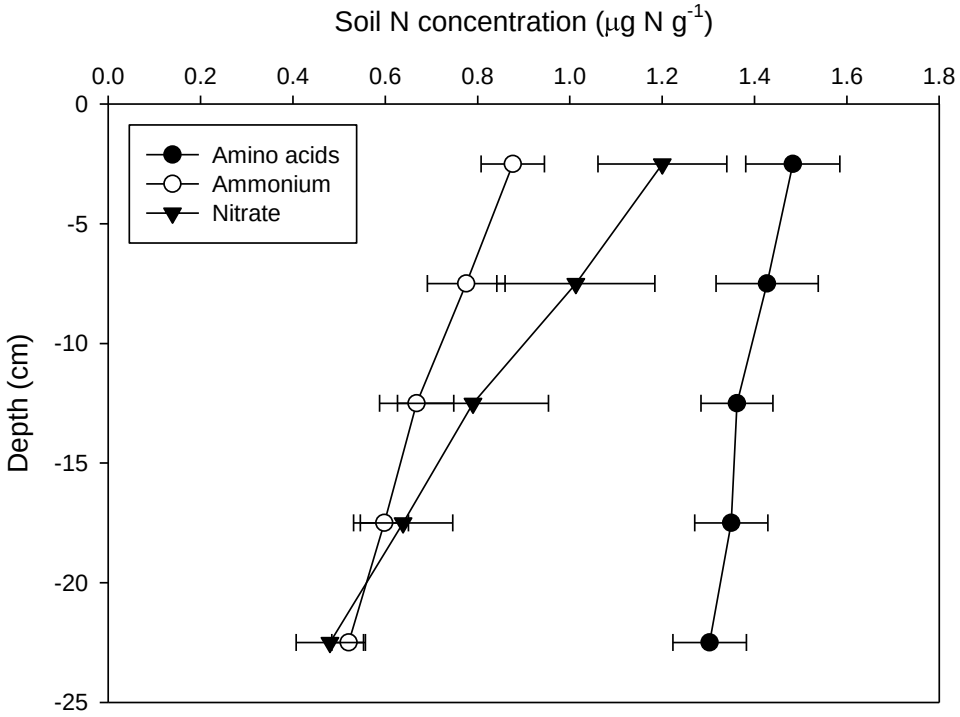

Figure 4. Variability of amino acid-N, ammonium-N and nitrate-N with depth in a grassland soil. Data points represent means ± SEM (n=12) of soil N concentration for each 5 cm depth increment.

3.5. Optimisation of a within-field sensor network for monitoring soluble N in soil

Figure 5 shows plots of the width of the 95% confidence interval for NO_3-N using variance components from the two sets of spatially nested samples. These were computed for n_l =1–10 loggers with n_e =2–15 sensors distributed equally among the loggers, 15 being the maximum number of sensor ports on the data logger (DL2e DeltaT, Cambridge, UK). The total cost for each configuration is shown on the abscissa of the plot, on the basis of unit costs of £2000 and £200 respectively for a data logger (DL2e DeltaT, Cambridge, UK) and an ELIT NO_3^- electrode with a coupled reference electrode (ELIT 8021, ELIT 003, Nico2000, Harrow, UK). The form of these curves reflects the distribution of the variance of NO_3-N over the spatial scales. Note that the width of the confidence interval is largest for the June observations, so discussion is focussed on these. Note also, as described in the Figure caption, that some points are excluded from the plot (small numbers of sensors on small numbers of loggers) to allow both graphs to be displayed with the same range of values on the ordinate.

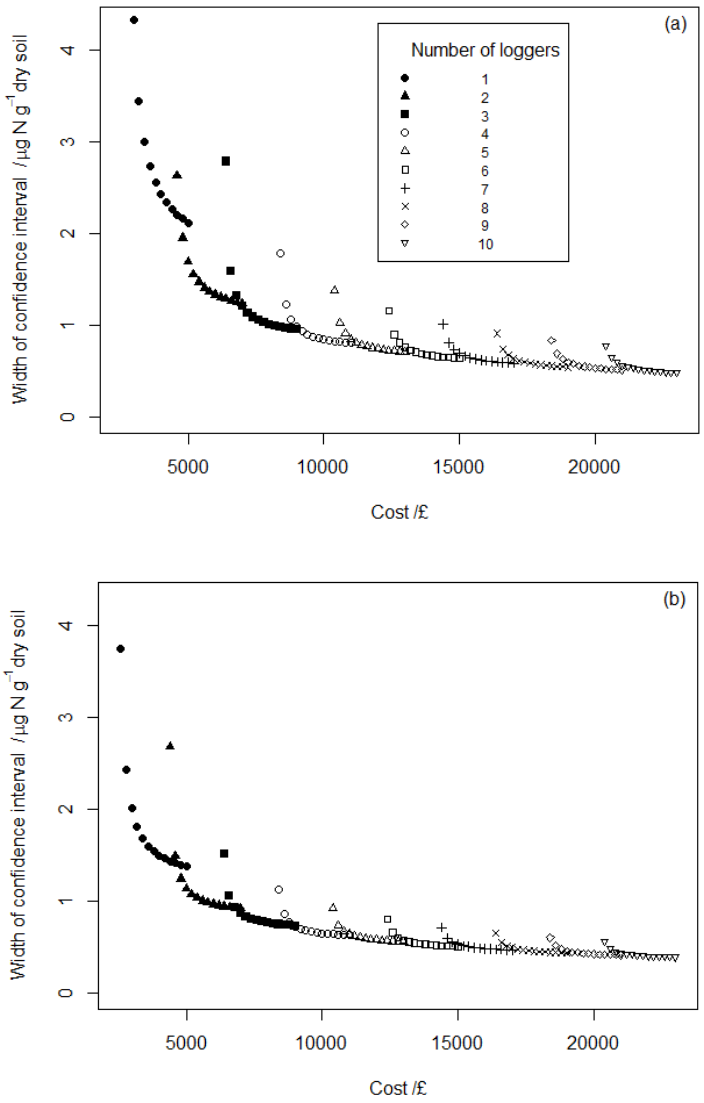

Figure 5. Width of the 95% confidence interval for alternative sensor arrays of different cost computed from variance components from nested sampling of nitrate N in (a) June (before fertiliser addition) and (b) July-August (after fertiliser addition). Note that the arrays comprise 1–10 loggers and a maximum of 15 sensors per logger. To allow a common range of values on the ordinates of these graphs, and to facilitate interpretation, arrays with fewer than five sensors in total have been excluded from Figure 5(a) and arrays with fewer than three sensors have been excluded from Figure 5(b).

The graphs show how both increasing the number of sensors per logger, and increasing the number of loggers, reduces the width of the confidence interval. Note that reducing this width substantially below 1 µg N g^{-1} dry soil would require substantial costs, with small marginal improvement on increasing the size of the array. To reduce the width of the

confidence interval to 0.5 µg N g^{-1} dry soil requires 10 loggers with 11 sensors each at a cost of £22,200.

The graphs allow different options for the design of arrays to be explored. If, for example, we required the width of the 95% confidence interval to be no wider than 1 µg N g^{-1} dry soil then the options include the use of 3 loggers with 11 sensors per logger, 4 loggers with 5 sensors per logger or 5 loggers with 4 sensors per logger. The costs of these options are £8,200, £9,000 and £10,800 respectively. This quality measure cannot be achieved with just one or two loggers. The rational choice of array configuration is therefore 3 loggers with 11 sensors on each. Consider an alternative situation where the budget was fixed at £5,000. This could be used to provide a single logger with 15 sensors on each, or two loggers with 5 sensors on each. The width of the confidence interval for these two options is ± 2.12 and ± 1.69 µg N g^{-1} dry soil respectively, so the second option is the rational choice.

The discussion above highlights that, with increasing budget, it is not necessarily rational to use the maximum number of sensors on a logger before changing to an array with an extra logger. However, once three or more loggers are in use, $n_l \geq 3$, an array of $15n_l$ sensors is always more efficient than any array of equal or lesser total cost with more than n_l loggers.

4. Discussion

4.1. Spatial variation of soluble N at within-field scales

An assessment of the spatial variation of amino acid-N, NH_4-N and NO_3-N at within-field scales was determined using a nested sampling approach. Short-range variation was found to be dominant, with at least 61%, 86% and 61% of the total accumulated variance amino acid-N, NH_4-N and NO_3-N, respectively, attributed to scales < 2 m. The aggregate-scale sampling revealed further large variation at the sub 1-cm scale, which was considerably higher than the variation attributed to the 1-cm scale for all N forms. Although short-range variation dominated, variation at larger scales was not negligible and the within-strata, between-mainstation scale was considered an important spatial component for all N forms.

It is likely that the observed variation at scales < 2 m is primarily due to the relatively random and uneven deposition of N from sheep excreta. Similar small-scale variation of NO_3-N in grazed pastures has been identified in previous studies, with semi-variograms exhibiting the range of spatial dependency < 5 m (White et al., 1987; Broeke et al., 1996; Wade et al., 1996; Bogaert et al., 2000), and a nugget variance of 60 % (Bogaert et al., 2000). These results

contrast with similar studies performed on arable soils, which were characterised by ranges of spatial dependencies for NO_3-N > 39 m (Van Meirvenne et al., 2003; Haberle et al., 2004).

Given that the linear dimension of a urine patch is approximately 40 cm, it is unlikely that the observed variation at the 1-cm and "aggregate" scales is driven by the uneven deposition of sheep excreta. Previous studies of spatial variation in soil N, in the context of within-field scales, have not investigated variation over such small scales. Variation at these scales is unlikely to have any significance for agronomic management as most soil sampling is conducted using large soil cores (ca. 2-10 cm diameter) with subsequent bulking of samples to ensure that small scale variation is encompassed. This small-scale variation is likely due to the inherent micro-heterogeneity of soil properties, for example, the abundance of plant roots and mycorrhizal hyphae (Stoyan et al., 2000), availability of labile organic matter (Parkin, 1987; Wachinger et al., 2000), earthworm channels and the composition and abundance of the microbial community (Grundmann and Debouzie, 2000; Nunan et al., 2002), which in turn will affect biogeochemical processes controlling soil N concentrations. The proportion of the total accumulated variance attributed to the 1-cm scale was much larger for amino acid-N and NH_4^+-N than NO_3-N which may be related to their relative diffusion coefficients, interactions with the solid phase (Owen and Jones, 2001) and the rapid rate of amino acid turnover and mineralisation in this soil (Jones et al., 2004; Wilkinson et al., 2014). The observed variation at larger spatial-scales within this study, could be due to the habit of sheep to frequent certain areas of the pasture such as paths, a drinking trough and areas of shade (Bogaert et al., 2000).

Variation with soil depth was also apparent, with all N forms showing a consistent reduction in concentration with increasing depth, although this was not considered a significant effect for amino acids. Decreasing concentrations of inorganic N down the soil profile is well characterised in the literature (Van Meirvenne et al., 2003; Wall et al., 2010) and can be attributed to inputs of N via leaf litter, rainfall, animal excreta and fertilisers to the soil surface as well as being the site where maximal root turnover exists.

There was also some suggestion of a spatio-temporal interaction as evidenced by small differences in the spatial dependencies of the N forms between the June and July nested sampling events. In the case of NO_3-N, the total accumulated variance was lower, with more of the observed variance attributed to scales > 2 m for the July sampling. This change may be attributed to the removal of sheep and the associated local inputs of N, combined with N fertilisation of the field (60 kg N ha^{-1}) that occurred 3 weeks prior to the second nested sampling event.

4.2. Optimisation of planning a within-field soil N sensor network

This study clearly demonstrates how nested sampling combined with geostatistical analysis can be used to explore how varying sensor-logger numbers and configurations affect the degree of accuracy of a field mean estimation. Furthermore, given knowledge of logger and sensor costs it is possible to rationalise planning decisions on a cost-accuracy basis. Given the unit costs of £2000 and £200 respectively for a data logger and NO_3^- ISE, the field mean for NO_3-N concentration could be estimated with a 95% confidence interval no wider than ± 1 μg N g^{-1} for a cost of £8,200. This would represent a significant cost to the farmer and may prevent anything significant uptake of the technology. The data logger and NO_3^- ISEs used for the cost calculation were chosen as they were used previously in this thesis (*Articles III* & *IV*). There is currently a wide range of similar devices, with a range of costs, currently on the market so the figures for the cost of implementing a sensor network described above should not be considered absolute. Furthermore, it is likely that the cost of the technology will continue to fall.

It is important to note that the data used for these calculations was derived from the nested sampling which used a soil corer of 1 cm diameter. As such, these calculations are based on the assumption that any given sensor used for the within-field network would have a similar sized sampling volume. Results derived from the aggregate-scale sampling exhibited variation at the sub 1-cm scale, which for NH_4-N and NO_3-N was an order of magnitude larger than the 1-cm scale. This will have significant implications when using sensors with sensing areas of size < 1 cm. More local replication at the sub 1-cm scale and hence an increase in the size of sensor arrays would be required for an acceptable level of accuracy to be achieved, resulting in increased costs. To explore optimisation of a network of sensors with sensing areas < 1 cm, further sampling using sample volumes of < 1 cm would be required. Ideally this would involve a similar level of replication, across all scales, to that which was used in the July nested sampling campaign. This evidence may also be quite instructive for optimising sensor design, as sensors with larger sampling areas will encompass more of this small-scale variation.

Within this optimisation, no consideration has been made to the observed depth effects. The resulting field mean is therefore, only applicable to the 5-10 cm depth. Rooting depth, and therefore, nutrient uptake, in fields adjacent to the study site has previously been observed to a depth of 30 cm (Jones et al., 2004). As such, any quantification of plant-available N derived from the sensor network should be adjusted for observed depth effects. In the case of cereals, which may root to depths >1.5 m, both topsoil and subsoil sensors will probably be required to

avoid bias and gain a representative pattern of soluble N within the field. Logistically, however, the deployment of sensors in subsoils represents a significant challenge.

4.3. Potential for precision agriculture

Integration of this approach with a PA framework requires some further consideration. PA, with regard to N management, has often focused on within-field spatial variation and the identification of management zones to enable variable rate fertilisation, which is often referred to as site-specific N management (SSNM) (Ferguson et al., 2002; Franzen et al., 2002; Bongiovanni and Lowenberg-DeBoer, 2004; Cui et al., 2008). As the field used in this study was broadly homogenous in its soil type and topography, which was reflected in the observed small variation of soil N at large scales, it was considered more suitable to treat the field as a single management unit with a singular mean value rather than impose MZs. However, the importance of temporal variation for N management should not be underestimated. Temporal variations in growing conditions, both within and between seasons may lead to considerable differences in optimum N fertiliser requirement and hence, inefficiencies in N fertiliser-use if temporal variations are not considered (Lark et al., 2003; McBratney et al., 2005; Shahandeh et al., 2005; Shanahan et al., 2008; Deen et al., 2014).

It is important to consider how the approach used in this study could be applied to field exhibiting significant random and non-random (i.e. a gradient) large-scale variation, which might benefit from SSNM. It is possible that management zones could be delimitated based on *a priori* knowledge of variables that may affect or indicate soil N status such as topography (Kravchenko and Bullock, 2000), soil type (Moral et al., 2011), yield variability (Diker et al., 2004) and farmers knowledge (Fleming et al., 2000). Alternatively, proximal or remote sensing, such as electromagnetic induction, may allow rapid and cost effective identification of large scale heterogeneity of soil physical properties (Hedley et al., 2004; King et al., 2005). However, the extent to which these variables correlate to soil N concentration is likely to be site specific and so may require some ground truthing. The need for management zones should become apparent from exploratory sampling but accurate delimitation of areas with significantly different soil N concentration may require sampling at a finer resolution by increasing the extent of the stratification accordingly. A further broad question which needs to be addressed with respect to management zones, is at what point does the magnitude and the spatial-scale of soil N variation become sufficiently large enough to justify site-specific agriculture?

The success of the approach used here to optimise a sensor network and that of any management zone based system requires temporal stability of spatial variation (Sylvester-Bradley et al., 1999; Shi et al., 2002). Given significant spatio-temporal interaction, the results from any sensor network could no longer be considered accurate or precise. In this study there was evidence of a slight spatio-temporal interaction which was related to the removal of sheep from the field and the application of N fertiliser. An alternative approach to that advocated here, would be the implementation of a grid network, with sensor arrays at each node to account for small-scale soil variation. This would enable temporal, large-scale spatial variation and their interaction to be monitored. Kriging techniques could then be used to produce dynamic maps of soil N concentrations which could be used to inform variable-rate fertiliser management. However, this approach is likely to require significantly more sensing units and hubs with a resulting cost increase.

5. Conclusions

In this study, the spatial variation of amino acids, NH_4^+ and NO_3^- within the soil of a grazed grassland field was investigated using a nested sampling approach and geo-statistical analysis. Variation at small scales (< 2 m) was shown to be dominant, with further large variance evident at scales < 1 cm. The observed variation was attributed to the random input of N to the soil via sheep excreta and the inherent heterogeneity of soil at the aggregate scale. Optimising the deployment of in-situ soil sensors, on the basis of accuracy and cost, was demonstrated using data derived from the nested sampling and showed that achieving accurate estimates of the field mean comes at a considerable cost. Whilst the cost of technology is likely to decrease, investigation of how best to integrate this approach within a PA framework to improve NUE is still required.

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Chapter 8

General discussion and future work

1. Discussion of experimental work

This PhD aimed to develop novel methods to enable real-time and in-situ measurement of soluble N in soil. Better quantification of soil N status is required to improve management of agricultural land receiving N fertilisers and manures, and increase our fundamental understanding of soil N processes. However, soil scientists, farmers, and agronomists are currently limited by a lack of non-destructive and user-friendly techniques that allow real-time and in-situ soil N determination. The research undertaken here set the following aims in order to address this issue:

6) To chronicle issues related to the use of N in agriculture and to review new approaches to soil N determination with a focus on in-situ monitoring (Chapter 2);
7) To investigate the use of microdialysis-based sampling for the determination of plant available N and in-situ monitoring of soil N dynamics (Chapters 3 & 4, *Articles I & II*);
8) To develop farmer-operated tools and methodologies which are user-friendly and could be used for the on-farm determination of soil N (Chapters 5 & 6, *Articles III & IV*).
9) To construct, develop and test a NO_3^- ISE for the real-time, in-situ monitoring of soil N (Chapter 6, *Article IV*).
10) To investigate how to optimise the field-scale configuration of an in-situ sensor network to facilitate both accurate and economical soil N monitoring (Chapter 7, *Article V*).

Below, the results and implications of the experimental work undertaken to satisfy the above aims are discussed.

Microdialysis is a technique that has been widely used in pharmacological research for the in-situ sampling of biological fluids (Nesbitt et al., 2013). Recently, several research papers have utilised this approach for the in-situ sampling of soil solution (Inselsbacher et al., 2011; Inselsbacher and Näsholm, 2012a). In *Article I*, we explored how microdialysis could be used to determine soil N availability. Diffusive-flux measurements of eight soils along a catena sequence were compared to conventional soil core batch extractions (using 0.5 M K_2SO_4 or distilled H_2O). The percentage contribution that amino acids, NH_4^+, and NO_3^- made to total plant-available N, were most similar to conventional distilled water extractions. However, the relative magnitude of the diffusive flux measurements did not always reflect the pool sizes as estimated by the soil extractions. Microdialysis was also used for in-situ sampling of amino acids, NH_4^+, and NO_3^- from the rhizospheres of *Zea mays* L. seedlings (*Article II*) grown in soil-filled rhizotubes. In a parallel experiment, direct sampling from root surfaces of seedlings

grown in sterile nutrient solution showed a significant spike of amino acid exudation from the zone 1 – 2 cm behind the growing root tip. However, this exudation spike was not identified by the microdialysis sampling from soil. This was attributed to extremely rapid uptake from the rhizosphere by both carbon-limited soil microbes and the roots themselves (i.e. resorption). The microdialysis sampling did show a significant decrease in soil solution NO_3^- concentration, which corresponded to the time the root tip grew past the probe and this was attributed to plant uptake.

These two studies highlight both the advantages of microdialysis sampling and its associated problems. It has been suggested that such a sampling procedure will better inform the availability of N for plant uptake as diffusion through the membrane is dependent not only on the concentration of the target solute but also its mobility through the soil, which, in turn, is dependent upon a range of physical and chemical variables (Inselsbacher and Näsholm, 2012b; Shaw et al., 2014). This is exemplified in *Article I*, where clear differences in N availability measured using pool size or diffusive flux were apparent across a wide range of soils differing in their chemical and physical soil properties. The other main advantages of the microdialysis approach is the small size of the probes and the ability to take multiple samples over an extended period time with minimal perturbation to the system being evaluated. This enables an assessment of soil N dynamics to be made with excellent spatial and temporal resolution as demonstrated in our study of rhizosphere N dynamics (*Article II*). This excellent spatial resolution can also be an issue as soil is an inherently heterogeneous medium, especially at small scales (Parkin, 1987; Nunan et al., 2002). This is often manifested in our microdialysis results as large errors around means, despite pre-sieving and mixing. This spatial variability has also been demonstrated in a previous microdialysis study (Inselsbacher et al., 2011).

The results of the microdialysis sampling were presented differently in *Article I* and *II*. In *Article I*, the diffusive-flux measurement was used, and in *Article II* absolute soil solution concentrations were calculated. Deriving soil solution concentrations from microdialysis relies on the application of a correction factor (i.e. the percentage recovery of N from a standard solution). It further assumes that this correction factor remains constant across a wide range of soils. It is highly likely that, due to inherent and obvious differences between soil and a standard solution, that the percentage recovery will vary between these mediums and between different soils (e.g. due to surface contact, inherent moisture content etc). However, results from *Article II* showed that the initial soil solution concentration of amino acids and NO_3^-, as assessed by centrifugal-drainage and microdialysis, were statistically similar. Whether this is true for other soils requires further investigation. The diffusive-flux measurement simply describes the rate

of solute diffusion into the microdialysis probe, which will be affected, not just by the absolute concentration, but also by a range of physical and chemical soil properties. As such, this method may better reflect N which is available for plant uptake. However, this measurement is biased towards solutes with a lower molecular weight, as these will diffuse most quickly across the microdialysis membrane creating a larger concentration gradient around the probe, and hence resulting in a faster rate of diffusion through the soil. The diffusive-flux is also dependent upon the type of microdialysis probe used, its molecular weight cut-off, pore size and the speed at which the perfusate is pumped through the probe. As such, absolute comparisons between different studies are difficult to make. Drawing conclusions for plant nutrition from the diffusive-flux measurement is also confounded by the modifying rhizosphere effect and active root uptake mechanisms (Shaw et al., 2014).

Whilst microdialysis may continue to offer new insights into soil N dynamics, using this approach for agronomic purposes will require considerable development prior to commercial adoption. Currently, the microdialysis samples need subsequent chemical analysis which precludes its on-farm use. It may be possible to combine microdialysis with an on-line measuring system but this will add further complexity and expense to a system that already requires a water reservoir and pump.

The literature review (Chapter 2) identified a lack of user-friendly tools that enable the on-farm quantification of soil N. As it became apparent that microdialysis is currently unsuitable for this application, the use of commercially available NO_3^- ISEs and UV spectroscopy for a soil NO_3^- rapid-test was investigated (*Article III*). Our results showed that manual extraction using distilled H_2O, combined with either NO_3^- ISEs or UV spectroscopy could accurately determine the NO_3^- concentration of the extracts. As such, both of these methods have the potential to be used as on-farm quick tests. Whilst UV spectroscopy has not previously been used in this context, the concept of on-farm rapid-tests are not new (Jemison & Fox, 1988; Hartz, 1994), but despite this, on-farm use is thought to be low. Using UV spectroscopy may require filtering of extracts prior to testing and using ISEs requires some pre-calibration. In addition, an assessment of soil moisture content is needed to calculate an accurate soil NO_3^- concentration. These issues, when combined with a lack of a suitable decision support system to generate fertiliser recommendations, perceptions of cost-benefit and farmer attitudes to new technologies may partially explain low uptake.

ISEs have many properties that are advantageous for in-situ soil monitoring. Previous work has demonstrated their use for direct soil measurements (Ito et al., 1996; Adamchuk et al., 2005) but until now there has been no evidence that they have been successfully used in-

situ and real-time monitoring of soil NO_3^-. In *Article IV*, we demonstrate the use of a novel NO_3^- ISE for in-situ and real-time monitoring of an agricultural soil, both in a field trial and under controlled conditions in the laboratory. Results from the ISEs were found to be statistically similar to conventional laboratory analysis of contemporaneous soil samples on 16 out of 19 occasions. These novel NO_3^- ISEs provide a new opportunity for in-situ and real-time measurement of soil N dynamics, which represents a significant step forward for analytical soil science and environmental monitoring. In our study, we found that temperature had a significant effect on the ISEs and the datalogger, which could not be fully compensated for. Therefore, further work is required to better understand the effects of temperature on the ISE-datalogging system and develop improved compensation calculations. As ISEs measure the soil solution, it will also be important to look at how differing soil moisture contents affect the ISE performance. It is likely that the ISEs may not operate in very dry conditions, which may limit their usefulness for long-term monitoring. Furthermore, interpreting how soil moisture-related changes in soil solution NO_3^- concentration affects the availability of NO_3^- for plant uptake requires further investigation, which may be achieved using microdialysis. In addition, the NO_3^- ISE gives no consideration for other plant-available N forms, especially those which may be predominantly held on the solid phase (i.e. NH_4^+).

Results from the microdialysis experiments (*Articles I & II*) and the in-situ NO_3^- ISE testing (*Article IV*) showed large variability around means, which may reflect inherent spatial variation at small scales. Using in-situ methods to estimate soil N status at field-scale may be confounded by variation at range of scales. In *Article* V, we investigated the spatial variation of soil N in a grazed grassland field in order to optimise the spatial and economic configuration of an in-situ sensor network. Our work established that at least 60% of the variance in amino acids, NH_4^+ and NO_3^- occurred at scales < 2 m, with significant variation occurring at the sub 1-cm scale. This data was used to demonstrate how an in-situ sensing network could be optimised on a cost-accuracy basis. Given the unit costs of £2000 and £200 respectively for a data logger and NO_3^- ISE, the field mean for NO_3^--N concentration could be estimated with a 95 % confidence interval no wider than ± 1 µg N g^{-1} for a cost of £8,200. However, these calculations are based on a sensing support size of around 1 cm. Sensors, such as the NO_3^- ISEs developed in this project, that operate at sub 1-cm scales will be exposed to further variation, and hence more local replication will be required at the sub 1-cm scale to achieve similar levels of accuracy, with a resulting cost increase. It is therefore clear that in-situ monitoring is likely to incur significant costs, and future work must focus on assessing the cost-benefit and determining the most effective way to use the real-time data to inform fertiliser management.

The novel NO_3^- ISEs and microdialysis sampling have some considerable advantages but also some disadvantages when compared to conventional destructive soil testing, and these are summarised briefly below and in Table 1. Both methods allow an in-situ assessment of soil N with minimal disturbance to the system that is being evaluated. The main advantage of the novel ISEs is that soil NO_3^- can be quantified in-situ and in real-time at a fine temporal resolution, without the need for any destructive sampling and laboratory analysis. This make them ideal tools for on-farm monitoring use, as once they have been set up they require no further input. One drawback of this approach is the potentially high start-up costs, although for long-term monitoring at a high temporal and spatial resolution it is likely that total costs would be lower than performing conventional soil sampling and analysis at the same resolution. A further disadvantage is that the novel ISEs are only capable of sensing NO_3^-, so information on other forms of plant available N is not captured. Microdialysis also has the advantage over conventional soil testing that sampling can be performed in-situ, with minimal disturbance. However, currently microdialysis samples requires subsequent analysis in a laboratory resulting is both an economic and time cost. Furthermore, running the microdialysis probes is a more active process compared to the ISEs due to the need for a pump system and sample collection. Microdialysis is able to assess a large range of soil solutes, including all forms of plant-available N. It also has the advantage over both the other methods that its unique method of sampling via passive diffusion may better reflect the availability of N forms for plant uptake.

Table 1. Comparison of microdialysis and novel NO_3^- ISEs with conventional soil extractions for the assessment of soil N

	Conventional salt extraction (1 M KCl/0.5 M K_2SO_4)	**Novel NO_3^- ISE**	**Microdialysis**
Sampling requirement	Destructive sampling of soil and transport to laboratory	Can be used in-situ. ISEs are sensors	Soil solution solutes sampled in-situ via passive diffusion
Analytical requirement	Vigorous shaking with strong salt solution followed by filtering/centrifuging and chemical analysis in laboratory	Analysis performed in-situ and stored on data logger. Calibration required to convert mV output to [NO_3^-]	Chemical analysis of samples in laboratory
N pool assessed	Exchangeable soluble N pool. Both organic and inorganic N forms	NO_3^- activity of the soil solution	Time integrated concentration/diffusive flux. Both organic and inorganic soluble N forms
Level of disturbance	High – soil is removed from system	Very low following initial placement into soil	Low – only small quantities of soil solutes removed
Temporal resolution	Poor. Each sampling event requires significant extra cost/labour	Excellent. ISE output can be recorded at < 1 hz	Good. Probes can be run continuously. Resolution limited by sample volume required for analysis
Spatial resolution	Large range of sample sizes possible i.e. > 1kg to < 1 g. Samples can be homogenised/bulked to reduce small scale spatial heterogeneity	Excellent. Diameter of sensing membrane < 1 mm. Subject to micro-scale heterogeneity	Excellent. Linear dimension of membrane 4 mm. Subject to micro-scale heterogeneity
Cost	High in terms of labour, especially if sampling is carried out at fine spatial and temporal resolution	High start-up costs, but value increases as temporal resolution increase	Medium start-up costs as pump also required. Costs increase with number of samples due to requirement for subsequent analysis
Relevance of results to plant nutrition	Possible changes in N pool sizes during sampling and analysis. Concentration may not equal plant availability	Soil solution NO_3^- activity may be what is 'sensed' by plants. Importance of interaction between variable soil moisture content/ N activity and plant availability needs further investigation	Results may better reflect plant availability as they are affected by many soil and environmental variables. However, N transformations occur in rhizosphere and plant uptake is selective/active

2. Limitations of this thesis

The vast majority of the experimentation performed in this thesis was carried out using grassland soils local to Bangor University. Whilst microdialysis diffusive flux measurements (*Article 1*), commercial NO_3^- ISEs and dual-wavelength UV spectroscopy (*Article 3*) were performed on soils up a catena sequence, the novel NO_3^- ISEs were tested solely on a Eutric Cambisol. As such, the transferability of these approaches to other, particularly, arable soils, is unknown. This is particularly concerning with regard to the NO_3^- ISEs. It is likely, given the high costs associated with using the novel ISEs for real-time in-situ monitoring, that uptake of this approach will be limited initially to more profitable forms of agriculture, such as arable cropping and horticulture. These soils are often different to grassland soils in many aspects, which may affect the performance of the novel ISEs and the design of in-situ sensor arrays. For example, soils under arable cultivation are often drier, especially in the upper profile, have lower organic content and have much less variance at small-scales when compared to grassland soils. Furthermore, soils that have a high proportion of clay, such as those used for arable agriculture in central and eastern England, are prone to cracking during the summer months. The performance of the novel ISEs in such soil remains very much an unknown. It is also possible that microdialysis may not function effectively in very dry soils as the some of the perfusate may be lost across the membrane and into the soil. Future testing of these methods must focus on a wider range of soils, particularly those that are used for arable cropping and horticulture.

A further limitation to the study is that the novel ISE is only capable of sensing NO_3^- and not other forms of plant available N. Whilst NO_3^- is often considered the most important N form in high-input arable soils, the contribution of NH_4^+ and organic forms may be significant, especially under low-input and grassland systems. The extent to which fertiliser recommendations can be improved from NO_3^- measurements only requires further investigation.

3. Future work

3.1. Method/technological development of microdialysis and novel ISEs

This body of research represents the early stages in the development of microdialysis and in-situ ISEs. Further work on both of these techniques is required to fully optimise their performance and increase their usefulness to both scientists and the agricultural industry. One of the disadvantages of the microdialysis technique is the need to collect samples at regular

intervals for subsequent analysis. To overcome this, automated sample analysis using systems such as 'lab-on-chip' (Beaton et al., 2012) or flow-injection analysis using electrochemical sensors (Kim et al., 2007) could be adapted for use with microdialysis. One further problem with the microdialysis sampling is that equilibrium between the perfusate and the soil solution is never achieved and extremely low flow rates are required to ensure concentrations in the perfusate are detectable. Significantly increasing the length of the membrane would increase the time for diffusion and hence improve recovery rates. The length of the membrane used in this thesis was 4 mm. Increasing this length may reduce its usefulness for investigating systems at fine spatial scales – such as the rhizosphere – but it may help to reduce the impact of micro-scale heterogeneities which may hinder field research. Another option is to operate microdialysis as a circulatory system with in-line analysis. This would avoid the need for sampling and may further boost recovery of soil solutes into the perfusate. However, whether this would enable equilibrium to be reached and how quickly the system would respond to changes in the intrinsic solution require further investigation.

As discussed in section 2 above, considerable further testing of the novel ISEs on a range of soil types and environmental conditions is required. The effect of soil moisture on both the performance of the ISEs and the interpretation of the results is particularly important. For the ISEs to function, the membrane needs to be able to interact with the soil solution, which may be impossible in very dry conditions. Consideration of how changes in moisture content may affect the interpretation of the ISE results is also required. Assuming no change in the amount of NO_3^- in any given volume of soil, a decrease in soil moisture content will result in results in an increase in soil solution NO_3^-. With knowledge of the soil moisture content it is possible to convert the soil solution concentration in to a bulk soil concentration, however, water matric potentials will differ between different soils with the same moisture content, which further complicates interpretation. Plants access soil nutrients by a combination of root interception, mass flow and diffusion (Barber, 1984). How the concentration/moisture content/matric potential interaction affects the availability of NO_3^- for plant uptake and the relative importance of root interception, mass flow and diffusion is poorly defined and requires further investigation. Microdialysis maybe an ideal tool to assess how soil moisture contents control the availability of soluble N forms for plant uptake via both diffusion and mass flow (Oyewole et al., 2014).

3.2. Using in-situ methods to improve fundamental understanding of soil N processes

Gaining a better understanding of soil N processes is often limited by a lack of in-situ and non-destructive techniques. As has been demonstrated in this thesis, microdialysis can be used to determine how soil N dynamics vary over time and space. However, whilst microdialysis estimations of pool size and diffusive-fluxes may be affected by the balance of consumptive and additive processes, they have not yet been used to determine absolute fluxes between pools. This could be done indirectly using an incubation approach or directly using isotopic labelling. Repeating the rhizosphere study *(Article II)* using ^{14}C-labelled plants may allow the degree of root exudation of amino acids in soil to be determined.

Whilst microdialysis may better inform on the availability of soil N, drawing conclusions for plant nutrition is confounded by a number of factors including rhizosphere priming effects. Exudation of labile C from root tips stimulates microbial growth, which in turn leads to a reduction in the availability of N for plant uptake (Kuzyakov, 2002). Microdialysis could be used to assess the effect of this C exudation on N availability by using a low molecular weight C substrate (such as glucose) as the perfusate. This would diffuse out through the microdialysis membrane into the soil, simulating root exudation and creating a 'rhizosphere effect'. Varying the composition, concentration and C:N ratio of the LMW C substrate with a range of soils may enable new insights into rhizosphere priming to be made.

The scope for using NO_3^- ISEs with the capability for in-situ measurements for research into soil N dynamics is significant. Staying within an agricultural context, reducing emissions of N_2O from soils receiving N inputs is of great importance (Mosier et al., 1998). There has been much research into the biogeochemical controls of emissions and potential mitigation options, but the research is limited by the ability to make continuous soil NO_3^- measurements inside gas sampling chambers. Destructive sampling within chambers causes disturbance to the soil which will affect gaseous emissions. As such, it is currently difficult to directly relate the concentration of soil NO_3^- to N_2O emissions. In-situ measurements of soil NO_3^- using ISEs could be used to address this issue and improve our understanding of the relationship between soil N dynamics and N_2O emissions. One potential mitigation option is the use of nitrification inhibitors to retard the production of NO_3^- from NH_4^+ (Zhang et al., 2015). Investigating the use of these inhibitors using both NO_3^- ISEs and microdialysis would likely result in an improved understanding of how they affect soil N cycling and enable optimisation of their use.

3.3. Using in-situ sensors for precision agriculture

We have demonstrated in-situ monitoring of soil NO_3^- using a novel ISE and have also explored how networks of these sensors could be spatially configured to provide accurate and economic data. Such real-time data may enable a shift away from predetermined and empirically-derived fertiliser recommendations based upon N requirements over a growing season and potential crop yields, to a more dynamic system that responds rapidly to changes in crop N demand and soil N availability. This would have the benefit of minimising N surpluses in the soil hence decrease losses therefore potentially resulting in increased NUE. However, using this data to improve fertiliser recommendations presents a significant future challenge. N management is confounded by the multiple biotic and abiotic variables that ultimately control the final yield of the crop and the efficiency at which N is used. It is important to understand the reasons for any observed variation of soil N availability, and how that will affect cop growth at that specific location. The observed variation may be due to differences in crop uptake rates, N inputs or N cycling dynamics as determined by the biogeochemistry of the soil. Changes in NO_3^- concentration may also simply reflect recent rainfall events (i.e. dilution or leaching) rather than biological uptake. Further, sensors are frequently deployed in the topsoil which may not reflect N availability at depth. This is of particular relevance in arable cropping systems where roots can penetrate to >1.5 m in the soil profile and where soil moisture often constrains N uptake from dry topsoils. It is also important to determine whether the concentration of NO_3^- is growth-limiting or whether other agronomic factors are limiting (e.g. pH, other nutrients, plant pathogens, bulk density). It may be the case that in certain areas, improving NUE comes not from adjusting N fertiliser application rates but improving other factors, such as the status of other key nutrients, soil drainage or soil compaction. Currently, there are no sensors for the in-situ determination of soil NH_4^+ and plant available forms of organic N, such as amino acids. Whilst in many high-input arable systems, NO_3^- is the most dominant N form, the importance of NH_4^+ and organic N should not be underestimated in grassland, low-input and organic farming systems. It is clear that as well as monitoring soil N it is important to determine plant N status in order to estimate crop N requirement as it has been shown that plant N uptake is controlled by both plant growth and availability of soil N (Devienne-Barret et al., 2000; Gastal and Lemaire, 2002). Such technology is now in commercial use in the form of tractor-mounted crop canopy scanners which can be coupled with variable rate fertiliser spreaders (Diacono et al., 2013).

Given the complexity of the plant-soil system, it is likely that modelling approaches will be the best way forward for generating fertiliser recommendations. The aim of a dynamic approach to fertiliser management should be to maintain the pool of plant-available N at a level that matches plant uptake. Modelling approaches using real-time data may optimise both the timing and amount of fertiliser needed. Take for example, a study conducted by van Alphen (2002), who used soil N modelling combined with real-time weather data to monitor soil N status. Spatial variation was incorporated through the use of management zones, which were defined in terms of water regimes and N dynamics. Early warning was provided when soil mineral N concentrations dropped below a critical threshold. Used as a trigger, this information served to optimise the timing of four consecutive N fertilisations. Compared to conventional management, fertiliser input was reduced by 15–27%, without affecting grain yield. This approach could be improved by incorporating real-time soil NO_3^- measurements using the novel ISEs. However, calculating a 'trigger' NO_3^- concentration will be very challenging and the following points must be considered:

- How does soil moisture content interact with soil solution NO_3^- to control the availability of NO_3^- for plant uptake? Using soil moisture sensors in combination with NO_3^- ISE would allow the calculation of NO_3^- concentration on a per kg of soil or per ha basis.
- Crop demand for N is variable during the growing season. For winter wheat, most N uptake occurs during a 2 month period in spring. Therefore, any trigger value will depend upon crop N demand at any given point in the growing season. Using crop canopy sensing techniques (Diacono et al., 2013) to assess plant N status may allow N demand, and hence a trigger concentration, to be determined.
- Presuming that within each management zone there are multiple sensors as part of an array, how many of these need to drop below the 'trigger' value before an application of fertiliser should be made? Assuming that any management zone is fairly homogenous in terms of its N dynamics, then the mean value of the sensors would be used as the 'trigger' value. However, if variance in NO_3^- concentration within a management zone becomes significant then a more dynamic approach to spatial variation may be needed.
- Soil solution NO_3^- concentration will fluctuate depending upon soil moisture content but also the balance of nitrification, denitrification and plant uptake. As such, the NO_3^- concentration may drop below the 'trigger' value for a short period

of time before recovering. How long must the value remain below the 'trigger value' before a fertiliser event is initiated? Monitoring soil moisture content will enable fluctuations in soil solution NO_3^- concentration due to soil moisture dynamics to be determined. Fluctuations in the intrinsic supply of NO_3^- may be accounted for by monitoring plots with no N inputs.

In addition, data gained from the in situ sensors could be supported by a tractor-mounted near-infra red scanning of the soil matrix which provides additional estimates of soil organic matter quality which can be linked to rates of N mineralization/supply (Gomez et al., 2008). This will rely on developing sensitive algorithms to support the "SMART" farming approach, where real-time data on plant/soil conditions are gathered from numerous sources and integrated using an "Internet of Things" approach. Ultimately, this may enable a step change towards a more dynamic approach of nutrient management. Based on the rate of recent advances in sensor technology, networks and data processing platforms, realistically, this approach could be commercially implemented on UK farms in the next 25 years.

4. Conclusions

This project aimed to develop in-situ and real-time methods of soil N determination to enable continuous monitoring of agricultural soil and improve understanding of soil N dynamics. The research was carried out with the ultimate aim that the techniques developed may eventually result in an improvement in the NUE of agriculture. The project has demonstrated the use of microdialysis as a novel in-situ method that better reflects the availability of N for plant uptake than conventional destructive sampling and soil extractions. Use of microdialysis to assess N dynamics in the rhizosphere also proved successful. It is likely that microdialysis will continue to offer new insights into the functioning of soil N processes and the factors which control the availability of N for plant uptake. The project has also developed a novel NO_3^- ISE that was used successfully for the in-situ monitoring of a grassland agricultural soil. In addition, the project demonstrated, using a geo-statistical approach, how the ISEs could be deployed to optimise field-scale monitoring of soil NO_3^-. This represents a significant step forward for analytical soil science and agricultural management. However, due to the significant cost of field-scale monitoring, it is likely that use of such an approach will be limited initially to agricultural land used for growing high value arable and horticultural crops. Further work is required to test the ISEs in a wide range of soil types and environmental conditions. Considerable research is also needed to determine how data generated from in-situ sensors can be used to improve fertiliser recommendations.

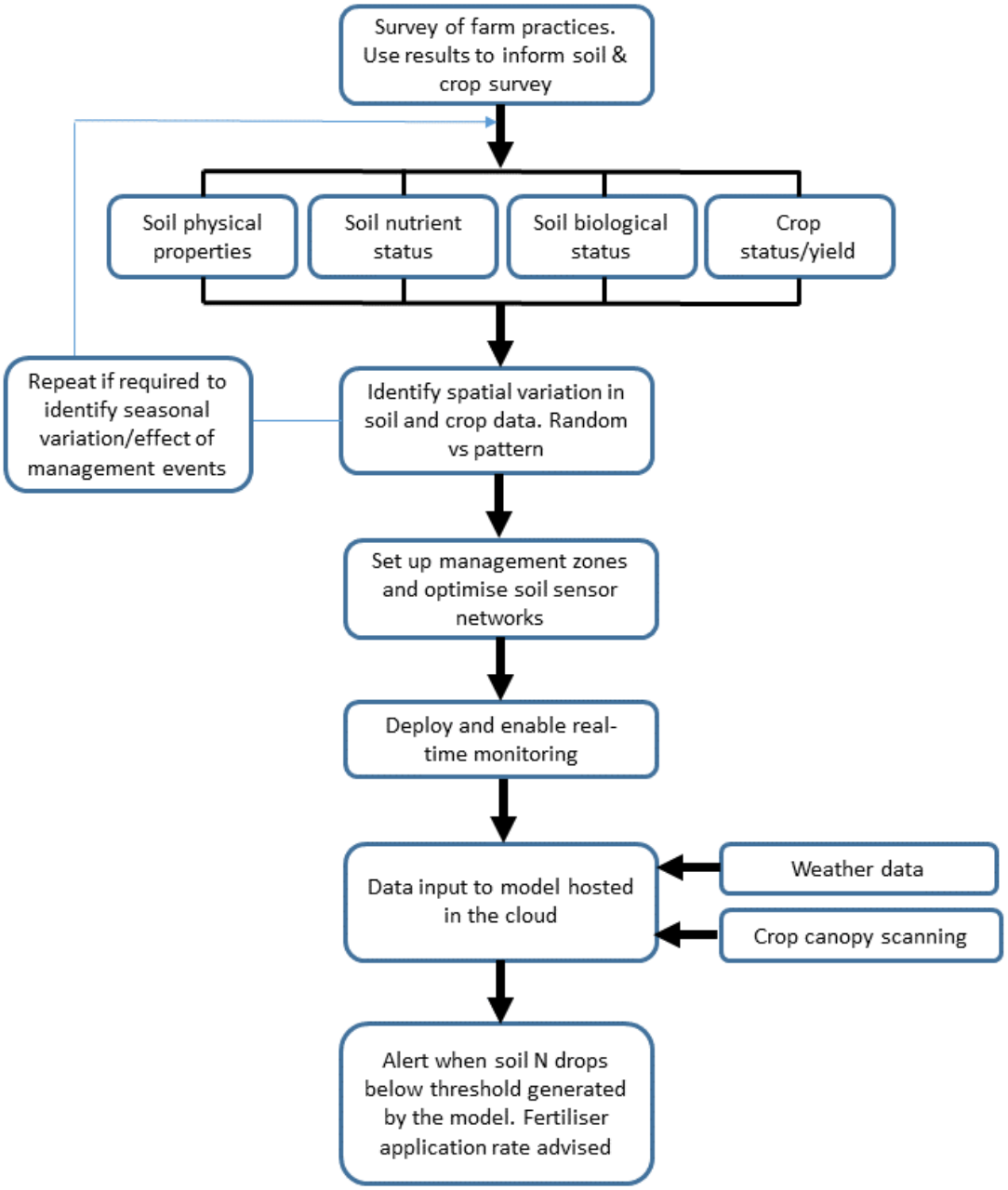

Figure 1. Flow diagram describing a new approach to N fertiliser management based on real-time data input into cloud based models.

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Appendices

Appendix 1.

Conference paper for oral presentation at the 18th Nitrogen Workshop, 2014, Lisbon, Portugal, 30th June – 3rd July 2014.

R. Shaw, A.P. Williams, D.L. Jones. (2014). Nitrogen dynamics in the rhizosphere. In: C. Cordovil (ed.). Proceedings of the 18th nitrogen workshop – The nitrogen challenge: building a blueprint for nitrogen use efficiency and food security. Lisbon, Portugal. pp 179 – 180.

Appendix 2.

Conference paper for oral presentation at the 25th general meeting of the European Grassland Federation. Aberystwyth, Wales, 7 – 11th September 2014.

R. Shaw, A.P. Williams, D.L. Jones. (2014). Nitrogen dynamics in the rhizosphere. In: A. Hopkins et al (eds.). Proceedings of the 25th general meeting of the European Grassland Federation. EGF at 50: The future of European grasslands. Aberystwyth, Wales. pp 273 – 275.

Appendix 3.

Protocol for constructing a nitrate-selective electrode and reference electrode

Appendix 4.

Photographs of experiments

Appendix 1.

Nitrogen dynamics in the rhizosphere

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School of Environment, Natural Resources and Geography (SENRGy), Bangor University, Wales

Abstract for oral communication

Background and Objectives

The fate and dynamics of organic N in the rhizosphere has been the focus of a large number of studies (Kuzyakov and Xu, 2013). Plants have been shown to both, exude amino acids from roots, and have the capacity for amino acid uptake (Jones and Darrah, 1994; Jones et al., 2009). However, determining the fate of amino acids exudates, and N dynamics, in the rhizosphere, has been limited by a lack of non-destructive sampling methods (Oburger et al., 2013). In a novel, *in situ* approach, microdialysis probes were used to monitor N dynamics in the rhizosphere of maize seedlings.

Materials and Methods

Microdialysis probes were inserted into a rhizotube filled with an agricultural Eutric Cambisol. Maize seedlings were grown, over a 68 hour period, in the rhizotubes, so that the growing root passed directly over the membrane of the microdialysis probe. The probes were perfused with de-ionized water, at a rate of 5 µl min^{-1}, and the dialysate was sampled over a four hour time period. Samples were then chemically analysed for total amino acids, ammonium and nitrate. In addition, the fate of amino acid inputs in the same soil was investigated using an isotopically-labelled amino acid mixture, at a range of concentrations representing that which could be found in the rhizosphere (1, 10, 100, 1000 µM). A 300 µl aliquot of amino acid mixture of each concentration was applied to 1 g (DWE) of field moist soil and incubated, at 20°C, for 1, 5, 10, 30 and 60 min. The same treatments were applied to sterilised soils and the results used as a control to determine the significance of abiotic mechanisms. Carbon dioxide traps were used to capture any $^{14}CO_2$ produced during mineralization and ^{14}C-labelled amino acids remaining in soil solution was collected using a centrifugation drainage technique (Hill et al., 2008). Measurements of ^{14}C, in the CO_2 traps and soil solutions, were performed with liquid scintillation counting. Net amino acid efflux from the roots of maize seedlings, grown in a sterile nutrient solution was also investigated using a nano sampling technique coupled with spectrofluorometric analysis.

Results and discussion

A significant exudate-derived concentration of amino acids, which peaked 1-2 cm from the root tip, was determined using the nano sampling method. However, results from the microdialysis sampling

showed the concentration of amino acids in the soil solution remained relatively constant throughout the experiment. The lack of amino acid spike in the soil solution, due to root exudates, may be explained by the fact that the depletion of the isotopically labelled amino acid mixture from the soil solution was extremely rapid. Figure 1 shows that only 10% of the 10 µM treatment remained in the soil solution after 1 minute. Nitrate concentration decreased following the growth of the root past the probe, which is likely due to plant uptake.

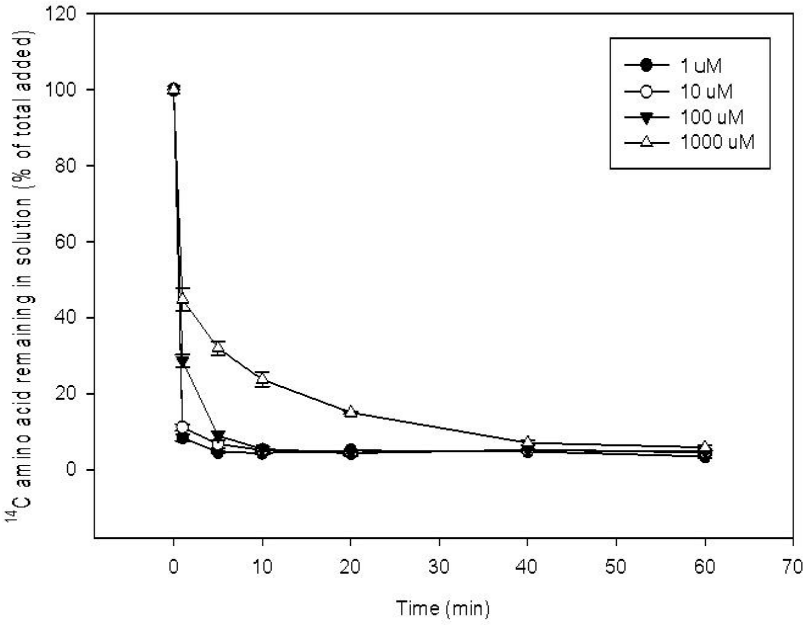

Fig. 1. Amount of ^{14}C-label remaining in soil solution after the addition of a ^{14}C-labelled amino acid pulse (1 µM – 1000 µM) to an agricultural soil. Values represent means ± SEM (n = 3).

Conclusions

This study demonstrates the feasibility of microdialysis sampling within the rhizosphere and suggests that LMW DON exuded by roots, is rapidly cycled by carbon-limited microorganisms in the rhizosphere.

Acknowledgements

The authors would like to thank EBLEX, Hybu Cig Cymru (HCC), Quality Meat Scotland (QMS) and DairyCo. for funding this PhD.

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Appendix 2.

Developing an *in-situ* sensor for real-time monitoring of soil nitrate concentration

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Abstract for oral communication

Abstract

Improving nitrogen-use efficiency is key to increasing the sustainability of livestock farming systems. Better management of nitrogen (N) inputs and waste resources is needed if significant improvements are to occur. However, farmers are currently limited by a lack of suitable, field-based tools for soil analysis and are overly reliant on limited, computer-based approaches. This project aims to develop an ion-selective electrode (ISE) capable of *in-situ*, real-time soil monitoring of soil nitrate (NO_3^-). Construction of the electrodes in the laboratory is simple, low-cost and reproducible and the ISEs conform to theoretical norms. Current and future work will focus on the testing the performance of the electrodes in soil solutions and soils, and comparing the results to a range of standard methods.

Keywords: sustainable farming, nitrogen-use efficiency, ion-selective electrode, soil testing

Introduction

Optimising the use of nitrogen (N) represents one of the major goals of sustainable livestock farming systems, from both an economic and environmental standpoint. While there have been thousands of studies investigating different management strategies for optimising N use on farms, translating this research into practical management advice and subsequent adoption by farmers has often been unsuccessful. Consequently, as evidenced by numerous recent reports, there is no doubt that we have a long way to go before N is used efficiently within the UK livestock sector (Wilkins, 2008; Rees and Ball, 2010; Spiertz, 2010). The lack of significant improvement is partially due to a paucity of farmer-operated, field-based tools for soil analysis and reliance on virtual (computer)-based approaches, which have limited adoption and lack precision (Cuttle and Jarvis, 2005). Ideally, adoption of simple field-based sensors that can monitor soil N in real-time will allow for more active management of fertiliser and waste resources, resulting in enhancement of fertiliser use efficiency, better timing of waste applications and reductions in environmental pollution.

Ion-selective electrodes (ISEs) have the potential to be used for real-time, *in situ* soil monitoring and have the advantages of being relatively inexpensive and easy to use (De Marco et al., 2007; Sinfield et al., 2010). However, current ISEs are not sufficiently robust enough for soil sensing and are subject to

drifting calibration parameters, fouling and short lifespans (De Marco et al., 2007). This project aims to develop a nitrate (NO_3^-) ISE which can overcome these challenges and bring about a step change in on-farm N management.

Materials and methods

ISEs function by measuring the potential difference between an electrode containing an ion-selective membrane and a reference electrode, which is not affected by the target ion. The activity of the target ion is related to the potential difference by the Nernst equation, which states that a ten-fold increase in activity of the target ion will result in a 59.1 mV change in electrode output. We construct the electrodes in our laboratory using a simple protocol and easily sourced materials. Briefly, both the NO_3^- and reference electrodes consist of a 1250 µl pipette tip, into which a PVC-based membrane is cast. The pipette tips are then back filled with an unspecified solution, a Ag/AgCl wire is inserted, and the tip sealed. The electrodes are then coupled with a millivolt meter or suitable data logger.

Initial work has focused on characterising the electrode's basic properties, including response time, the effect of interfering ions, developing a temperature compensation calculation and standardising the manufacturing protocol to obtain a reproducible calibration (Buck and Lindner, 1994). Currently, we are testing the sensors in soil solutions and comparing the results to a standard colorimetric analytical method (Miranda et al., 2001) to assess the accuracy and precision of the sensors for NO_3^- determination. In addition, we are monitoring soil NO_3^- levels in the laboratory and assessing the performance of the sensors against a range of soil sampling techniques, including, small tension lysimeters, centrifugal drainage and conventional soil core analysis. Following completion of this work, the sensors will be deployed in a grass/clover field trial in March–August 2014 to determine the differences in soil nitrate dynamics between different clover densities and inorganic N fertiliser amendments.

Results and discussion

Construction of the electrodes has proved succesful and reproducible as seen in Figure 1. These electrodes have a limit of detection of 47 µM and a near nernstian slope of 61.7 mV dec^{-1}. Figure 2 shows how electrode output (mV) is affected by temperature, with the magnitude of the change dependent upon the NO_3^- concentration of the sample. This is expected as the Nernst equation contains a temperature term, so the electrode outut can be easily adjusted using a simple calculation. Chloride is a well known interfering ion (Miller and Zhen, 1991) and the effect on these electrodes was determined using the fixed interference method (Buck and Lindner, 1994). The electrodes were recalibrated in the prescence of 100 mM Cl^- and the selectivity coefficient was found to be 0.04.

Ongoing work and the results of the field trials will be reported on in September, as well as recommendations for industry as to how to take this promising technology further.

Figure 1. Calibration of NO_3^- ISE in standard NO_3^- solutions. Electromotive force (EMF) is the electrode output and pNO_3^- is the negative log of the NO_3^- activity. Data points represent means (*n*=6) ± *SEM* and the curve represents a modified Nernst equation.

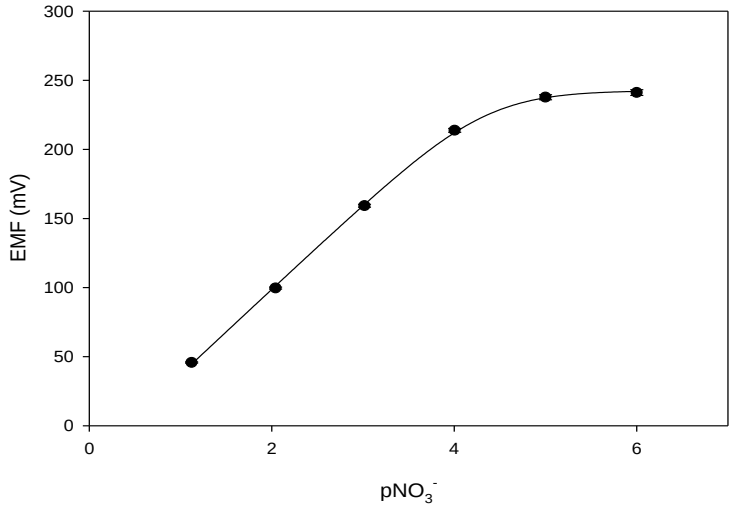
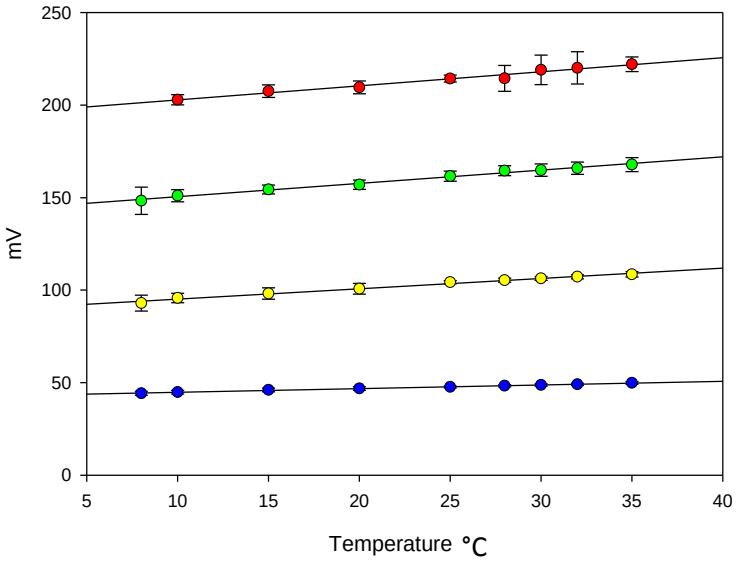


Figure 2. The effect of temperature on NO_3^- ISE output at different concentration of NO_3^- (red – 0.1 mM, green – 1 mM, yellow – 10 mM, blue – 100 mM). Data points represent means (*n*=6) ± *SEM*.



Acknowledgements: The authors would like to thanks Hybu Cig Cymru (HCC), the Engish Beef and Lamb Executive (EBLEX), DairyCo. and Quality Meat Scotland (QMS) for funding this project.

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Appendix 3.

Protocol for constructing a nitrate-selective electrode and reference electrode

Equipment required:

- Pipette tips: 1250 µl xl graduated (TipOne, Starlab, Milton Keynes, UK)
- Pipette tips: 5000 µl (epT.I.P.S. 100 – 5000 µl, Eppendorf, Hanburg, Germany)
- Pure silver wire (1 mm diameter)
- Standard electrical wire
- Silicon sealant
- Parafilm

Chemicals required:

- Tetrahydrofuran (THF)
- PVC (high molecular-weight polymer)
- Methyltriphenyl phosphonium bromide (MTPB)
- 2-nitrophenyl octyl ether (2-NPOE)
- Nitrocellulose
- Methyltridodecylammonium nitrate (MTDDA)
- Polyethylene glycol (PEG) 1500
- KTpCIPB
- Potassium chloride (KCl)
- Potassium nitrate (KNO_3)
- Double junction reference electrode inner filling solution (Thermo Scientific)
- Double junction reference electrode outer filling solution (Thermo Scientific)

Preparing the membrane cocktails

Weigh out the following chemicals into a glass vial. In a fume hood, add 2 ml of THF using a glass pipette to the vial. Stopper the vial and use a vortex to assist the dissolving process.

Nitrate membrane:

- MTDDA: 0.012 g
- MTPB: 0.002 g
- PVC: 0.046 g
- Nitrocellulose: 0.01 g
- 2-NPOE: 0.130 g

Reference membrane:

- KTpCIPB: 0.002 g
- PEG 1500: 0.046 g
- PVC: 0.045 g

Casting the membrane

In the fume hood, remove the vial stopper and dip a pipette tip into the cocktail so that the tip fills via capillary action to a depth of 3 mm. Replace pipette tip into the rack and leave for 24 hrs to allow the THF to evaporate and the membrane to harden.

Preparing the Ag/AgCl wire

Cut a length of standard electrical wire (no more than 1 m) and solder a 7 cm length of Ag wire to one end. Take a 5000 µl pipette tip and using a sharp knife, make a transverse section and remove the bottom 35 mm. Fill this will silicone sealant and push the Ag/AgCl wire as far as it will go through this so that the Ag/AgCl wire passes through the hole at the end of the tip. Remove all traces of silicon sealant from the Ag/AgCl wire. Leave to dry for 2 days. Set up a battery pack and dipole switch to according to Figure 1 to create a circuit that can be used to electroplate the Ag wire with AgCl. Attach the free end of the wire to the circuit and place both of the silver wire into a 50 mM KCl solution. Alternate the current direction using the dipole switch until the Ag wire is fully covered with a black AgCl coating. Remove the wire and place on blue roll until dry. Repeat the electroplating procedure to ensure complete coverage of the wire.

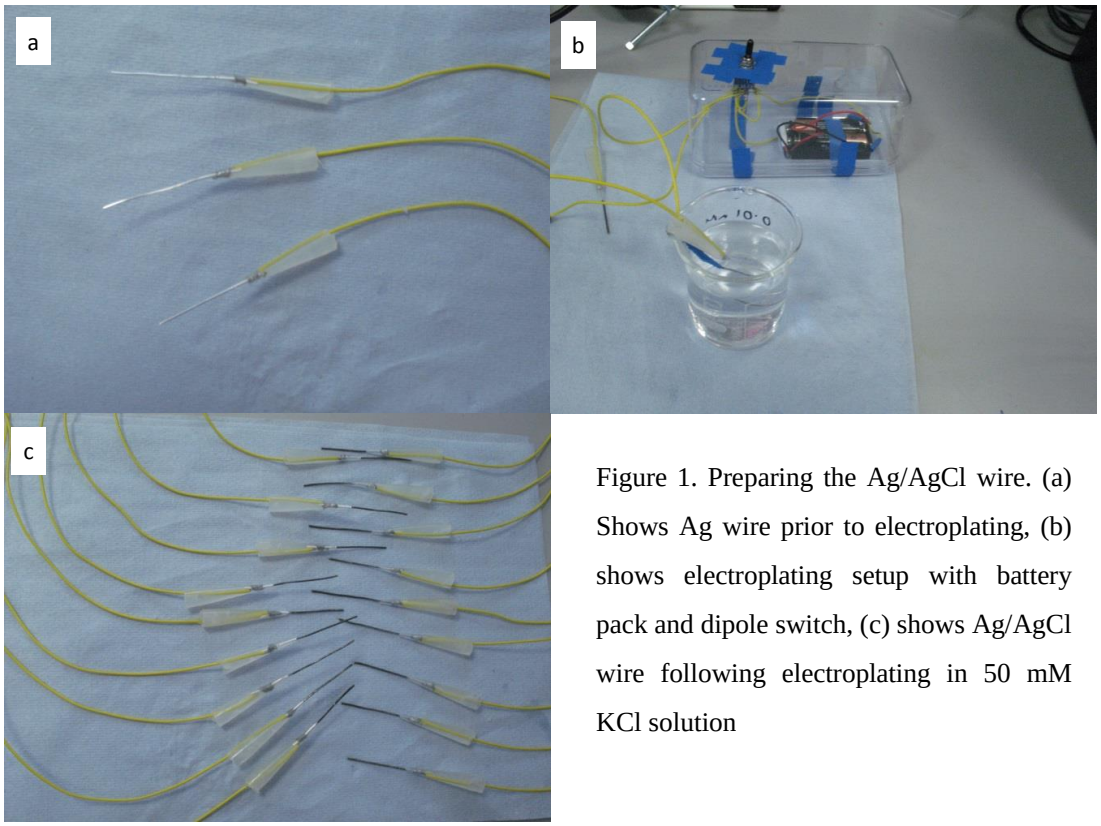

Figure 1. Preparing the Ag/AgCl wire. (a) Shows Ag wire prior to electroplating, (b) shows electroplating setup with battery pack and dipole switch, (c) shows Ag/AgCl wire following electroplating in 50 mM KCl solution

Backfilling and sealing the electrodes

NO_3^- electrode

Prepare a solution containing 100 mM KNO_3 and 100 mM KCl. Take a tip into which a NO_3^- sensing membrane has been cast. Using a syringe and suitable needle, fill the tip with the above solution to approx. 0.5 cm from the top. Ensure that there are no air bubbles in the solution. Take a Ag/AgCl wire and place it into the back-filled tip. Secure this to the tip using parafilm. This can be reinforced with insulating tape to improve durability and water tightness if needed.

Double junction reference electrode

Take a tip into which a reference membrane has been cast. Using a syringe and suitable needle, fill the tip with the inner reference electrode solution to approx. 0.5 mm from the top. Ensure that there are no air bubbles in the solution. Take a Ag/AgCl wire and place it into the back-filled tip. Secure this to the tip using parafilm. This can be reinforced with insulating tape to improve durability and water tightness if needed. Take a second reference tip and half fill it with the outer reference electrode solution. Ensure that there are no air bubbles in the solution. Place the inner tip inside the outer tip and seal as described above. Connect the reference electrode to a NO_3^- electrode and place in a 100 mM KNO_3 solution for

24 h prior to calibration. For ease of use and for deployment in soils the NO_3^- and reference electrodes can be taped together for ease of use.

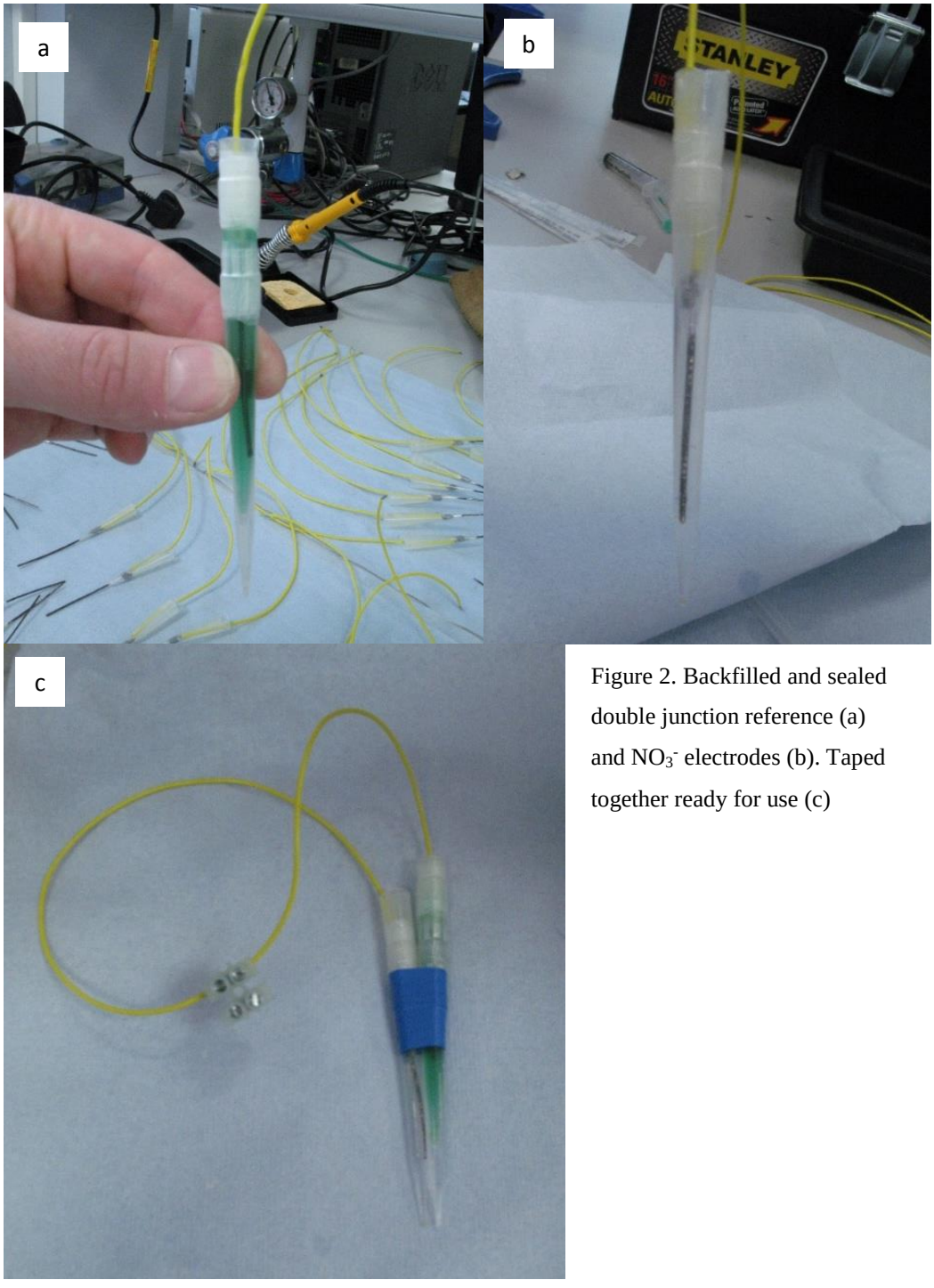

Figure 2. Backfilled and sealed double junction reference (a) and NO_3^- electrodes (b). Taped together ready for use (c)

Loss of NO_3^- from the back-filling solution of the reference electrodes

Testing of the reference electrode described above showed that loss of NO_3^- from the back-filling solution caused a significant increase in the [NO_3^-] of a standard solution over time as shown below in figure 3. Because of this issue, the reference electrode was replaced with a commercially available Lithium-Acetate double junction lithium acetate reference electrode (ELIT 003n; NICO2000 Ltd., Harrow, Middlesex, UK).

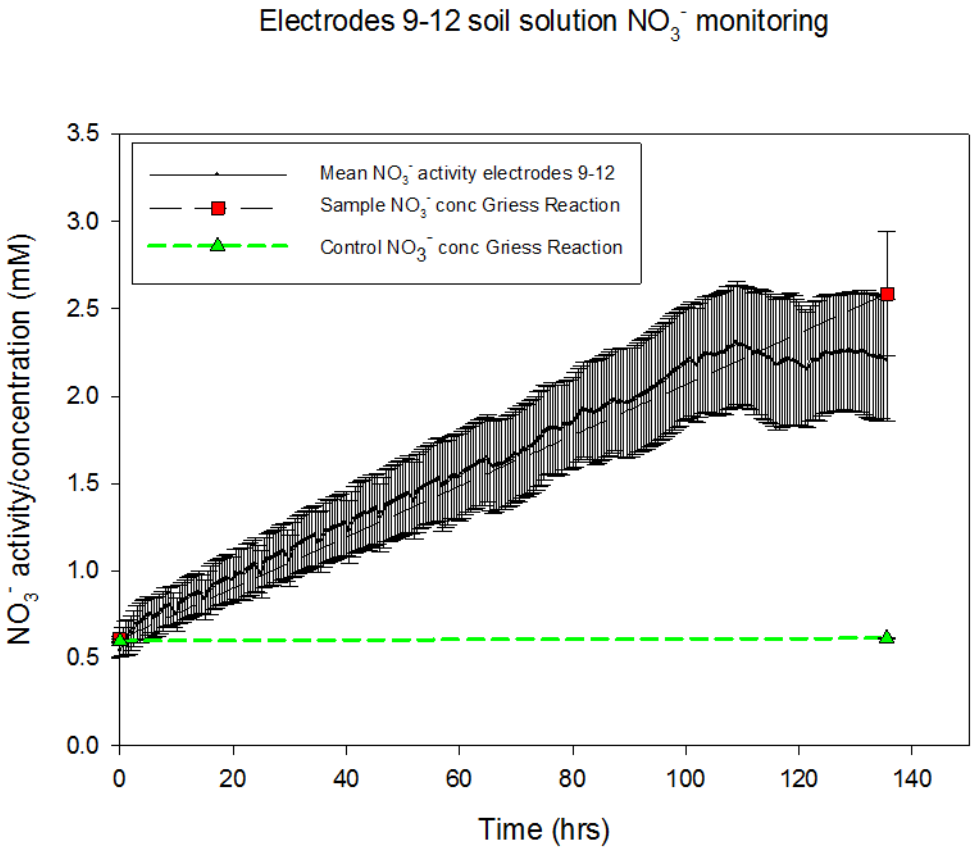

Figure 3. Change in NO_3^- concentration of a 0.6 mM standard solution over 136 h as measured by the Griess reaction (control and sample) (Miranda et al., 2001) and a NO_3^- ISE (sample only).

Appendix 4.

Photographs of experiments

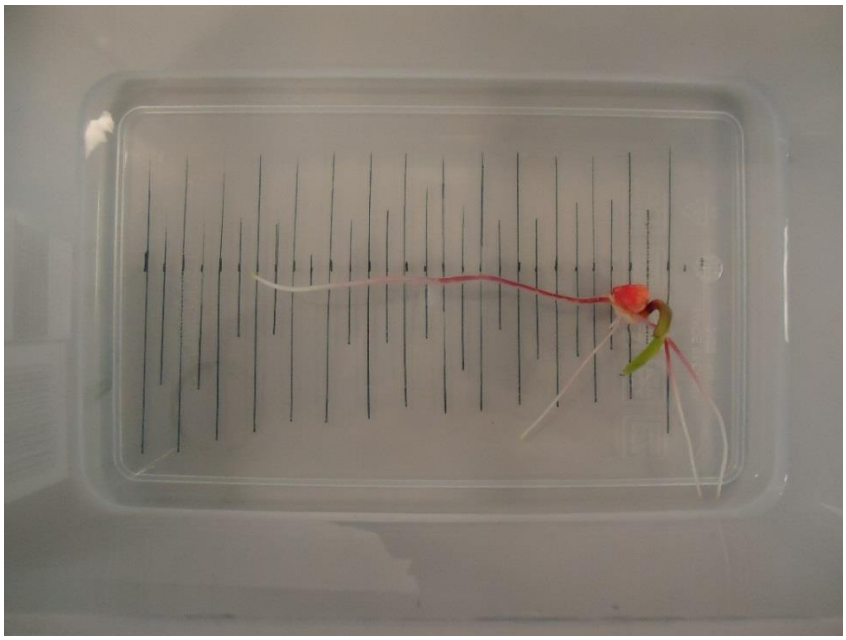

Figure 1. Experimental setup to measure amino acid exudates from seedlings of Zea mays L. *Samples were taken directly from the root surface at 1 cm intervals using a 1 µl pipette and subsequently analysed on a Nano Drop^{tm} ND 3300 Fluorospectrometer (NanoDrop Products, Wilmington, USA) (Article II).*

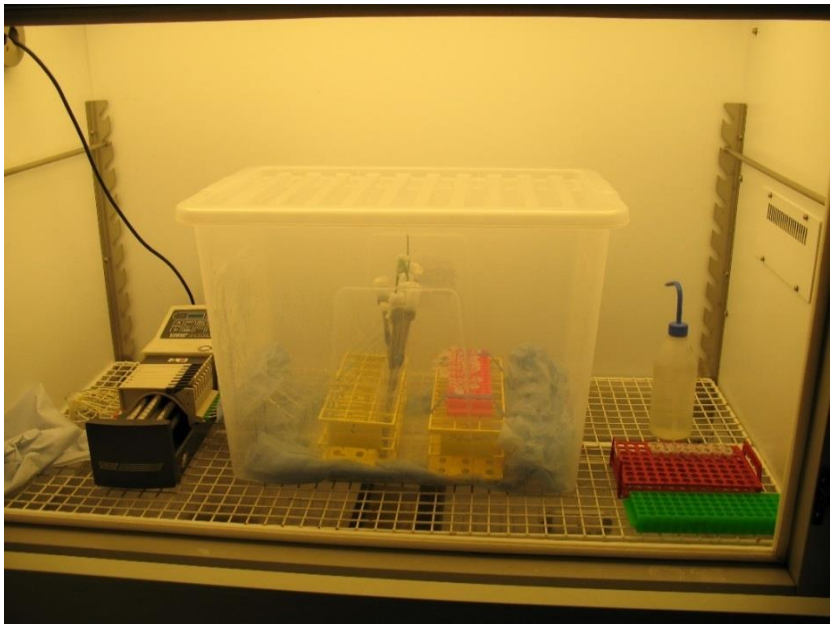

Figure 2. Experimental setup used for microdialysis monitoring of N dynamics in the rhizosphere of growing maize seedlings (Article II).

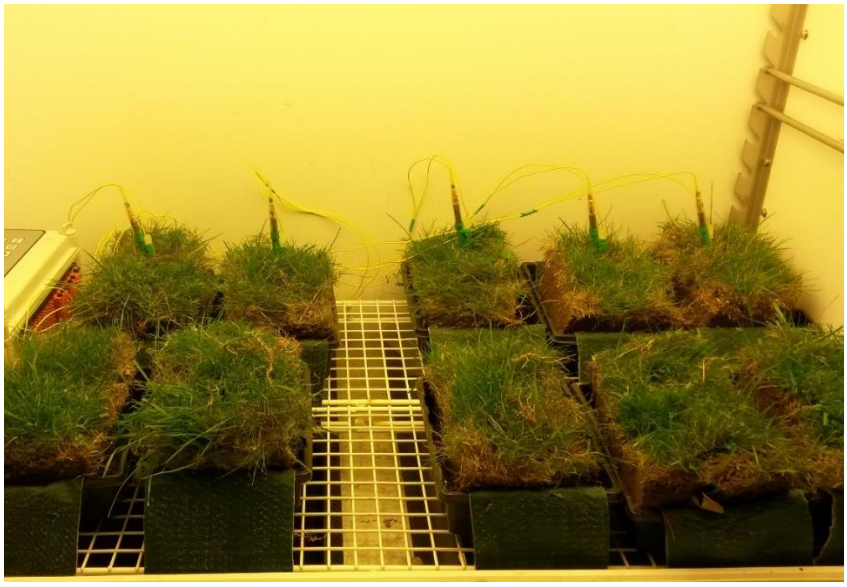

Figure 3. Experimental setup for laboratory testing of NO_3^- ISEs under controlled environmental conditions (Article IV)

Figure 4. Close up of a NO_3^- ISE placed in a turf for real-time monitoring (Article IV).

Figure 5. NO_3^- ISEs deployed in situ for field testing. The cut turf was replaced to ensure the NO_3^- ISEs were completely buried (Article IV)

Figure 6. A NO_3^- ISE removed from the soil following 6 days of continuous, real-time monitoring (Article IV).