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Sensing Nitrogen Status in Mosses

by

Catherine Jane Cooke

A thesis submitted in partial fulfilment
of the requirements for the degree of

Doctor of Philosophy
at the University of London

2006
The aim of this research was to assess how mosses took up nitrogen (N), assimilated this N and sensed N status. Research into the short term foliar uptake and assimilation of N investigated the rapid induction of nitrate reductase activity (NRA) in two species of moss Mnium hornum and Sphagnum fimbriatum. NRA was induced in both species in just 15 minutes. Tissue nitrate (NO3⁻) content was measured over this period and it was shown that the species had contrasting NO3⁻ storage capabilities. Both species also differed in their long term N use. S. fimbriatum was a less nitrophilous species and died after approximately 80 days N-deprivation but M. hornum had a tremendous capacity to sustain itself and survived for in excess of 450 days. It was suggested from electrophysiological measurements and microscopy that S. fimbriatum had a lower NO3⁻ storage capacity due to the lack of a distinct vacuole or storage compartment for NO3⁻. M. hornum had both cytosolic and vacuolar compartments and was more able to store NO3⁻, even in the longer term, being more similar in structure to a higher plant. Ammonium (NH4⁺) induced NRA in the absence of NO3⁻ in both mosses. Electrophysiological measurements, using pH-selective microelectrodes, showed a depolarisation of the membrane potential and acidification of intracellular compartments in moss cells upon addition of NH4⁺; an example being a pH change from pH 7.11 to 5.84 in the cytosol of M. hornum. N uptake mechanisms in these mosses supported the classical pH-stat model and current thinking on plant N transporter mechanisms in higher plants whereby the addition of NH4⁺
caused an acidification of the cytosol, in turn inducing NRA to stabilise intracellular pH. This project brought together the biochemistry, physiology and localised changes in metabolite pools and pH to explain why these mosses behaved differently.
To my Nana Jean who would have been very happy to know that I had enjoyed doing this.
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It's a little bit funny this feeling inside
I'm not one of those who can easily hide
I don't have much money but boy if I did
I'd buy a big house where we both could live

If I was a sculptor, but then again, no
Or a girl who makes potions in a lab near Soho
I know it's not much but it's the best I can do
My gift is my song and this one's for you

And you can tell everybody this is your song
It may be quite simple but now that it's done
I hope you don't mind
I hope you don't mind that I put down in words
How wonderful life is while you're in the world

I sat on the roof and examined the moss
Well a few of the chapters well they've got me quite cross
But the sun's been quite kind while I wrote this song
It's for people like you that keep it turned on

So excuse me forgetting but these things I do
You see I've forgotten if they're green or they're blue
Anyway the thing is what I really mean
Yours are the sweetest eyes I've ever seen

(adapted from Elton John and Bernie Taupin (1969) – ‘Your Song’)

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CHAPTER 1

INTRODUCTION

1.1. Introduction

Nitrogen (N) is an essential element for plant growth and metabolism. As such it is one of the elements required in relatively large amounts by plants and is regarded as a major growth limiting nutrient in both an ecological and an agricultural context (Lee et al. 1983; Miflin and Habash, 2001).

Experiments to study N status have been extensive and varied in higher plant systems. It is taken up into the cell, metabolised into amino acids and then used for proteins and enzymes which are fundamental to cellular function (Allen et al., 1974; Haynes and Goh, 1978). Much research work has gone into understanding the mechanisms underpinning the production of high yielding crops, with better protein content and increased biomass due to more effective use of N fertiliser by plants, in the agricultural industry. The improvement of N use efficiency is a major goal of crop improvement (Miflin and Habash, 2001). In an environmental context recent research initiatives, such as the global nitrogen enrichment (GANE) programme (www.nerc.ac.uk/funding/thematics/gane), looking into the effects of increases in anthropogenic N on the environment due to the intensification of agriculture and industry in the UK, have highlighted
several important issues. Not only do we still know very little about how native UK plants specifically respond to increased N but impacts on the environment are only just being revealed.

1.2. Measuring N in plant systems

The available sources of inorganic N to higher plants in soils is NO$_3^-$ or NH$_4^+$ (Lee et al., 1983). Plants utilise these forms differently. NO$_3^-$ is taken up in higher plants via the roots where it is either directly assimilated or translocated via the vasculature of the plant to the shoots to be reduced in the cytoplasm, it can also be stored in plant cell vacuoles. NH$_4^+$ is also taken up by roots but is considered to be assimilated directly in root tissue due to its toxicity, it is not thought to undergo long distance transport and is not stored (Pearson and Stewart, 1993). Plants have developed mechanisms to cope with low N supply including very sensitive and selective uptake systems that allow the possibility to grow using different N sources (Tischner, 2000). Ombrotrophic bogs, where Sphagnum species tend to dominate, are amongst ecosystems with the lowest natural N availability (Baxter, Emes and Lee, 1992). These systems provide negligible NO$_3^-$ for higher plant growth and NH$_4^+$ is generally considered the dominant form of N (Woodin and Lee, 1987). Sphagnum species had been considered to have adapted to low concentrations of both NO$_3^-$ and NH$_4^+$, with high rates of supply of either of these ions having the potential to affect Sphagnum growth adversely (Press and Lee, 1982). However in a study into the effects of an experimentally applied increase in NH$_4^+$ on growth and amino-acid metabolism of Sphagnum cuspidatum from a site in the South Pennines, that had
been subjected to atmospheric pollution deposition for a period of at least the last 200 yr, growth was stimulated above that of the untreated control tissue at both 0.1 and 1.0 mM NH$_4^+$. The largest growth stimulation was at the lowest dose (Baxter, Emes and Lee, 1992).

One of the most widely studied enzymes involved in the regulation of nitrate assimilation is nitrate reductase (NR). NR was first isolated and characterized more than 50 years ago, and each decade of study of this key enzyme of N assimilation has been associated with a new understanding of its structure and function (Campbell, 1996). Measuring NR activity alongside assimilatory products including NO$_3^-$, NH$_4^+$, amino acids and proteins has given a suite of data that help us to understand and measure plant N status and how it may be sensing N in the external environment; for example mosses are known to store NO$_3^-$ in times of plenty (Pearson and Soares, 1998). Factors such as changes in tissue N and δ-$^{15}$N have all been used to try and track the pathway of N throughout the plant as N is taken up and assimilated (Marsh et al., 2004; Power et al., 2004). Mosses can vary their total N content having wide intra- and interspecific ranges that can vary from as low as 4 to 12 mg g$^{-1}$ DWt in Racomitrium lanuginosum (Baddley and Lee, 1994) or from 10 to 50 mg g$^{-1}$ DWt, as in Tortula muralis (Pearson et al., 2000).

A technique that has been adapted to look at the measuring the transport of N across cell membranes in plants is the field of electrophysiology (Miller et al., 2001). Single barrelled microelectrodes measure voltage and when inserted into cells measure the membrane potential, in mV, between the inside and outside of
the cell. Ion-selective microelectrodes can also measure the ion gradients across
membranes. These techniques can be used in this way on mosses as work into
the transport mechanisms involved in N uptake in lower plants is somewhat
limited. In higher plants, two families of N transporter proteins can provide an
entry route for NO$_3^-$ into cells, these are called nitrate transporters (NRT) 1 and 2
(Crawford and Glass, 1998; Forde, 2000; Williams and Miller, 2001).

1.3. Mosses as a system for sensing N

Mosses represent a simpler system than higher plants without the need to
understand soil processes, transfer and transformation within the plant as they
lack roots and a vasculature for translocation. Ectohydric mosses are a useful
system for studying the effects of increased N in the environment as they are
largely rootless and absorb water rapidly both into the cells and into the moss
carpet through their external leaf structure (Richardson, 1981). In this way
ectohydric mosses obtain much of their external nutrients and primarily take up
N from the atmosphere or in run-off from groundwater, with minimal uptake of
N from the soil. By lacking an extensive root system and vasculature mosses do
not have the complication of the translocation of metabolites and they are
therefore a simpler model system to work with when compared with higher
plants. However, in field based studies mosses are subject to inputs from both
dry and wet atmospheric deposition and run-off in rainwater, so atmospheric N
inputs require careful consideration and quantification and can be complicated by
other factors, such as fluctuations in pH. The two moss species chosen for this
study *Mnium hornum* and *Sphagnum fimbriatum* are both ectohydric mosses.
For this thesis only ectohydric mosses were used to minimise inputs from sources other than atmospheric deposition in order to prevent the complication of inputs from uncontrolled sources and focus on known inputs of N.

1.4. Uptake of N from the atmosphere into the leaf

Higher plants, including trees, can take up N in the form of NOx, directly from the atmosphere into their leaves through the stomata (Taiz and Zeiger, 1998). Stomata regulate the influx and efflux of gases in leaves of higher plants, and these structures are not prevalent or highly developed in mosses and the mechanisms for nutrient uptake are not widely understood. Another barrier to water, and therefore solute, uptake and loss is the cuticle. Terrestrial vascular plants and a few bryophytes are covered with a complex mixture of lipids, usually called epicuticular waxes (Koch et al., 2004). A major function is to serve as a barrier against uncontrolled water loss (Schönherr, 1976). Applying inorganic-N to plant leaf surfaces the epicuticular wax layer of the plant can also influence the uptake of compounds into the leaf and the composition of any barrier layer in mosses is important when investigating atmospheric N uptake.
Figure 1.1. Simplified diagram, of the fate of NOx, NO$_3^-$, NH$_3$ and NH$_4^+$ and entry into the leaf and subsequent assimilation. The question marks indicate uncertainty about the fate of NO after it enters the apoplast.

N is taken up after deposition on to the leaves either as NO$_2$ and NO$_3^-$ provide the supply of ions to be metabolised in the cytoplasm, with NO also requiring consideration but the fate of this compound is unknown after it enters the cytoplasm (Mansfield, 2002). The enzymes nitrate reductase (NR) and nitrite reductase (NiR) are involved in the regulation of internal N pools and provide the 'sinks' that might be driving uptake of NO$_3^-$ and subsequent conversion into NH$_4^+$ and amino acids via the glutamine synthetase pathway (GS$_2$) and glutamate synthase (GOGAT) cycle (Taiz and Zeiger, 1998). NH$_4^+$ can also be taken up...
via the apoplast and converted to amino acids via the GS₁ pathway. These amino acids then go on to form proteins in the cells (Figure 1.1).

The uptake of N and subsequent reduction of $\text{NO}_3^-$ to $\text{NH}_4^+$, and incorporation into amino acids and then proteins, is vital to plant nutrition, growth and survival. Major advances in investigating the factors that regulate these processes are helping us to understand how N uptake is regulated (Campbell, 1996) and two major enzymes involved most specifically with N uptake are nitrate reductase (NR) and nitrite reductase (NiR). NR was isolated and characterised almost 50 years ago and research into its structure and function still continues. The enzyme is involved in one of the most important steps of $\text{NO}_3^-$ assimilation, the reduction of $\text{NO}_3^-$. 

1.5. Atmospheric N and the external environment

As many mosses take up their nutrients, including N, through their external leaf surfaces the main supply of N for uptake is from the atmosphere. This occurs either from dry or wet deposition or to some extent from run-off and throughfall if it is a woodland species. To investigate how plants take up N from the atmosphere it is important to quantify the amount of N naturally available in the environment and then simulate the exposure of vegetation to these N concentrations in the laboratory. Plant atmosphere exchange processes are complex with atmospheric pollutants, of which $\text{NO}_3^-$ and $\text{NH}_4^+$ are included for N, transported by wind and turbulence from source points such as factories and agriculture (Figure 1.2).
Pollutants can undergo changes in the atmosphere and interestingly the phase change from dry to wet deposition, gas to aerosols, for sulphur and N compounds allows the formation of highly efficient condensation nuclei for cloud droplet formation which are efficiently removed from the atmosphere by precipitation as wet deposition (Fowler et al., 2004). It should be noted that these particles deposit quite slowly to terrestrial surfaces, so that in the absence of precipitation they have relatively long atmospheric lifetimes, extending to several days (Fowler et al., 2004). This longevity contributes to the increasing N in marine, terrestrial and atmospheric environments globally.

![Figure 1.2. Atmosphere-surface exchange of N compounds – wet deposition.](image)

Anthropogenic N deposition from burning fossil fuels in factories and from agriculture, including the application of fertilisers, has resulted in an accumulation of N in atmospheric, terrestrial and marine environments.
The transfer of pollutants to terrestrial environments is influenced by frictional drag with moss canopies having less frictional drag than tree canopies (Fowler *et al.*, 2004). Frictional drag also contributes to deposition dynamics according to the extent of epicuticular waxes on leaf surfaces with rough surfaces trapping deposited particles. Bryophytes tend to have less extensive cuticles than higher plants and examination of the cuticle in both species should gauge how much of an effect frictional drag has on N uptake from atmospheric N deposition (Bates, 2000). However, mosses growing under tree canopies may still experience enhanced N deposition due to throughfall enriched in N compared to rainfall in open situations.

Total annual emission of fixed N from natural processes, prior to the industrial and agricultural revolutions globally was about 35 Tg N, divided roughly evenly between reduced and oxidised forms (Figure 1.3) (Fowler *et al.*, 2004). Total annual emissions in 2000 amounted to 124 Tg N which exceeded natural sources by almost four-fold. Monitoring these increases is important with regard to global affects and changes to the environment.
Figure 1.3. Atmosphere-surface exchange of fixed N (Tg N). Total values on the left (black text) and anthropogenic (pink text) N in brackets. Values are annual and global. Values of primary interest are NOx and NH\textsubscript{3} inputs for the purposes of this research. Fossil fuel combustion: double blue arrow; soils and agriculture: double green arrow; wet and dry deposition onto the land and ocean; double yellow arrows. Adapted from Fowler et al., 2004.

Experiments that apply concentrations of inorganic-N in the laboratory to simulate conditions found in the field are few, and often it is not the major aim of plant biochemical research to apply realistic concentrations when looking for short term responses. It is not uncommon with studies of NR in higher plants for N to be applied at quite high concentrations, for example 15 mM (Leleu and Vuylsteke, 2004) and 5 mM (Bungard et al., 1999). This ensures that NR is induced in order to study the effects of increased N supply. The highest concentration of artificially applied N used by Woodin and Lee (1987) on Sphagnum mosses to investigate NRA was 1 mM and concentrations of N in rainwater in central London can reach 1 mM when both dry and wet deposition...
are collected over a length of time (personal communication John Pearson, University College London, Department of Biology).

1.6. The effects of increased atmospheric N deposition on mosses

Most research into the effects of increased N inputs on the environment has focussed on field based experiments. When using these systems plant community composition is regulated not only by plant performance under certain edaphic and climatic conditions but also depends on plant-plant, plant-microorganism, plant-parasitic fungi and plant-herbivore interactions (Näsholm 1997). Environmental N deposition occurs in a range of different forms such as NO₂, NO₃, NH₃ and NH₄⁺ in various physical states, for example gaseous or wet deposition, and these N compounds may each have diverse effects on different plants. Measuring N deposition effects in field based experiments and then drawing conclusions from the data can prove complex.

Field studies on the effects of increased N deposition on ecosystems in the UK have demonstrated that increased N can be a significant factor contributing to the decline and loss of moss species throughout Britain (Baddeley et al., 1994; Power et al., 1998; Lee and Caporn, 1998; Lee, 2000; Carroll et al., 2000; Pearce and van der Wal, 2002; Curtis et al., 2005). To take one example the decline of *Racomitrium* on montane heaths in northeast Scotland demonstrated how sensitive this ecosystem was to even low concentrations of atmospheric N deposition, with raised tissue N content and severely reduced shoot growth (Pearce and van der Wal, 2002). *Racomitrium* tissue N in low dose plots was
significantly greater than that in high dose plots and this was thought to be the result of growth dilution as shoot growth was significantly reduced compared to the other treatments (Pearce and van der Wal, 2002). This lack of tissue N accumulation may reflect physiological damage caused by the raised N supply which may take the form of tissue membrane damage resulting in greater solute loss from *Racomitrium* treated with the high N dose (Pearce and van der Wal, 2002). *Racomitrium* also appeared pH sensitive, with high N concentrations applied at pH 3 interfering with its ability to accumulate added N, thus reducing uptake efficiency compared to that applied at pH 5 (Soares and Pearson, 1997). Solutions of low pH have been known to similarly harm boreal mosses such as *Hylocomium splendens*, reducing nutrient content, segment length and biomass (Bates, 2000). A similar response in *Racomitrium* may explain the low tissue N content in high dose plots and the growth reduction in all N treated plots (Pearce and van der Wal, 2002). The research also highlighted the difficulties in determining whether the loss of the moss was due to the indirect effects of N deposition, such as the increase in higher plant cover reducing light availability to the moss, or the direct effects of N deposition disrupting moss growth. Pearce and van der Wal (2002) acknowledged the need to understand the effects of N addition on the complex of vegetation and soil processes and the relative importance of grazers in causing the decline of the heath.

### 1.7. Moss as bioindicators of climate change

Findings from such initiatives as the GANE programme have highlighted that the effects of increases in anthropogenic N on the environment, due to the
intensification of agriculture and industry in the UK, have implications for climate change as well as reductions in plant biodiversity. Research for mitigating climate change in agriculture is being developed to counter or reduce greenhouse gas emissions. For example work at Rothamsted Research includes the introduction of a decision support system for assisting with advice on N fertiliser application to decrease wastage of fertiliser N through better accounting of N coming from soil and better timing of application in relation to crop requirements (Climate change and land management, Rothamsted Research, Brochure, 2005). N fertiliser production is a dominant source of CO₂ emissions, so any saving in N inputs through improved N-use efficiency could make a contribution to climate change mitigation and reducing amounts of surplus inorganic-N in the global N cycle would reduce knock-on effects on biodiversity.

Understanding how plants specifically sense N, and respond to changes in N, is still being researched and there is a considerable amount of information available, specifically with regards to higher plants and particularly crop plants, in order to try and regulate N uptake and maximise N use efficiency. Mosses require more rigorous investigation to bring the research up to the same level of knowledge. Importantly the effects of increased N deposition on plants and the environment are only just being revealed and field simulation experiment have shown that some mosses are particularly sensitive to deposited N (Baddeley et al., 1994; Power et al., 1998; Lee and Caporn, 1998; Lee, 2000; Carroll et al., 2000; Pearce and van der Wal, 2002; Curtis et al., 2005). Mosses are a vital part of upland communities and in many places Sphagnum species are the most important peat forming plants. Several other species are dependent on the
condition produced by a *Sphagnum* blanket bog so if the bog conditions are changed then there will be knock-on effects on other species and potentially a loss of general biodiversity. These include the carnivorous sundews (*Drosera*) and cranberry (*Vaccinium oxycoccus*) (GANE programme outline, 2001). Aldous (2002) also highlighted the low nutrient demands of *Sphagnum* bogs with measurements of low tissue nutrient concentrations, high nutrient use efficiency, tight nutrient cycling and spatially distinct nutrient pools from vascular plant, but very low toxic thresholds for high atmospheric N deposition in field studies. Commentary from Bridgham (2002) outlined that from Aldous’ work *Sphagnum* mosses can be considered a keystone species on an ecosystem scale. Understanding *Sphagnum* bog ecological strategies to low nutrient availability was proposed to be essential not only for predicting peatland dynamics but also for predicting their future role in global carbon budgets and global change. Other evidence that enhanced reactive N deposition affects semi-natural terrestrial ecosystems comes from historic increases in plant tissue N concentrations, correlation between tissue N concentrations and present-day total atmospheric N deposition, changes in plant amino acid composition and effects on N assimilation. Lee and Caporn (1998) outlined that the ecological significance of such changes in biomarkers, or biological tracers, was uncertain in 1998, highlighting the importance of monitoring such changes in response to climate change. The use of a moss as a biological indicator or ‘bioindicator’ for climate change and pollution has been proposed most recently for the moss *Grimmia laevigata* (Fernandez et al., 2005). A bioindicator is an organism that contains information on the quality of the environment, because a change in the organism can be linked to a change in its habitat (Markert et al., 2003). Fernandez et al.,
(2005) suggest that the life history, growth habitats and extreme desiccation tolerance of *Grimmia laevigata* make this moss an ideal research tool and a candidate for a biological indicator of climate change and pollution. It is hoped that such a system could be developed using mosses to understand how they sense N and this may having implications for sensing such far-reaching goals as monitoring climate change and pollution.

1.7.1. Sensing N Status

Amino acids and proteins have been proposed as metabolites that may regulate the uptake of N and be involved in how plants sense N status (Lea and Miflin, 2003). Experiments with mosses looking at changes in amino acids, where excess N was applied on a plant community scale, have been carried out. A large increase in the amino acid, arginine, in the shoots of peat mosses was found after application of N, indicating a nutritional imbalance of the species (Tomassen *et al.*, 1999). There is also evidence that the amount of NO$_3^-$ delivered to the leaves of higher plants in the transpiration stream is determined by xylem-loading from cortical cells within the roots, the rate of loading being determined by feedback regulation from amino acids delivered to the roots via the phloem (reviewed by Crawford and Glass, 1998, Rennenberg *et al.*, 1998). Some amino acids have been shown to be inhibitors of NR, glutamine and asparagine have been shown to be the most potent inhibitors (Sivasankar and Oaks 1995, Dzuibany *et al.*, 1998). Indeed there has been some focus on the role of glutamine as a signal and inhibitor for NR (Lee *et al.*, 1992) but the evidence is conflicting and no definite link between glutamine and NR inhibition or glutamine as a signal of N status.
has been shown. Measuring tissue amino acid concentrations are therefore important parameters to consider when investigating N sensing in mosses. With respect to protein, high concentrations in proteins in plants may indicate that a plant is in an N sufficient state and lead to the accumulation of free amino acids. When a plant is N, and therefore NO$_3^-$ deficient it will generally have lower concentrations of NR protein and lower NR activity in the leaves but it may still have about the same NR activation state (high in the light, low in the dark) as N and NO$_3^-$ sufficient plants (Kaiser and Huber, 2001). Measuring protein concentrations is important in light of the research carried out by Andrews et al., (2006) which suggests that protein might act as a signal to regulate matter partitioning between the shoot and root of higher plants. Little is known about this in moss species and further investigation will prove important when drawing conclusions from the data but it should be noted that leaf tissue has primarily been analysed in this study.

Another innovative area which can be used to study sensing N status in mosses is the field of electrophysiology. N transporter proteins can provide an entry route for NO$_3^-$ into cells (Crawford and Glass, 1998; Forde, 2000; Williams and Miller 2001) and the use of microelectrodes can begin to develop an understanding of the transporter involved in regulating N uptake and sensing N.

1.8. Aims and Objectives of Research.

This research has the following overall hypotheses and objectives:
1. Two moss species (*S. fimbriatum* and *M. hornum*) from contrasting habitats may have differing metabolic responses to artificial applications of elevated inorganic-N and these differences may be used to assess the N status of the moss.

2. Mosses show a range of differing sensitivities to atmospheric N pollution and the use of moss as a bioindicator could prove useful in monitoring climate change and pollution.

3. Microelectrode measurements in moss, like higher plants, can be used to measure moss N status including membrane transporter activity and N storage capacity.

1.9. Thesis Overview

This Chapter provides an introduction to research on sensing N-status in plant systems and reviews existing literature in the field. In Chapter 2 the materials and methods used throughout the research are covered. In Chapter 3 the uptake of N in mosses and the rapid initial induction of nitrate reductase (NR) are discussed. In Chapter 4 long term N deprivation research in mosses is described. In Chapter 5 fieldwork on atmospheric N inputs is reported. In Chapter 6 measuring N status in mosses using microelectrodes is investigated. Chapter 7 reviews all the research and relates it to the current understanding of sensing N status in mosses.
CHAPTER 2

MATERIALS AND METHODS

2.1. Moss sample sites

Two moss species, *Sphagnum fimbriatum* and *Mnium hornum*, were sampled from two different sites in the UK – Goyts Moss and Mardley Heath respectively. These species were chosen for their contrasting habitats and potential use of N; *Sphagnum fimbriatum* being more able to thrive in acidic environments, such as bogs, which may be dominated by NH$_4^+$, and *Mnium hornum* being more prevalent in woodland areas where NO$_3^-$ may dominate.

![Image of Sphagnum fimbriatum at Goyts Moss](image)

*Figure 2.1* Photograph showing *Sphagnum fimbriatum* at Goyts Moss. Leaf detritus from surrounding vegetation of birch and oak trees visible.
The peat bog moss, *Sphagnum fimbriatum* (*S. fimbriatum*) was collected from Goyts Moss in Derbyshire (GPS coordinates SK 01057 73858 elevation 232m) (Figure 2.1). Goyts Moss is located in the Goyt Valley where the landscape ranges from the heather and grasses of upland moorland pastures, to the woodland and enclosed farmland of the valley where the sample site was located. The site was sheltered by surrounding birch and oak woodland. Sampling took place throughout the year and the time of collection was recorded.

Figure 2.2 Photograph showing *Mnium hornum* at Mardley Heath on woodland floor.

The woodland floor moss, *Mnium hornum* (*M. hornum*), was collected from Mardley Heath in Hertfordshire (GPS coordinates TL 24384 18387 elevation 59m) (Figure 2.2). The site is located next to the A1M motorway with 90 acres of open heath and mixed woodland; the woodland is dominated by oak, birch and ash. Parts of Mardley Heath were once devastated for gravel extraction and the
area is now the subject of a heathland restoration project. Sampling took place throughout the year and time of collection recorded.

2.2. Controlled environment conditions

*M. hornum* and *S. fimbriatum* were set up in controlled environment (CE) conditions in a 3 m by 4 m growth cabinet equipped with cool white fluorescent lamps (Model 830, Philips) mounted above a clear glass barrier, with an upward airflow distribution system that used sufficient outdoor air to provide near ambient CO$_2$ conditions inside the cabinet. The cabinet air temperature was maintained at 20 °C (s.d. ± 2/1 °C) during the light/dark period. The photosynthetically active radiation (PAR) at plant level had a photon flux density of 150 μmol m$^{-2}$ s$^{-1}$ (s.d ± 10 μmol m$^{-2}$ s$^{-1}$) during the 16 h photoperiod. The PAR was set-up to mimic a shaded woodland and this was achieved by positioning the trays of moss on the lower shelves of the growth cabinet. The relative humidity in the cabinet was maintained at 70% (s.d ± 10%).

2.2.1. *Sphagnum fimbriatum* - cultivation

Individual *S. fimbriatum* plants were separated out from the grass and leaves and cut away from the necrotic tissue at the base of the plant matter. The plants were then placed on top of a 5 cm layer of pre-watered peat. To sustain the tissue, prior to undergoing N-starvation, the *S. fimbriatum* was planted out into a large Perspex box (50 cm length, 35 cm width, 40 cm depth), with perforated holes for
drainage and a secure lid, which was then placed in a large tray to collect and remove the run-off underneath, and placed in the CE cabinet (Figure 2.3). The moss was watered by misting with dH₂O and the box and lid securely fastened to prevent water loss and tissue desiccation. This enabled the moss to be cultivated continuously for in excess of 6 months.

**Figure 2.3** Photograph showing *Sphagnum fimbriatum* in CE cabinet undergoing cultivation (left) and later N-starvation (right).

### 2.2.2. Sphagnum fimbriatum – N-starvation conditions

N-starvation involved replanting the *S. fimbriatum* into cylindrical netlon cylinders, (5 cm diameter and 8 cm depth), until the weight of the plants held them in a monolith (Figure 2.3). These were then placed into a plastic tray containing N-free rain solution (KCl 2.95 μmol dm⁻³, MgSO₄·7H₂O 4.44 μmol dm⁻³, CaCl₂·2H₂O 7.39 μmol dm⁻³, Na₂SO₄ 16.35 μmol dm⁻³, NaH₂PO₄·2H₂O 0.42 μmol dm⁻³) as described in Press et al., (1986) and then inside a large
Perspex box (50 cm length, 35 cm width, 40 cm depth) with lid, and misted daily with dH₂O whilst in the CE cabinets.

2.2.3. *Mnium hornum* – cultivation and N starvation conditions

Once collected from the field the *M. hornum* was planted into plastic trays containing a 2 cm thick layer of peat. The trays were perforated with holes in the base to aid drainage and a tray placed underneath to collect and remove the runoff and put in the CE cabinets as described for *S. fimbriatum* above (Figure 2.4). These trays were watered and misted with dH₂O and covered over with transparent film to prevent moisture loss and tissue desiccation.

![Figure 2.4. Photograph showing Mnium hornum in CE cabinet undergoing N-starvation.](image)
2.3. Treatments, enzyme and metabolite assays

Nitrate reductase activity (NRA) *in vivo* and *in vitro*, assays were carried out at the same time as tissue extraction for the determination of nitrate (NO$_3^-$) concentration. For both moss species the NRA and NO$_3^-$ concentration was first established using a dH$_2$O misting spray for the duration of a time trial experiment. Sampling took place with the first sample being taken 15 minutes after the misted application. A new misting application was then applied 2 hours after the first misting and sampling for enzyme activity and tissue metabolites again took place 15 minutes following the treatment. This was repeated after time 0 hr every 2 hr to either 6 or 8 hr. For any inorganic-N applications or other treatments an initial dH$_2$O misting spray was applied at time 0 hr in order to establish control NRA and NO$_3^-$ concentration. Treatments that were applied to the plants, using the same methods as for the control, included: 0.5 mM KNO$_3$, 0.25 mM Ca(NO$_3$)$_2$, 0.5 mM NH$_4$Cl, pH 3 H$_2$SO$_4$, 10 mM K$_2$SO$_4$, 1 mM CaSO$_4$ and 10 mM butyric acid as aerial mists of 200 ml solution. All inorganic N treatments were at the same pH (4.5) unless stated otherwise. Sampling took place for nitrate reductase activity (NRA), and at the same time tissue was extracted for the determination of nitrate (NO$_3^-$) concentration and total amino acids and protein. Measurements were the average of 5 replicates for each sampling time.

2.3.1. Nitrate reductase (*in vivo*)

*In vivo* NRA was measured according to a modified method of Stewart *et al.*, (1979). A known weight, usually 0.1 g FWt of material, was vacuum infiltrated
for 2-3 min with 5 ml of 0.1 M phosphate buffer (pH 7.5) containing 1 % propan-1-ol and 1 % KNO₃. The modification was one of optimising the assay for both species with regards to propanol and KNO₃ concentration in the incubation buffer. Both species showed maximum activity when propanol and KNO₃ where used at 1% v/v or w/v respectively as opposed to 1.5% for both in the assay of Stewart et al., (1979). The vials containing the tissue and assay medium were placed in a vacuum desiccator and evacuated with a Millipore pump at 25-30 kPa for 2-3 min, releasing pressure 3-4 times. This was done to fully infiltrate the moss tissue with the phosphate buffer solution. A blank was prepared with 5 ml 0.1 M phosphate buffer (pH 7.5) containing 1 % propan-1-ol and 1 % KNO₃ minus the tissue. Tissue was incubated at 25 °C, in the dark, for 60 min and the NO₂⁻ content determined by adding 0.5 ml 1 % w/v sulphanilamide in 1 M HCl and 0.5 ml 0.02 % N-naphthyl ethylene diamine dihydrochloride (NEDD) to 0.5 ml incubation medium. Colour was allowed to develop for 20 min and absorption read at 540 nm (using a Beckman DU-7 Spectrophotometer) and compared with known standards of NO₂⁻ concentrations.

2.3.2. Nitrate reductase (in vitro)

In vitro NRA was measured according to a modified method of Kaiser et al., (2000) and Man et al., (1999). A known weight of material was homogenised in a pestle and mortar with liquid N, sand and Polyvinyl polypyrrolidone (PVPP). Extraction buffer containing: 0.1 M Tris (pH 8.5), 1 mM Na₂EDTA, 20 μM Leupeptin, 10 mM DTT, 1 mM NaMoO₄, 10 μM FAD, 0.1% w/v nonidet detergent and 0.1% v/v mercaptoethanol was added, the whole being kept on ice.
The plant extract was then spun at 4°C for 20 min in a bench top microfuge at 13,000 rpm. The supernatant was decanted off and 100 µl was added to a 1 ml Eppendorf tube along with 300 µl 0.1 M phosphate buffer at pH 7.5, 50 µl 0.1 M KNO₃ and 50 µl 10 mM NADH. A blank was made by substituting the enzyme extract with 100 µl extraction buffer. The Eppendorfs were incubated in a water bath at 25°C, in the dark, for 60 min and the reaction was terminated by the addition of 0.5 ml 1 % w/v sulphanilamide in 1 M HCl and 0.5 ml 0.02 % N-naphthyl ethylene diamine dihydrochloride (NEDD) to 0.5 ml incubation medium. Colour was allowed to develop for 20 min and absorption read at 540 nm (using a Beckman DU-7 Spectrophotometer) and compared with known standards of NO₂ concentrations.

2.3.3. Tissue nitrate concentration measurements

Tissue NO₃⁻ content was measured by extracting known weights of tissue in 5 ml methanol. Methods were adapted from that reported by Gilliam et al., (1993) After 24 h, 0.8 ml of methanol extract, and 0.2 ml of 0.085 M K₄Fe(CN)₆ • 3H₂O mixed with 0.2 ml of 0.25 M ZnSO₄ • 7H₂O was spun at 4 °C for 10 min in a bench top microfuge at 13,000 rpm. 0.1 ml sample supernatant was then added to 0.37 ml of 0.1 M phosphate buffer (pH 8.0), 0.01 ml of 2.29 mM FADNa₂, 0.01 ml of 1.13 M NADPHNa and 0.1 U NR (<title>Aspergillus</title> species – Roche EC 1.6.6.2.). The reaction mixture was incubated at 25 °C for 60 min and the NO₃⁻ content determined by adding 0.5 ml 1 % w/v sulphanilamide in 1 M HCl and 0.5 ml 0.02 % NEDD in dH₂O to 0.5 ml incubation medium. Colour was allowed to develop for 20 min and absorption read at 540 nm (using a Beckman
DU-7 Spectrophotometer) and compared with standards of NO$_3^-$ put through the enzymatic reaction.

### 2.3.4. Amino Acids

Total amino acid content was measured using the ninhydrin colourimetric analysis as described by Ferguson et al., (1974). After 24 h, 0.02 ml of the methanol extract (see Section 2.3.1 – 2.3.3), and 0.28 ml of 0.8 M citrate buffer (pH 4.84) with 0.24 ml of 1% ninhydrin solution was pipetted into 1.5 ml Eppendorfs and boiled for 20 min. The reaction mixture was allowed to cool and 0.6 ml 60 % ethanol added. Colour was allowed to develop for 20 min and absorption read at 570 nm (using a Beckman DU-7 Spectrophotometer) and compared with known standards of amino acid (glutamine) concentrations.

### 2.3.5. Total Soluble Protein

Total soluble protein content was measured using colourimetric analysis as described by Bradford (1976). After 24 h, 50 µl of methanol extract, and 50 µl of dH$_2$O was added to 2.5 ml of Bio-rad reagent (diluted 1:4 with dH$_2$O and filtered through Watman no. 1 paper). The solution was left for 20 min and the absorption read at 595 nm (using a Beckman DU-7 Spectrophotometer) and compared with known standards of protein (bovine albumin or bovine serum albumin) concentrations.
2.4. Fieldwork – Mardley Heath

Following research on *M. hornum* in a laboratory controlled environment the species was identified as a suitable candidate for use in field experiments in preference to *S. fimbriatum* due to its higher desiccation and N-deprivation tolerance. A period of two months fieldwork was carried out to study the effects of placing the moss, with a known and low N status, back into the field where there were high N inputs. Two sites were chosen and trays of *M. hornum* were placed at these locations. Duplicate rain gauges were also set up to collect rainwater and measure N inputs and the effects of wet deposition at each site. *M. hornum*, at established N-depletion status (N-dep 151), was placed at both sites. NRA, NO₃⁻ concentration, total N and cations and heavy metals were measured in the plant tissue. Nitrogenous compounds, cations and heavy metals were also measured in the rainwater (see Section 2.5.). The sites chosen were 20 m from the side of the A1M Motorway at Mardley Heath (GPS coordinates TL 24355 18509 elevation 115m) (Figure 2.5) and in the woodland, 700 m from the motorway (GPS coordinates TL 24954 18349 elevation 105m) (Figure 2.6). The site adjacent to the motorway is an established site for monitoring the effects of automotive N inputs from the A1M (Marsh *et al.*, 2005). The woodland was chosen as a notional control site and to investigate the contribution of the A1M to atmospheric N inputs further downwind.
Figure 2.5 Photograph showing tray of *Mnium hornum* (N-dep 151) at the A1M site. The motorway barriers can be seen at the top, the tray of moss (bright green square) at the bottom left and two rain gauges (MO1 immediately above the tray and MO2 to the right).
Figure 2.6. Photograph showing tray of Mnium hornum (N-dep 151) at the Woodland site. The early tree cover can be seen (April), the tray of moss (bright green square) at the bottom right and two rain gauges (W1 and W2) Trays of moss were set up here from the start of April until the end of May 2005. These months were chosen as some of the wettest in the year in order to keep the plants hydrated.
2.5. Further Chemical Analysis

In *S. fimbriatum* and *M. hornum* further chemical analysis was utilised to determine the N-status of the tissue. Total N, natural abundance $^{15}$N status and cations and heavy metals were measured in the tissue of both species. This analysis was most applicable to the fieldwork material. To analyse rainwater inputs from field trials the aforementioned parameters were measured along with ion analysis of NO$_3$-N, NO$_2$-N and NH$_4$-N.

2.5.1. Foliar N and $^{15}$N atom analysis

An ANCA-SL (Automated Nitrogen Carbon Analysis for Solids and Liquids) / 20-20 Integra-CN (Europa Scientific Instruments Inc.) was used to measure total N and $^{15}$N. Moss tissue was dried in an oven and then ground to a fine powder using a methanol washed pestle and mortar. This process was repeated over a period of a few days to homogenise the material and ensure that it was completely dried. Samples of known weight of dried moss tissue were loaded into tin capsules and dropped into a furnace at 1000 °C while in an atmosphere of oxygen. The tin ignited and burnt exothermically, and the temperature rose to about 1800 °C, oxidising the sample. Complete oxidation was ensured by passing the combustion products through a bed of chromium trioxide at 1000 °C using a helium carrier gas. A 15 cm layer of copper oxide followed by a layer of silver wool completed the oxidation and removed any sulphur. The products were then passed through a second furnace containing copper at 600 °C where excess oxygen was absorbed and nitrogen oxides were reduced to elemental N.
Water was removed in a trap containing anhydrous magnesium perchlorate and carbon dioxide in a trap containing Carbosorb™. The gas stream passed into a gas chromatograph where components of interest were separated and then bled into a mass spectrometer where the isotope species were ionized then separated in a magnetic field. The isotopic species were detected separately and from their ratios, the level of $^{15}$N calculated. Calibration of the system was made using known standards allowing both total N and $^{15}$N content to be obtained from each sample. An Isotope Ratio Mass Spectrometer (IRMS) measured the ratio of heavy and light isotopes of a sample and compared this to a standard. It is difficult to measure the absolute abundance of the isotopes with a sample because of the small differences and problems such as sample heterogeneity, sample preparation and analyser fluctuations. To compensate for these problems, the isotope ratio of a sample ($R_{\text{sam}}$) was compared to a known standard ($R_{\text{std}}$). Therefore any fluctuations in the standard were also repeated in the sample. $R$ is expressed as the ratio of the heavy to light isotope. The differences in ratios were calculated in $\delta$ notation and had units of per million or $\%o$.

$$\delta\ (%o) = \left( \frac{R_{\text{sam}}}{R_{\text{std}}} - 1 \right) \times 100\ (%o)$$

For N, the accepted standard is that of atmospheric N at ($^{15}$N / $^{14}$N) 0.0036765. The abundance of an element, expressed in atom % is expressed as follows:

$$\text{Abundance, } A = \left[ \frac{R_{\text{sam}}}{1 + R_{\text{sam}}} \right] \times 100$$

The natural abundance of N is $^{15}$N = 0.3663 atom % and $^{14}$N = 99.6337 atom %. The Integra-CN was set-up for dedicated N isotope analysis. The collectors measured the masses of N 28, 29, 30. Values were expressed as foliar % N and $^{15}$N atom %.
2.5.2. Ion Analysis

For ion analysis of rainwater the Skalar SAN\textsuperscript{PLUS} System was used to carry out continuous colourimetric flow analysis (Skalar Analytical BV). The Skalar SAN\textsuperscript{PLUS} System is a segmented continuous flow analyser. Automatic segmented flow analysis is a continuous flow method of chemical analysis in which a stream of reagents and samples, segmented with air bubbles, is pumped through a manifold to undergo treatment such as mixing, heating, dialysis etc. before entering a flow cell to be detected. Air segmentation is used to eliminate cross contamination and to provide an aliquot to mix different reagents. In this way NO$_3$-N, NO$_2$-N and NH$_4$-N were measured in rainwater. This type of analysis is used to investigate ion concentrations in rainwater, streamwater chemistry, soil chemistry, groundwater run-off and many other systems to quantify concentrations in the external environment (from personal communication Wendy Gregory at Rothamsted Research (CPI Division)).

2.5.3. Total Major and Trace Element Analysis

To measure total major and trace elements in tissue and rainwater an Accuris Inductively Coupled Plasma Emission Spectrometer (ICP-ES) was used (Applied Research Laboratories). Moss tissue was dried in an oven and then ground to a fine powder using a methanol washed pestle and mortar. This process was repeated over a period of a few days to homogenise the material and ensure that it was completed dried. Sample materials, of known weight of dried moss tissue were then analysed. Rainwater was acidified before analysis was carried out.
The ICP-ES consists of a sample introduction system (nebulizer, spray chamber and pump), the plasma and spectrometer. The sample introduction system brings the sample solution in the form of an aerosol, to the plasma. The plasma is a highly ionised, very hot gas, which is stable and chemically inert with temperatures near 10,000 K. Light from the plasma is accepted by the spectrometer and separated into components and detected by photomultiplier tubes, the individual components are then recorded.

2.6. Microelectrodes

Microelectrodes were used to measure transport of N across cell membranes in both species of moss. These microelectrodes are glass micropipettes which are pulled into a fine tip at one end and filled with an aqueous salt solution. The simplest microelectrodes are single-barrelled and when inserted into cells they measure the membrane potential, in mV, between the inside and outside of the cell. Ion-selective microelectrodes are double-barrelled and they can be used to measure the ion gradients across membranes in addition to the membrane potential. Intracellular measurements using this technique have been used to study the compartmentation of nutrients, intracellular signalling, and transport mechanisms (Miller, 1994).
2.6.1. Ion-Selective Microelectrodes – background and set up

An ion-selective microelectrode contains an ion-selective membrane in the tip of the glass micropipette that is responsive both to the membrane potential and the activity of the ion sensed by the selective membrane. Ion-selective microelectrodes were used to measure ion concentrations both intra and extracellularly.

![Figure 2.7](image)

*Micrographs courtesy of D.J. Walker*

**Figure 2.7.** Electron micrograph of double-barrelled microelectrode showing the twisted tip. Inset: end view showing the open ends of each barrel. The external diameter of the tip is 1.0 \( \mu m \). Scale bars: \( a = 0.5 \) mm, \( b = 0.6 \mu m \).
Electrical signal
Ion-sensing barrel with ion-selective sensor at tip backfilled with electrolyte
Cell voltage recording barrel backfilled with 100 mM KCl
External solution in chamber
Cytoplasm
Vacuole

A = mV
B - A = [Ion]

Metal contact: AgCl coated silver
Half cell backfilled with 100 mM KCl (same for Half cell A and Half cell B)
Reference ground electrode backfilled with 100 mM KCl
Agar plug
Cell wall

Figure 2.8. Diagram of a liquid membrane double-barrelled ion-selective microelectrode suitable for intracellular recording. Adapted from Miller and Wells, 2006. A: cell voltage recording barrel. B: ion-sensing barrel with ion-selective sensor in the tip. The output of B is subtracted from the output of A and converted to ion activity using a calibration curve. V: voltmeter

Intracellular measurements give important information on nutrient compartmentation and transport mechanisms including measuring energy gradients for ion transport (Miller and Wells, 2006). The simultaneous measurement of intracellular measurements and membrane potential are achieved
using a double-barrelled microelectrode which combines the ion-selective and voltage-measuring electrodes in a finely extruded glass micropipette (Figure 2.8). Advantages of using ion-selective microelectrodes are that: they offer a non-destructive method of measuring ions within cells, they do not change the activity of the ion being measured and they allow the simultaneous measurement of the electrical and chemical gradients across membranes. The measurement of a voltage requires a complete electrical circuit that includes the measuring device, a voltmeter (V) or electrometer (E).

An ion-selective sensor at the tip of the microelectrode contains a chemical cocktail (sensor) which gives a voltage output of different values when placed in solutions containing different activities of the ion. Subsequently when the electrode is inserted in the cell, the voltage measured gives an indirect indication of the ion activity inside the cell. To obtain the output for the ion alone, the cell voltage is subtracted using a double-barrelled electrode in which the ion-sensing electrode is combined with a cell-voltage-measuring electrode (Figure 2.9). Both output voltages are measured against a reference ground electrode in the external solution. The ion activity is determined from the calibration curve (see Appendix 1) after subtracting the membrane potential (mV).
Setting up microelectrodes for use (see Figures 2.8 and 2.9) involved backfilling the microelectrode with electrolyte solution using a plastic syringe and a MicroFil™ flexible needle (World Precision Instruments Inc.). The filamented glass has a glass fibre attached to the inner wall; this fibre assists backfilling by providing a hydraulic conduit along which the solution can flow. For NO$_3^-$-selective microelectrodes the ion-sensing barrel was back-filled with an electrolyte of 100 mM KCl and 100 mM KNO$_3$ and reference barrel with 100 mM KCl. For pH-selective microelectrodes the ion-sensing barrel was filled with an electrolyte of pH 4 buffer and reference barrel as described previously. The ion-sensing barrel was connected directly into a microelectrode holder (model ESP-F10N, Harvard Apparatus Ltd.) filled with the appropriate
electrolyte using a plastic syringe with a stainless steel hypodermic needle. The shorter reference barrel was connected with silver wire (0.38 mm diameter silver wire, AGW1510, World Precision Instruments Inc.) from the microelectrode to a 100 mM KCl filled microelectrode holder. The microelectrode holders contained an Ag/AgCl pellet with a 2 mm socket which was connected to the jack of the headstage amplifier of a high impedance differential electrometer (model FD223, World Precision Instruments Inc.). The output from the electrometer was passed via an analogue to digital converter and data acquisition card (PC-LabCard model PCL-818H, Advantech Co. Ltd.) to an IBM-compatible personal computer. Data were displayed, analysed and stored using VISER software (Versatile Ion-Selective Electrode Recording software, Version 4.0, I. R. Jennings). The headstage/holder/electrode assembly was clamped to an 'x-y-z micromanipulator' (Goodfellow Cambridge Ltd.) to allow accurate positioning of the microelectrode tip. A half-cell connected to plastic tubing with an agar plug (100 mM KCl, 3.2 % (w/v) purified agar) was filled with 100 mM KCl; this 'ground electrode' provided the connection between the ground of the electrometer and the sample solution. Measurements were also obtained with single-barrelled microelectrodes where the same set-up was used, excluding the ion-selective barrel, to measure the membrane potential difference (mV) between the outside and inside of the cell in order to research transporter activity.
Figure 2.10. Arrangement of set-up for intracellular electrophysiological recordings. MS: microscopy; BRef: Bath reference electrode; Hc: Half cell; Mi: Microelectrode; PI: Perfusion Influx; C: Chamber; PC: Perfusion Control; PO: Perfusion Outlet; P: Plant

Further experimental details involved the construction of a suitable chamber so that the tissue could be perfused with nutrient solution throughout the experiment and at the same time enable the anchoring of plants for microelectrode penetration. In Figure 2.11 you can see a Perspex chamber designed for this purpose. Perfecting a successful chamber was essential in order to achieve successful recordings in the moss. The chamber was designed to allow the steady perfusion and efflux of solutions through the chamber during experimentation. The plant was secured using plastic tubing. Control of the rate of solution perfusion was enabled through the perfusion control mechanism fed via syringes mounted on the Faraday cage connected with plastic tubing. Plants
at known N-dep, which had previously only been watered with dH₂O and were bathed in N-free solution were perfused with 0.5 mM KNO₃, 0.25 mM Ca(NO₃)₂, 0.5 mM NH₄Cl, pH 3 H₂SO₄, pH 3 HCl and 0.25 mM CaCl₂. These treatments were made up in N-free rain solution and pH'd accordingly (at pH 4.5 unless stated otherwise). To study the contribution of any cell wall transporter activity some plants were pretreated with 10% SDS (sodium dodecyl sulphate) for a minimum of 24 hrs to disrupt the membrane and cell structure (Biswal and Mohanty, 1976). In order to penetrate moss cells the plants were observed under the microscope and the microelectrode positioned, using the micromanipulator, next to the exterior of the cell prior to impalement. Prior to use ion-selective microelectrodes require calibrating and this is outlined in Section 2.6.6 (see also Appendix 1 for typical calibration curve).

2.6.2. Ion-Selective Microelectrodes - Manufacture

Making ion-selective microelectrodes involves the pulling of glass micropipettes, silanisation of the inside surface of the ion-selective electrode or barrel, backfilling the barrel and then calibrating the electrode. Microelectrodes are prepared to give dimensions suitable for impaling the target cell type (see Figure 2.8.). For use in moss longer, finer tips were most successful for cellular penetration.
2.6.3. Ion-Selective Microelectrodes – Pulling

Double-barrelled microelectrodes were made by twisting pre-fused filamnented borosilicate glass (Hilgenberg Glass). The barrels of the glass had different diameters (external diameters 1.0 and 0.75 mm, internal diameters 0.58 and 0.35 mm respectively), the wider barrel being used as the ion-selective barrel and the narrower the reference barrel. The glass was clamped into the chucks of the micropipette puller (Narishige model PE-2, Figure 2.12.) with two protective sleeves of plastic tubing surrounding the ends to secure the glass in place. To twist the glass the electrode puller firstly heated and softened the glass until the lower chuck fell 4 mm. Heating was continued for a further 30 s, paused for the two barrels to be twisted around one another 360° in 1 min by the electric motor, and then heating and pulling was resumed. The micropipettes were then removed from the chucks using tweezers and placed carefully onto a steel plate in a fume hood. The narrower barrel was then broken back using a razor blade so that it was shorter than the larger diameter barrel (see Figure 2.9.).
Figure 2.11. Modified Narishige Electrode Puller (Inset: shows puller modification to give 360 twist). U: upper chuck; L: lower chuck; H: heating element; M: magnet; C: control unit (for adjustment of magnet and heater strength); B: metal block stepped to allow the lower chuck to fall through precise heights.

The micropipettes on the steel plate in the fume hood were then placed under a heating lamp (140 °C) to dry for 45 min. Once dried the inside of the glass micropipette was given a hydrophobic coating, to allow the formation of a high
resistance seal between the glass and the hydrophobic ion-selective membrane (see next Section 2.6.4).

2.6.4. Ion-Selective Microelectrodes – Silanising

The pipettes were silanised using 1 % (w/v) dimethyldichlorosilane (DCDMS, 40140, Fluka Chemicals) in chloroform (AnalR grade, BDH Laboratory Supplies). Two drops of silanising mixture were placed at the blunt end of the ion-selective barrel of each micropipette using a plastic syringe and metal needle. Care was taken not to silanise the reference barrel. The solution quickly vapourised giving the ion-selective barrel a hydrophobic coating. The micropipettes remained under the heating lamp for a further 45 min to complete the silanisation process.

2.6.5. Ion-Selective Microelectrodes – Backfilling with NO₃⁻/pH sensor

Before backfilling with sensor a check was made to ensure that no liquid residue was present in the microelectrode tip. The components of the NO₃⁻-selective sensor cocktail were mixed according to the proportions in Table 2.1. The components of the pH sensor cocktail were mixed according to Table 2.2. Sensors were dissolved in approximately 0.5 ml of the solvent tetrahydrofuran (THF) or until the sensor cocktail had dissolved. Cocktails were stored at 4 °C in screw-capped glass specimen vials with tetrafluoroethylene/silicon liners (98893,
Alltech Associates Applied Science Ltd.). Vials were stored inside light-tight pots containing self-indicating silica gel (Fisher Scientific UK Ltd.).

Table 2-1. Nitrate-selective microelectrode sensor membrane cocktail (Miller and Zhen, 1991)

<table>
<thead>
<tr>
<th>Component</th>
<th>% (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTDDA-NO₃, 91664, Fluka Chemicals.</td>
<td>6</td>
</tr>
<tr>
<td>Methyltriphenylphosphonium bromide. M-7883, Sigma-Aldrich Company Ltd.</td>
<td>1</td>
</tr>
<tr>
<td>2-Nitrophenyl octyl ether. 73732, Fluka Chemicals</td>
<td>65</td>
</tr>
<tr>
<td>Nitrocellulose. 7184002, Whatman International Ltd.</td>
<td>5</td>
</tr>
<tr>
<td>PVC. 81392, Fluka Chemicals</td>
<td>23</td>
</tr>
</tbody>
</table>

Table 2-2. pH-selective microelectrode sensor membrane cocktail (pH range 2 – 9)

<table>
<thead>
<tr>
<th>Component</th>
<th>% (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H⁺ Ion Ionophore II 95297, Fluka Chemicals.</td>
<td>35</td>
</tr>
<tr>
<td>Nitrocellulose. 7184002, Whatman International Ltd.</td>
<td>6</td>
</tr>
<tr>
<td>PVC. 81392, Fluka Chemicals</td>
<td>16</td>
</tr>
</tbody>
</table>

A plug of sensor cocktail was introduced to the silanised barrel of the double-barrelled micropipette using a 1 ml glass syringe and 38 mm long 29 gauge stainless steel hypodermic needle (Scientific Laboratory Supplies Ltd.). The finished microelectrodes were then transferred, tip down, into a silica-gel dried
sealed container with the micropipettes attached to the inner wall using Blu®-tack (Bostik). This container would be used for long term storage of the microelectrodes, in the dark. After 48 h the solvent phase of the cocktail evaporated to form a solvent cast membrane in the tip of the micropipette.

2.6.6. Ion-Selective Microelectrodes – Calibration

The ion-selective barrel gives an output voltage dependent on the activity of ion at the microelectrode tip, and requires calibration before use. Calibration of the ion-selective microelectrodes was carried out as in Figure 2.9. replacing the chamber with a funnel. The tips of microelectrodes were immersed in a series of calibration solutions of known ionic concentration held in the U-bend glass funnel (Soham Scientific) which allowed sequential solutions to be flushed through during the course of the calibration series. The composition of the calibration solutions can be found in Tables 2.3. and 2.4.

Table 2-3. Composition of solutions to calibrate nitrate-selective microelectrodes for intracellular measurements (Miller and Zhen, 1991).

<table>
<thead>
<tr>
<th>Nitrate activity (mM)</th>
<th>pNO₃</th>
<th>HEPES</th>
<th>KNO₃</th>
<th>KH₂PO₄</th>
<th>Ionic strength</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mM)</td>
<td>(mM)</td>
<td>(mM)</td>
<td>(mM)</td>
<td>(mM)</td>
</tr>
<tr>
<td>0.01</td>
<td>5</td>
<td>5</td>
<td>0.0121</td>
<td>50.0</td>
<td>140</td>
</tr>
<tr>
<td>0.1</td>
<td>4</td>
<td>5</td>
<td>0.122</td>
<td>50.0</td>
<td>140</td>
</tr>
<tr>
<td>1.0</td>
<td>3</td>
<td>5</td>
<td>1.22</td>
<td>50.0</td>
<td>141</td>
</tr>
<tr>
<td>10.0</td>
<td>2</td>
<td>5</td>
<td>12.20</td>
<td>46.5</td>
<td>140</td>
</tr>
</tbody>
</table>
All solutions were at pH 7.9 to ensure the major anion determining ionic strength, other than nitrate, was $\text{HPO}_4^{2-}$. $\text{pN}_3$ is the negative $\log_{10} [\text{NO}_3^{-}]$ activity. HEPES, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic) acid.

**Table 2-4. Composition of solutions for use with pH-selective microelectrodes.**

<table>
<thead>
<tr>
<th>pH</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>20 mM $\text{KHC}_8\text{H}_4\text{O}_4$ (potassium hydrogen phthalate)</td>
<td>120 mM KCl (potassium chloride)</td>
<td>10 mM NaH$_2$PO$_4$$\cdot$2H$_2$O (sodium dihydrogen orthophosphate)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.0</td>
<td>20 mM $\text{KHC}_8\text{H}_4\text{O}_4$ (potassium hydrogen phthalate)</td>
<td>120 mM KCl (potassium chloride)</td>
<td>10 mM NaH$_2$PO$_4$$\cdot$2H$_2$O (sodium dihydrogen orthophosphate)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.0</td>
<td>20 mM MES (2-[N-Morpholino]ethanesulfonic acid)</td>
<td>120 mM KCl (potassium chloride)</td>
<td>10 mM NaH$_2$PO$_4$$\cdot$2H$_2$O (sodium dihydrogen orthophosphate)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.0</td>
<td>20 mM MOPS (3-[N-Morpholino]propanesulfonic acid)</td>
<td>120 mM KCl (potassium chloride)</td>
<td>10 mM NaH$_2$PO$_4$$\cdot$2H$_2$O (sodium dihydrogen orthophosphate)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.5</td>
<td>20 mM TAPS (N-[Hydroxymethyl]methyl-3-amino-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
propanesulfonic acid)

120 mM KCl (potassium chloride)

10 mM NaH$_2$PO$_4$·2H$_2$O (sodium dihydrogen orthophosphate)

All solutions used were analytical grade chemicals and were weighed out into clean beakers. Distilled water was used and the pH of each solution adjusted to the appropriate value with 1M NaOH. Solutions were made up to volume in a clean volumetric flask and stored in a clean glass bottle.

The slope of the calibration curve is temperature sensitive and both calibrations and intracellular measurements were carried out at the same temperature. If the temperature of the calibration solutions is 4 °C and the cell is at 20 °C, the slope of the electrode calibration for a monovalent ion will be 55 mV per decade change in activity, not the 58 mV expected at 20 °C. Further information on microelectrode theory can be found in Appendix 1.

2.7. Imaging

Light microscopy, SEM (scanning electron microscopy) and chlorophyll fluorescence imaging were all used to elucidate the structural similarities and differences between the two species. Imaging techniques gave a good insight into the structure and extent of compartmentation in *M. hornum* and *S. fimbriatum* and chlorophyll fluorescence imaging was used as a tool to measure
the effectiveness of photosystem II as an indicator of the N status and health of the plant tissue in its varying stages of N-dep.

2.7.1. Light microscopy

Light microscopy was used to investigate the structural similarities and differences between the two species. Imaging of fresh tissue was carried out using an Axiophot (Zeiss) light microscope connected to a digital camera DC 300 FX (Leica) and images were acquired using IM 50 (Leica) software. To determine and compare the relative compartmental volumes within the plants neutral red staining of vacuoles was used. The leaves were stained in 0.1 M phosphate buffer (pH 7.5) containing 0.02% (w/v) neutral red and left overnight before viewing with a light microscope (methods adapted from Marschall et al., 1998). To investigate the effect of 10 % SDS on the plants, moss was also stained with neutral red and images examined using light microscopy using the same methods. Magnification was noted.

2.7.2. Scanning Electron Microscopy (SEM)

To look at the surface layer and compartmentation in both species SEM images were obtained. Some plants were misted with dH₂O 12 hrs before preparation and others misted with pH3 H₂SO₄ 12 hrs before preparation to look at the effect of these treatments on plant structure and wax cuticle layer. Samples were imaged using a Jeol LVSEM 6360 Scanning Electron Microscope (SEM)
operating in High Vac mode. Preparation of the samples was performed in the Gatan Alto 2100 Cryo unit attached to the electron microscope. Fresh leaf tissue was selected and an area 2 mm x 3 mm was mounted on a cryo stub using tissue-Tek (OCT compound) Sakura. This was plunged into LN₂ slush and transferred under vacuum to the main preparation chamber maintained at a temperature of -185 °C. If freeze fracturing was to be carried out a pre-cooled metal knife was introduced to the main preparation chamber of the cryopreparation unit. Any residual pieces of tissue were shaken off the stage before sublimation was carried out. Any ice crystals were removed by sublimation (raising the temperature to -90 °C) for approximately 120 s. The sample temperature was lowered back to -185 °C and when stable was coated for 60 s using an Au target. The sample was imaged using a SEI detector and accelerating voltage of 5 kV. Magnification and dimensions were noted on individual images.

2.7.3. Chlorophyll Fluorescence Imaging

Nitrogen deficiency has been associated with decreases in the fluorescence parameters $F_q/F_m$, $F_v/F_s$ and $F_\text{v}/F_m$ in sunflowers and many other higher plants (Ciompi et al., 1996). A fluorescence imaging system (Figure 2.13.) capable of measuring the parameter $F_s/F_m$ was used to assay its suitability in detecting the N status of a plant. *M. hornum* and *S. fimbriatum* were used at varying stages of N-dep to investigate the relationship between N-status and PSII. The following protocol was obtained from personal communication Gordon Forbes at Rothamsted Research (CPI Division).
The fluorescence imaging systems' most important components are those involved in fluorescence excitation and image capture. Fluorescence excitation requires a powerful light source, in this case a high powered parabolic 1000 W LX1000CF lamp (LOT-Oriel) capable of emitting c. 13000 µmol photons m\(^{-2}\) s\(^{-1}\) without filter interference. Light generated is transmitted to the sample by focussing through a 100 mm lens (LOT-Oriel) into a 1 m x 8 mm diameter liquid light guide (LOT-Oriel). Light exiting the guide is directed onto the sample below by a cold mirror (LOT-Oriel) placed at a 45 ° angle to the end of the light guide. All of the components required to transmit light from source to sample are attached to a 0.6 m optical rail (Edmund Scientific).

Immediately after light has been directed through the 100 mm lens there is a lab built pneumatic shutter arrangement to allow modulation of light intensity in the system. This utilises a servo controller board (Milford Instruments) to which 4 servos were attached, these allow for operation of the high/low-light shutter and the systems auto-sampler mechanism.

Images are captured using a CCD camera. The camera component of the system is an UltraPix SI 250 16 Bit Cooled CCD camera (EG&G Wallac LSR). The camera is powered through connection to the PC used to display, store and analyse data acquired.

Plants are placed under the CCD camera and PSII operating efficiency measured by generating images representative of \(F_q\) and \(F_m\). These images are then exported to the analysis software ImageJ (NIH) and fluorescence quantification
undertaken. This software package is capable of removing the black image background and calculating the mean overall fluorescence. This value is an indicator of the current PSII operating efficiency. Data was then exported to Excel (Microsoft) for graphical analysis.

Figure 2.12. Arrangement of equipment for chlorophyll fluorescence imaging. L: high-powered lamp; S: servo controller to allow light level and auto-sampler control; LG: light guide; C: CCD camera; OC: optical cabinet; PC: computer.
CHAPTER 3

UPTAKE OF NITROGEN IN MOSSES AND THE RAPID INITIAL INDUCTION OF THE ENZYME NITRATE REDUCTASE

3.1. Introduction

3.1.1. N-deprivation, hydration status and establishing controls

An established method for measuring the effect of a particular nutrient on plant metabolism is to limit the supply of the nutrient to the plant before re-supplying it at a higher concentration in order to investigate the response. Higher plants have an extensive root system and the withdrawal of NO$_3^-$ or NH$_4^+$ from plant nutrient solution, or media bathing the roots, is a commonly used technique to investigate the response of plants to limited nutrient supply, especially in plant electrophysiology (McClure et al., 1990; Glass et al., 1992; Wang et al., 1994; Sueyoshi et al., 1995; van der Leij et al., 1998). Other research used includes measuring the kinetics of induction of the enzyme nitrate reductase (NR) activity. The influence of N form directly on NR activity in leaf tissue was investigated in Clematis vitalba by using N-deprived controls. Some leaf discs were fed basal nutrient solution minus N for 10 d prior to sampling (Bungard et al., 1999) and
were then treated with inorganic-N and NRA quantified. Also in barley deprived of nutrients the NADH specific NR, found in higher plants, was typically induced by re-supply of NO$_3^-$ to the roots (Sueyoshi et al., 1995). Expression of NRA was below the limits of detection prior to induction and the withdrawal of NO$_3^-$ from the solution for several days was a technique to deprive the plants of N and act as an N-deprivation control.

The reported length of time of N-deprivation in experiments with higher plants is very variable. For example, in the model plant *Arabidopsis thaliana*, where seedlings were grown in buffer and received no additional N beyond what was available from the seed reserve, plants could be maintained for 10-12 days from germination, with shoots developing the first true leaves and roots. However, seedling growth was noticeably retarded after 7-8 days an indication of N-deprivation when compared to control seedlings that were supplied N (Meharg and Blatt, 1994). This is an example of how difficult the establishment of N-deprivation can be in a higher plant system without detriment to the functioning of the plant.

Throughout any regime of N-deprivation hydration status is critical. If a plants hydration status is compromised this can lead to inaccuracies when interpreting data thought to be from an equivalent data set. For example, tissue fresh weight is very dependent on the water content of the plant. When comparing the nutrient economy of two contrasting moss species continuous hydration was an important factor when assessing growth stimulation and the net uptake of nutrients, especially in the moss *Brachythecium rutabulum*, in a study by Bates
Work has been carried out on the importance of hydration status in both *S. fimbriatum* and *M. hornum*. In *M. hornum* it was observed that the surfaces of the leaves of the plants were slightly water repellent, although it had a well developed central conducting strand in the stem, but the single-celled structure of the leaves caused the plants to dry out quickly and leaves were only fully expanded in moist weather or for a limited period after rain (Proctor et al., 1998). Water supply in *S. fimbriatum* is critical due to the semi-aquatic environment in which the plant usually grows and plants have a less developed protective cuticle than higher plants. Therefore hydration status in both mosses is taken into consideration throughout experimentation in this research. In order to investigate the effects of inorganic-N treatments on *M. hornum* and *S. fimbriatum* leaf tissue, and limit additive effects such as background N inputs and environmental variables, initial experiments were carried out to establish controls. The experimental set-up had been designed to stabilise temperature, humidity, light intensity and day length, as well as only watering the plants with an N-free rain solution (Press et al., 1986). NRA and NO$_3^-$ concentrations were measured in both mosses over several weeks in order to establish when they had reached stable enzyme activity and tissue concentrations whilst undergoing N-deprivation. Previous studies on *Sphagnum capillifolium* and *Sphagnum fuscum* used a similar approach, when describing the effects of NO$_3^-$, NH$_4^+$ and temperature on NRA, and sampled tissue after at least 72 h acclimation (Woodin and Lee, 1987). They found NRA per unit mass was highest in the capitulum and declined down the stem. Therefore in this study the upper part of the plant in *S.fimbriatum* and the upper canopy of *M. hornum* was assayed for consistency of results.
3.1.2. Nitrate Reductase biochemistry

As work on NR is so extensive this section will focus on established theory on NR related to NO$_3^-$ reduction and some of the areas where further research is being carried out.

**Figure 3.1.** Diagrammatic representation for foliar uptake and assimilation of atmospheric N in *S. fimbriatum* moss. Photographic image of *S. fimbriatum* hyaline cells and living green cells (left), and photographic image of *S. fimbriatum* leaf (right).

NR is a dimeric enzyme with each subunit containing a 100 kDa polypeptide and one each of molybdenum (MoCo or Mo-pterin), cytochrome b (heme-Fe), and FAD (flavine adenine dinucleotide) (reviewed by Campbell, 1996). NR has two active sites NAD(P)H and the molybdenum cofactor (Figure 3.1). At the first active site the NAD(P)H domain donates electrons to FAD (FADH$_2$) which
begins the transport of electrons (indicated with red line) via the heme-Fe or cytochrome domain (Cyt.). The electrons at the second active site, the molybdenum cofactor (MoCo), finally reduce $\text{NO}_3^-$ to $\text{NO}_2^-$. It is widely accepted that NR is located in the cytosol, as represented in Figure 3.1, but there is some evidence that NR can also be located at the outside of the plasma membrane (Tischner, 2000).

NR has two-site, ping-pong, steady state kinetics, where the enzyme ‘ping-pongs’ between oxidised and reduced forms, as $\text{NAD(P)}H/\text{NAD(P)}^+$ bind at the electron donor active site and $\text{NO}_3^-/\text{NO}_2^-$ bind at the electron acceptor site (Campbell, 1996). The activation state of the enzyme is tightly regulated and the induction of NR has been widely studied. NR displays a cycle in its activity, becoming more active in the light when photosynthesis is functioning and returning to low activity in the dark. The precise mechanisms, involving changes in the phosphorylation of NR and a specific NR-inhibitor protein (NIP) as regulatory components are currently being elucidated. NIP are small molecular weight 14-3-3 proteins which are known to be essential components in signalling pathways. The activation/inactivation of NR involving NIP is a key regulatory process in N uptake. It has been proposed that inactivation may occur by a change in NR conformation that interrupts electron transport along the enzyme (Kaiser and Huber, 2001).

Figure 3.2 illustrates the regulation of activity of NR involving phosphorylation and dephosphorylation and NIP proteins. NR can be found in three forms,
moving clockwise round the figure starting at the top: active free NR, active phosphorylated NR and inactive phosphorylated NR combined with NIP.

Figure 3.2. Simplified model of the regulation of the activity of nitrate reductase (NR) (blue) which involves both phosphorylation (blue) with NR kinase (purple), and dephosphorylation with one or several NR inhibitor proteins (NIP) (green).

The regulation brings about activity/inactivity of the enzyme to coincide with the daily cycle of light and darkness. Adapted from Mansfield (2002).

In a review by Tischner (2000) he summarises the induction and inhibition factors effecting NR. The widely held view is that NRA is inducible by NO$_3^-$ and inhibited by NH$_4^+$ in a majority of plant systems. NRA is inducible by NO$_3^-$ (Shaner and Boyer 1976; Kronzucker et al., 1995) and the induction requires light in green tissues (Tischner 1976; Li and Oaks 1994; Ferretti et al., 1995; Cabello et al., 1998). It is NO$_3^-$ that is considered to be the primary signal for the
induction of NR with \textit{de novo} synthesis of the NR protein occurring within minutes of exposure to NO$_3^-$ and NR mRNA increasing five to 100-fold (Gowri \textit{et al.}, 1992, Long \textit{et al.}, 1992). In plant systems it is more usual for NH$_4^+$, which in comparatively low concentrations is potentially toxic to plants (Bungard \textit{et al.}, 1999), to inhibit NRA as shown by reduced NRA and a reduced capacity to store NO$_3^-$ (Press and Lee, 1982; Woodin and Lee, 1986; Aslam \textit{et al.} 1997; Soares and Pearson, 1997). With regard to mosses, in three species of moss NH$_4^+$ applications caused decreases in NRA, organic acids and cations whereas NO$_3^-$ treatments caused the reverse response (Soares and Pearson, 1997). There is also evidence that the NR gene is induced following the deactivation of a repressor after depletion of N-sources such as NH$_4^+$ (Samuelson \textit{et al.}, 1995) or organic-N compounds. The presence of NO$_3^-$ is therefore not an absolute prerequisite for expression of the NR gene. Some data are available showing that very small amount of NO$_3^-$ are sufficient for induction (Tischner \textit{et al.}, 1993; Samuelson \textit{et al.}, 1995) and that either the N flux or plant N status control NR expression (Samuelson \textit{et al.}, 1995).

There are a suit of factors associated with the regulation of NR activity, its activation and inactivation; include NO$_3^-$ itself, carbon and N metabolites, light, phytohormones, and CO$_2$ concentration (Kaiser and Brendle-Behnisch, 1991; Huber \textit{et al.}, 1992; Kaiser and Huber, 1994; Li \textit{et al.}, 1995; Sivasankar and Oaks, 1996; MacKintosh, 1998; Morcuende \textit{et al.}, 1998). It is important to compare NR activity either \textit{in vivo} or \textit{in vitro}, as an indication of N assimilation, as changes in NR activity measured \textit{in vitro} are not always associated with changes in NO$_3^-$ reduction rates \textit{in vivo}. This suggests that NR can be under strong
substrate limitation (Kaiser and Huber, 2001). Another important factor to consider is the degradation and half-life of the NR protein which appears to be affected by NR phosphorylation and NIP binding the factors affecting these mechanisms still requiring further study (Kaiser and Huber 2001) (Figure 3.2).

$\text{NO}_3^-$ is one of the main sources of N for many plants and is not only a nutrient and a signal for NR induction but it also acts as a signal for the initiation of various processes (Crawford 1995). For example carbohydrate metabolism can be affected by $\text{NO}_3^-$ with sucrose synthesis being promoted instead of starch synthesis when $\text{NO}_3^-$ is in abundance (Tischner, 2000). Experiments focussing on $\text{NO}_3^-$ sensing are rare. In barley the effect of $\text{NO}_3^-$ pulses on N-free cultivated plants measured in conjunction with NR expression was investigated by Tischner (1993). Under these conditions concentrations of 120 nmoles g$^{-1}$ FWt were required to start NR mRNA synthesis. The work again supported evidence for $\text{NO}_3^-$ being a signal, however not much is known about how this $\text{NO}_3^-$ is sensed by the plants. Little work on $\text{NO}_3^-$ sensing has been carried out specifically in mosses, due to the uptake of $\text{NO}_3^-$ being primarily through the leaf and across the plasma membrane whereas most work, such as the studies described above on higher plants systems, work on roots as this is their primary mode for $\text{NO}_3^-$ uptake. More recently work carried out on a role for shoot protein in shoot-root dry matter allocation in higher plants suggests that protein might act as a signal to regulate dry matter partitioning between the shoot and root of higher plants rather than $\text{NO}_3^-$ (Andrews et al., 2006). Experimental data in this thesis investigated these theories further, however the mosses used in this study used leaf tissue only.
3.1.3. Tissue NO$_3^-$ concentration measurements; uptake and role in N cycle

In higher plant systems NO$_3^-$ is taken up by the roots reduced and stored in the vacuoles or translocated to the shoot for reduction and vacuolar storage (Tischner, 2000). Variation in soil NO$_3^-$ levels affects the rate of plant growth and the concentration of NO$_3^-$ stored in higher plants (Crawford and Glass, 1998). When provided with an unlimited supply of NO$_3^-$, root and shoot NO$_3^-$ concentrations can reach up to 100 mM, most of which is stored in the vacuole (Miller and Smith, 1996). Tissue NO$_3^-$ concentrations give an overall measure of the NO$_3^-$ stored in all of the plant tissue, the vacuolar and cytoplasmic compartments combined, and it is only by using techniques such as electrophysiology that specific compartmental concentrations can be established (see Chapter 6). Microelectrode results suggest that cytoplasmic NO$_3^-$ concentrations are somehow held constant perhaps by the competing processes of influx, efflux, xylem and vacuolar loading and NO$_3^-$ reduction in higher plants (Miller and Smith, 1996; Crawford and Glass, 1998). Measuring tissue NO$_3^-$ concentrations and corresponding NR activity in experiments can give a good indication of how N-deprived plants re-supplied with known concentrations of inorganic N respond to increased N supply.

3.1.4. Amino Acid concentration measurements; uptake and role in N cycle

Tissue amino acid pools are associated with NR inhibition, alongside NH$_4^+$, (Aslam et al., 1997). Changes in amino acid concentration may therefore provide a clear picture of the N status of a plant and indicate feedback-regulation
of NRA and N uptake rate. As outlined in Chapter 1 experiments with mosses looking at changes in amino acids, where excess N was applied to the shoots of peat mosses, showed a large increase in arginine which indicated a nutritional imbalance in the moss (Tomassen et al., 1999). The amino acids glutamine and asparagine have been shown to be the most potent inhibitors of NRA (Sivasankar and Oaks 1995, Dzuibany et al., 1998) and glutamine has been proposed as a signal and inhibitor for NR (Lee et al., 1992) but the evidence is conflicting. To investigate N sensing in mosses amino acids were subsequently measured as historically amino acids have been studied as a regulatory metabolite for N uptake and metabolism.

3.1.5. Protein concentration measurements and role in N cycle

Protein concentrations were measured to see if any changes were detectable in plants deprived of N and re-supplied with inorganic N. High concentrations of protein could indicated whether plants were in an N-sufficient state, leading to the accumulation of free amino acids; which were then also measured in conjunction with NRA and NO$_3^-$'. Measuring these parameters were important in establishing whether protein could act as a signal and inhibitor for NR in N sensing in mosses. As outlined in Chapter 1 protein has most recently been proposed as a signal in regulating matter partitioning between shoot and roots of higher plants (Andrew et al., 2006) and changes in concentrations of NR during N sensing and N uptake could be an important parameter for N sensing in mosses.

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3.1.6. Aim

We hypothesised that two moss species from contrasting habitats would have differing responses to artificial applications of elevated inorganic-N. Those from a nutrient-poor environment (*S. jimbriatum*) being inhibited with respect to metabolic function, and those from a relatively nutrient-rich environment stimulated (*M. hornum*) with respect to metabolic function. To establish how both species took up N from the external environment, and assess their capacity to assimilate and metabolise this additional N, analysis of tissue N-containing metabolites and NR activities were measured in the laboratory. In this chapter experiments are described for plants that were set-up in controlled environments and starved of N. Nitrate reductase activity (NRA) both *in vivo* and *in vitro* and tissue NO$_3^-$ concentrations were measured to assess the rate of N assimilation in the mosses.
3.2. Results and Discussion

3.2.1. Establishing hydration status

Both *S. fimbriatum* and *M. hornum* are subject to slow dehydration in all but very humid atmospheric conditions and as most of the results are presented on a metabolite concentration or enzyme activity per g FWt it was necessary to investigate the effects of desiccation in a time course experiment. NRA and tissue NO$_3^-$ concentrations in N-deprived *S. fimbriatum* misted with dH$_2$O or untreated are shown in Figure 3.3. There was no overall increase in NRA when the *S. fimbriatum* monolith was misted at regular intervals with 200 ml dH$_2$O. When the plants were untreated NRA was very variable in the trial and there was an overall increase in NRA activity. Similarly NO$_3^-$ concentration was most stable when misted with dH$_2$O and when untreated *S. fimbriatum* showed an increase in NO$_3^-$ concentration. This increase in NO$_3^-$ concentration in the tissue can be attributed to the decrease in water content of the tissue and subsequent alteration of the FWt:DWt ratios of the mosses as they lost water content.
Figure 3.3. Time course of a) NRA and b) NO₃⁻ concentration in *S. fimbriatum*.

Treatments are misting at every sampling point with dH₂O (dashed line) or initial misting at time 0.00 h and then untreated at subsequent sampling points (solid line). Data points are an average of 5 replicates. SE bars are shown.
Figure 3.4. Time course of a) NRA and b) NO$_3^-$ concentration in *M. hornum* when undergoing misting at every sampling point with dH$_2$O (dashed line) or initial misting at time 0.00 h and then untreated (solid line). Data points are an average of 5 replicates. SE bars are shown.

NRA of *M. hornum*, when trays were misted with 200 ml dH$_2$O at regular intervals, showed no overall increase in activity (Figure 3.4). If the plants were untreated the results showed more variability in NRA with a slight increase in NRA in the *M. hornum* plants when compared with dH$_2$O misted plants. The
tissue NO$_3^-$ concentration was very stable when misted with dH$_2$O, however when the plants were untreated there was an increase in the concentration of NO$_3^-$. As in _S. fimbriatum_ this increase in NO$_3^-$ concentration in the tissue can be attributed to the decrease in water content of the tissue and subsequent alteration of the FWt:DWt ratios of the mosses as they lost water content.

The effect of diurnal variation was accounted for by sampling the plants at time periods known to be at stable activities. This was achieved by sampling the plants during the day / night cycle in the CEH cabinet and choosing the time course sampling points accordingly (data not shown). No diurnal variation was observed in either species of moss during establishment of controls (see Figure 3.3 and 3.4). This could be attributed to the effect of rapid acclimation of the plants to daylight in the controlled environment. In order to accurately represent the standard biochemistry and enzyme activities measured in the laboratory it was therefore important to establish controls as both moss species were very sensitive to hydration status. In a study on the desiccation-tolerant moss _Tortula ruralis_ the importance of hydration status was also emphasised when the enzyme NR declined in plants experiencing water deficits (Mahan _et al._, 1998). Upon the alleviation of water stress NRA recovered to control levels between one to seven days. Both moss species were subsequently kept fully hydrated throughout the experimental period and controlled sampling achieved using misting with dH$_2$O. FWt:DWt ratio (water content) of the plants was also monitored at every sampling (see Section 3.2.2.).
3.2.2. Establishing FWt:DWt data for *S. fimbriatum* and *M. hornum*

![Graph a](image)

\[ y = 22.767x \]
\[ R^2 = 0.9129 \]

![Graph b](image)

\[ y = 5.8771x \]
\[ R^2 = 0.9159 \]

**Figure 3.5.** FWt:DWt of a) *S. fimbriatum* (open squares) and b) *M. hornum* (open diamonds). Data points are individual measurements. Lines show the fitted regression lines are drawn using Excel (Microsoft).
Figure 3.5. shows a strong positive linear relationship between FWt:DWt for *S. fimbriatum* and *M. hornum* with a correlation coefficient close to 1. Inaccuracies in the data were overcome by using plants in a fully hydrated status prior to sampling (see Section 3.2.1). On the strength of this, and for the purposes of comparability with higher plant systems, values throughout the thesis have been quoted on a FWt basis. Conversion from FWt to DWt can be calculated from the regression statistics shown in Figure 3.5.

### 3.2.3. Components of N cycle measured

The NR activity *in vivo* and *in vitro*, tissue NO₃⁻, amino acid and soluble protein concentrations were measured to investigate the pathway of N uptake and assimilation in both moss species. Unfortunately extracting intact NR enzyme for the NR *in vitro* assay in *S. fimbriatum* and *M. hornum* was not achievable in the time frame of the research therefore the NR *in vivo* assay has been used throughout to represent the uptake and assimilation of N. The benefits of measuring NR, both *in vivo* and *in vitro*, are discussed further in Chapter 7. Although the NR *in vitro* assay is useful to measure both maximum activity and inactive forms the NR *in vivo* assay serves as a useful assay for measuring relative differences in varying conditions and is certainly far more useful in this respect than the *in vitro* assay (Woodin and Lee, 1986). NRA throughout the thesis will refer to NRA *in vivo* unless stated otherwise.
3.2.4. *Sphagnum fimbriatum* response with inorganic-N treatment

N-deprived *S. fimbriatum* was treated with KNO$_3$, Ca(NO$_3$)$_2$ and NH$_4$Cl, all at 0.5 mM with respect to N, to investigate the effect of increased N and different N forms on the biochemistry of a plant species native to a nutrient poor environment. Following KNO$_3$ treatments (Figure 3.6. a-d), NRA was rapidly induced after only 0.25 h, with NRA reaching an activity after 4.25 hrs of 3.3 μmol NO$_2^-$ h$^{-1}$ g$^{-1}$ FWt. There was also a corresponding rapid initial increase in NO$_3^-$ concentration but this then declined with subsequent treatment. Amino acid concentration did not show any large changes over the course of the treatment however there was a slight increase in soluble protein after 4.25 hours but looking at other inorganic N treatments this did not appear to be a common phenomenon. These graphs are representative of several treatment trials whereby the maximum NRA was reached after 4.25 hrs. Other experiments were carried out for long time courses whereby maximum NRA induction was reached after 4.25 hours. The data shown in this Chapter give the most representative traces for that particular moss species according to the treatment applied based on a larger data source (data not shown). As this work focussed on the rapid initial induction of NRA and the uptake of N later effects from prolonged exposure to elevated concentrations of N are not discussed further.
Figure 3.6. Response of *S. fimbriatum* (N-dep 29 days) to treatment with dH₂O (white bar) and 0.5 mM KNO₃ (black bars). Time course of a) NRA and corresponding b) NO₃⁻ concentration (conc.), c) Amino acid conc. and d) Soluble protein conc. Data points are an average of 5 replicates. SE bars are shown.
Following Ca(NO$_3$)$_2$ treatments (Figure 3.7 a-d), similar trends in rapid initial induction of NRA activity, rapid increase in NO$_3^-$ concentration followed by a decline, and no large changes in amino acids or proteins were observed, although control concentration of protein in this tissue were higher overall than for the KNO$_3$ treated trial. NRA was also slightly higher and the initial NO$_3^-$ concentration increase slightly lower but overall the units and trends in response were the same. Comparing results for Figures 3.6 and 3.7 it appears that K, but not Ca, was limiting in addition to N. The increase in soluble protein is also greater when K is supplied along with N. This could be explained by the need for K in normal protein synthesis and enzyme activation (Walker et al., 1998). It should be noted however that protein concentrations were higher overall in the plants supplied with Ca(NO$_3$)$_2$ as the control concentration of protein was around 4 fold higher than that contained in the KNO$_3$ supplied plants prior to treatment. Therefore these observations are related in observable differences throughout the course of the inorganic-N supplied to the plants during the time course.

Interestingly, following NH$_4$Cl treatments in *S. fimbriatum* (Figure 3.8 a-d), NRA rapidly increased after 0.25 h with NRA reaching peak activity after 2.25 hrs (2.3 μmol NO$_2^-$ h$^{-1}$ g$^{-1}$ FWt.). This increase in NRA occurred in the absence of additional NO$_3^-$ as the concentrations of NO$_3^-$ in the tissue were at or below the control concentration following a dH$_2$O misting. Amino acid concentration did peak at 2.25 hrs, the time corresponding to the peak NRA but it is unclear if there is a correlation between these two measurements and would require further study. Protein concentrations were at or below the control level.
Figure 3.7. Response of *S. fimbriatum* (N-dep 35 days) to treatment with dH₂O (white bar) and 0.25 mM Ca(NO₃)₂ (grey bar). Time course of a) NRA and corresponding b) NO₃⁻ concentration (conc.), c) Amino acid conc. and d) Soluble protein conc. Data points are an average of 5 replicates. SE bars are shown.
Figure 3.8. Response of *S. fimbriatum* (N-dep 33 days) to treatment with dH$_2$O (white bar) and 0.5 mM NH$_4$Cl (hatched bar). Time course of a) NRA and corresponding b) NO$_3^-$ concentration (conc.), c) Amino acid conc. and d) Soluble protein conc. Data points are an average of 5 replicates. SE bars are shown.
KNO$_3$, Ca(NO$_3$)$_2$ and NH$_4$Cl treatments rapidly induced NRA under these experimental conditions in *Sphagnum fimbriatum* after just 15 minutes. Similar studies in the moss *Sphagnum capillifolium*, at concentrations of 1 mM KNO$_3$ solution, took 12 hrs to reach maximal NRA (Woodin and Lee, 1986) after acclimation to a light and temperature regime for a minimum of 72 hrs prior to experimentation. The rapid initial induction of NR in *Sphagnum fimbriatum* could be due to the longer term N-deprivation, supported by the low basal NRA and NO$_3^-$ concentrations, with plants readily taking up inorganic-N for use in plant metabolism. Another factor involved could be a deficiency in K, with a re-supply stimulating plant metabolism, perhaps even as a combined effect of both N and K deficiencies. NR is supposedly a substrate inducible enzyme and an established indicator of the uptake and assimilation of NO$_3^-$ (Campbell 1996), however NR was induced by NH$_4^+$ in *S. fimbriatum* in the absence of additional NO$_3^-$. Studies on *Clematis vitalba* support these findings and will be discussed at length in 3.2.5. (Bungard et al., 1999). Tissue NO$_3^-$ concentrations were also initially increased with inorganic-NO$_3^-$ treatments but there appeared to be no long term storage. These responses are indicative of a species with low NO$_3^-$ storage capacity.
3.2.5. *Mnium hornum* response with inorganic -N treatment

N-deprived *M. hornum* was treated with inorganic KNO₃, Ca(NO₃)₂ and NH₄Cl to investigate the effect of increased N in different N forms on the biochemistry of a plant species native to a nutrient rich environment. *M. hornum* also responded to KNO₃ treatments with a rapid initial induction of NRA (Figure 3.9 a-d). There was a corresponding increase in NO₃⁻ concentration in *M. hornum* tissue following sequential treatment. NRA was maintained post-‘plateau’ after 2.25 hrs (13.9 µmol NO₂⁻ h⁻¹ g⁻¹ FWt.) and NO₃⁻ concentration also increased with subsequent KNO₃ treatments and was at the highest concentration after 4.25 hrs (113.0 µmol NO₃⁻ g⁻¹ FWt.). These responses are indicative of a nitrophilous species that maintains a NO₃⁻ storage pool. Amino acid and protein concentrations increased after treatment with KNO₃. There was a decline in amino acid concentration after 4.25 h to 25 µmol g⁻¹ FWt, however this could be an anomalous result, as at 6.25 h the converse was true with amino acid levels returning to those observed previously and NO₃⁻ mapping this trend. Protein levels increased post-treatment. With Ca(NO₃)₂ treatments (Figure 3.10 a-d), there was a rapid initial induction of NRA activity and increase in NO₃⁻ concentration with evidence of storage. There was a slight increase in amino acid and proteins concentrations from the control level but no large changes were observed. NO₃⁻ concentrations were lower but overall the units and trends in response were the same.
Figure 3.9. Response of *M. hornum* (N-dep 13 days) to treatment with dH₂O (white bar) and 0.5 mM KNO₃ (black bar). Time course of a) NRA and corresponding b) NO₃⁻ concentration (conc.), c) Amino acid conc. and d) Soluble protein conc. Data points are an average of 5 replicates. SE bars are shown.
Following NH₄Cl treatments in *M. hornum* (Figure 3.11 a-d.), NRA also rapidly increased after 0.25 hrs with NRA reaching peak activity after 2.25 hrs (3.8 μmol NO₂⁻ h⁻¹ g⁻¹ FWt.). This occurred in the absence of additional NO₃⁻ as the concentrations of NO₃⁻ in the tissue were at or below the control level (a dH₂O misting). Amino acid and protein concentrations did increase after treatment with NH₄Cl and these values were consistent with those observed in *M. hornum* with other inorganic N treatments. KNO₃, Ca(NO₃)₂ and NH₄Cl treatments rapidly induced NRA under these experimental conditions in *M. hornum* after just 15 minutes. Tissue NO₃⁻ concentrations were also initially increased with inorganic- NO₃⁻ treatments and there was evidence for long term storage and pooling of NO₃⁻ in the tissue. This is indicative of a nitrophilous species with NO₃⁻ storage capacity.
Figure 3.10. Response of *M. hornum* (N-dep 30 days) to treatment with dH$_2$O (white bar) and 0.25 mM Ca(NO$_3$)$_2$ (grey bar). Time course of a) NRA and corresponding b) NO$_3^-$ concentration (conc.), c) Amino acid conc. and d) Soluble protein conc. Data points are an average of 5 replicates. SE bars are shown.
Figure 3.11. Response of *M. hornum* (N-dep 43 days) to treatment with dH₂O (white bar) and 0.5 mM NH₄Cl (hatched bar). Time course of a) NRA and corresponding b) NO₃⁻ concentration (conc.), c) Amino acid conc. and d) Soluble protein conc. Data points are an average of 5 replicates. SE bars are shown.
3.2.6. *Sphagnum fimbriatum* and *Mnium hornum* - inorganic -N treatment

*S. fimbriatum* and *M. hornum* showed similar responses with respect to NRA *in vivo* under these experimental conditions. The NR *in vivo* assay has provided a useful measure of the activity of the enzyme in both mosses if they were exposed to inorganic-N in the external environment. NRA was higher in *M. hornum* than in *S. fimbriatum* but the trend of rapid initial induction of NR following treatment with inorganic-N treatment was the same. Interestingly, both species support the findings in *C. vitalba* where NRA *in vivo* was induced with NH$_4^+$ in leaves in the absence of NO$_3^-$ (Bungard *et al.*, 1999). In *C. vitalba* leaves, supplied with 2.5 mM NO$_3^-$, NRA increased with N supply up to 2.5 μmol NO$_2^-$ h$^{-1}$ g$^{-1}$ FWt from the control NRA concentration and was then either unaffected or decreased slightly with additional N supply. Control levels, where no NO$_3^-$ was supplied, were consistently low in the plants. The NRA activity and response to inorganic-NO$_3^-$ in *C. vitalba* is comparative and at similar values in both moss species. When 2.5 mM NH$_4^+$ was supplied to the leaves of *C. vitalba* the NRA actually increased to a greater extent than NO$_3^-$ supplied plants (approximately 5.0 μmol NO$_2^-$ h$^{-1}$ g$^{-1}$ FWt) with maximum activities of 6.4 μmol NO$_2^-$ h$^{-1}$ g$^{-1}$ FWt. occurring in leaves supplied with 5.0 mM NH$_4^+$ (Bungard *et al.*, 1999). Although inorganic-NH$_4^+$ does stimulate NRA in *S. fimbriatum* and *M. hornum* in the absence of additional NO$_3^-$, the activities in both moss species do not show the same pattern and are half those values observed when inorganic-NO$_3^-$ is applied. These effects are rare as in most higher plants NR is actually inhibited by NH$_4^+$ treatments (Aslam *et al.* 1997). With regard to mosses, in three species of moss NH$_4^+$ applications caused decreases in NRA, organic acids and cations.
whereas $\text{NO}_3^-$ treatments caused the reverse response (Soares and Pearson, 1997). These responses are typical of those found in most higher plant systems and raise many questions about the NRA in \textit{S. fimbriatum} and \textit{M. hornum} in this research. However, few studies have looked at the responses of NRA over such a short time-course and it is possible that these conflicting results are a consequence of the selection of sampling times used in previous studies. Short term activation of NRA could occur through removal of the NIP (14-3-3 inhibitor molecule) (Kaiser and Huber, 2001). In the longer term loss of activity may occur through enzyme protein turnover or breakdown as NR is also regulated by \textit{de novo} synthesis (Campbell 1996; Kaiser and Huber, 2001).

Unfortunately for \textit{S. fimbriatum} and \textit{M. hornum} in this study attempts to assay the active and inactive forms of NR by \textit{in vitro} methods proved unsuccessful and were abandoned due to lack of ability to extract a stable and consistent enzyme activity in both species of moss. NRA induction studies carried out by Soares and Pearson (1997) used plants which were not deprived in the long term of N, with their plants being more nutrient replete than those used in trials of \textit{S. fimbriatum} and \textit{M. hornum} which may explain the inhibition of NR upon addition of $\text{NH}_4^+$ due to the availability of basal $\text{NO}_3^-$ which was the preferred source of N for metabolism in these species. There is also the potential from K-deprivation to occur alongside N-deprivation, in \textit{S. fimbriatum} and \textit{M. hornum} as discussed previously, with the combined supply of inorganic-KNO$_3$ containing both K and N having a larger stimulatory effect on moss metabolism overall; in part due to the role K plays in protein synthesis and enzyme activation. Further investigation of this unusual induction in \textit{S. fimbriatum} and \textit{M. hornum}, with
A hypothesis to explain the response of induction of NR with NH$_4^+$ treatment is an alternative role that has been proposed for NRA in maintaining intracellular pH. NH$_4^+$ is an obvious source of H$^+$ ions (Raven, 1988) and its uptake and assimilation could cause a pH imbalance in the cell. The study of Kaiser and Brendle-Behnisch (1995) lends support for this idea, they have shown that pH changes in leaf tissue, invoked by feeding leaves with weak acids, can activate NR. With the reduction of NO$_3^-$ to NH$_4^+$ in the cell producing OH$^-$, and direct uptake and assimilation of NH$_4^+$ producing H$^+$ there is a mechanism to maintain cell pH homeostasis through plant N use (Kaiser & Brendle-Behnisch, 1995; Raven, 1988). An alternative method to investigate this hypothesis uses pH sensitive microelectrodes which measure the intracellular pH in moss cells in response to inorganic-N treatments to try to further understand the mechanisms underlying these processes (see Chapter 6).

Other interesting observations by Bungard et al., (1999) in C. vitalba were the pooling of NO$_3^-$ in the leaves with levels reaching 20 μmol NO$_3^-$ g$^{-1}$ FWt. when treated with 5 mM NO$_3^-$ (Bungard et al., 1999). NO$_3^-$ was below the detection limit of 0.1 μmol NO$_3^-$ g$^{-1}$ FWt when treated with NH$_4^+$. In this study the pool of NO$_3^-$ in control S fimbriatum and M. hornum was relatively high despite the mosses being kept for 30 days in an N-deprived state (approximately 5 μmol NO$_3^-$ g$^{-1}$ FWt). An explanation for the rapid induction of NRA in these mosses could be explained by release of NO$_3^-$ from a storage compartment. The
presence or absence of a storage compartment for NO$_3^-$ in both mosses was investigated further using electrophysiology.

**Figure 3.12.** Response in leaf tissue of *Hordeum vulgare L* (N-dep 7 days) to treatment with 15 mM KNO$_3$ (black bars), time 0 is control. Time course of a) NRA in vitro and corresponding b) NO$_3^-$ concentration. Data re-plotted from Sueyoshi *et al.* 1995.
It is difficult to find a comparative study to explain the rapid induction of NRA in the mosses as compared to a higher plant system. However work on factors affecting NO$_3^-$ uptake and transport and the influence on NR expression in barley (Hordeum vulgare L.) leaves gave some useful data (Sueyoshi et al., 1995). It should be noted that the KNO$_3$ was applied to the roots and there was some time delay in the supply of NO$_3^-$ reaching the shoots and subsequently the leaves (Figure 3.12). In barley following continuous 15 mM KNO$_3$ treatment to the roots, both NRA induction and storage of NO$_3^-$ reached its maximum after 24 h (Figure 3.12). NR was extracted for the in vitro assay in these experiments therefore the NRA cannot be directly compared with the in vivo assay results for S. fimbriatum and M. hornum. However, treatment of barley with 15 mM NO$_3^-$ increased NO$_3^-$ concentrations to amounts found in M. hornum after just 4.25 h. It can be suggested that M. hornum is therefore more like a higher plant in its storage of NO$_3^-$, when compared to S. fimbriatum which had a much lower NO$_3^-$ storage capability.

In both mosses there are changes in amino acids and protein, but overall there is no clear pattern in the short term (see Figures 3.6 – 3.11) probably due to the time required to synthesise complex compounds. Some amino acids have been shown to be inhibitors of NR, glutamine and asparagine have been shown to be the most potent inhibitors (Sivasankar and Oaks 1995, Dzuibany et al., 1998) and further experimentation to measure specific amino acids, perhaps using HPLC could be an interesting area to work on. Considering the scope of the project this work was not dealt with. Amino acid concentration measurements in higher plant studies have measured the changes in partitioning between the leaf and
roots in response to changes in inorganic-N supply and have proved successful in providing data that support amino acids as compounds that signal the N demand of the shoot to the roots (Rennenberg et al., 1998). However, no clear cut single amino acid as yet been put forward as a signal molecule and some have argued that it may be the ratio of certain amino acids that are important (Rennenberg et al., 1998). Such changes, if they occur, make interpretation of changes in amino acids complicated.

A potential area to consider for further work would be to measure the activity, synthesis and degradation of specific enzymes involved in N uptake and assimilation. However, attempts to measure immunologically reactive NR protein levels or NR mRNA levels using heterologous DNA probes were unsuccessful in the moss Tortula ruralis (Mahan et al. 1998). It was suggested that the Tortula NR may be significantly different from that found in algae and higher plants (Mahan et al., 1998). A difference in moss NR could also provide an explanation for the difficulties encountered when trying to optimise the NR in vitro assay in Sphagnum fimbriatum and Mnium hornum. A more successful experiment used Western blot analysis of nitrite reductase (NiR) protein concentrations to demonstrate that NR activity in C.vitalba supplied with either NO$_3^-$ or NH$_4^+$ stimulated the entire NO$_3^-$ assimilation pathway (Bungard et al., 1999). In C.vitalba supplied with NH$_4^+$ the high levels of activity involved transcription and protein synthesis rather than an increase in enzyme activation state. N form did not influence GS activity or protein levels in C.vitalba (Bungard et al., 1999). They were therefore independent of NR activity and NiR protein levels. The co-regulation of NR and NiR and the independent regulation
of these enzymes from those involved in NH$_4^+$ assimilation has been reported previously (Seith et al., 1994, Migge and Becker, 1996). In *C. vitalba*, and in *S. fimbriatum* and *M. hornum* this relationship seems to be true when the NO$_3^-$ assimilation pathway has been stimulated by NH$_4^+$ and in this work suggests that mosses may be an ideal model for also measuring short term activation of NiR.

In order to draw firmer conclusions from the biochemical profiling in *S. fimbriatum* and *M. hornum* the data was collated and analysed from over 25 time courses, not including the control trials (see Chapter 4). Values were also converted to % changes in enzyme induction of tissue concentrations to allow a more direct comparison of the data.

### 3.2.7. Epicuticular waxes in *Sphagnum fimbriatum* and *Mnium hornum*

To further understand the rapid initial induction of NRA and increases in tissue NO$_3^-$ concentration in the mosses SEM work was carried out to look at how effectively the misting treatments penetrated the plant. N uptake from the atmosphere into leaves involves penetration across the plant cuticle. Few studies have been undertaken of cell wall composition in non-vascular land plants (Popper and Fry, 2003).
Figure 3.13. Scanning electron micrographs of the upper epidermal surface of *Sphagnum fimbriatum*. Treated with a) dH₂O at pH 4.5 b) H₂SO₄ at pH 3.

Leaves from plants set-up in controlled environment N-dep 7 days. White bar = 5 μm.

The cuticle of terrestrial vascular plants and some bryophytes consists mainly of two hydrophobic components, the biopolymer cutin and a mixture of lipids which are usually called waxes (Koch *et al.*, 2004) the external wax being called
epicuticular wax forming the outermost boundary layer for the plant and representing a multi-functional interface between plant and environment. SEM images were taken of *S. fimbriatum* and *M. hornum* when treated with dH$_2$O and acid to see the effect on the wax layer. There have been numerous reports of changes in the structure and properties of leaf surfaces, without visible injury symptoms, in response to acid mists and rain. These changes may be indicative of long term weathering and degradation of leaf cuticle in response to repeated rain and mist events and may occur at lower acidities than those required for visible injuries (Ashenden, 2002). To observe degradation effects on the wax layer that may occur in the field, and influence the uptake of solutes such as inorganic-N, dH$_2$O at pH 4.5 and sulphuric acid (H$_2$SO$_4$) at pH 3 were misted onto leaves to see if there were changes in structure with simulated high acidity rainfall.
Figure 3.14. Scanning electron micrographs of the upper epidermal surface of *Mnium hornum*. Treated with a) dH₂O at pH 4.5 b) H₂SO₄ at pH 3. Leaves from plants set-up in controlled environment N-dep 7 days. White bar = 5 µm.

Figure 3.13 a) shows that *S. fimbriatum* has oval structures, or pores, surrounded by smooth interconnecting tissue with some feathery wax, accumulations of white, in places across the cell, but it is not extensive as that seen in higher plants. An SEM study by Mozingo, Klein Zeevi and Lewis (1969) on *Sphagnum*
*imbricatum* revealed that the pores are covered by a membrane and some of the membranes rupture to form true pores. Pore rupturing was observed in some of the SEM images taken but it is still not known whether membrane rupturing is a natural phenomenon or the result of cryopreparation. The pores allow rapid absorption of water. Treatment with dH$_2$O or acid did not influence the amount of pore rupture in *Sphagnum fimbriatum*. The leaves misted with acid b) show a degradation of the cellular structure and the cells appear to have ‘dissolved’ revealing some internal organelle structure. The wax that was present in a) seen as white feathery deposits is also not present in the interconnecting spaces. This qualitative analysis supports the reports of abraded waxy cuticles and changes in the microscopic organization of leaf surfaces in several species exposed to mists of acid (Ashenden, 2002).
Figure 3.15. Scanning electron micrographs of freeze-fractured cross section across the upper epidermal surface of *Mnium hornum*. Treated with a) dH$_2$O at pH 4.5 b) H$_2$SO$_4$ at pH 3. Leaves from plants set-up in controlled environment N-dep 7 days. White bar = 5 μm.

The leaf of *M. hornum* is only one cell thick, except in the midrib region and moss leaves lack the stomata of higher plants (Watson, 1957; Taiz and Zeiger, 1998). Gases and water vapour can therefore readily exchange between the leaf
cells and the atmosphere. The cuticle can be clearly seen as an even layer which covers the outer surfaces of the external cells. Figure 3.14 a) shows that the upper epidermal surface of *M. hornum* has smooth regular cells more typical of those observed in higher plants with extensive feathery wax across the cells and in the spaces that connect the cells. Upon application of acid b) the wax gives the appearance of having ‘melted’ and is flattened on the surface of the plant with wax accumulating in the spaces between cells. In Figure 3.15 this accumulation of wax can be seen more clearly at the section where the cells have been freeze-fractured and interconnecting ‘troughs’ have no wax accumulation in a) and a thick flattened covering of wax in b).

These effects are evidence for the degradation of the protective barrier around internal plant tissues. Changes in cuticular permeability can affect the uptake of pollutants, rates of water loss and also uptake of inorganic-N into the cells. Barker and Ashenden, (1992) found a significant correlation between water permeability of detached cuticles and pH of fog which holly leaves had been exposed to. Foliar fertilisation and leaching can occur as a consequence of these processes. In both mosses, although the SEM analysis is qualitative, it can be said with some confidence that cuticular permeability would be affected by wet acid deposition in the field and the effects could be far reaching. Suggested methods to investigate these effects further could include the measurement of cuticular thickness, following treatment with inorganic-N or acids, on freeze-fractured SEM images using a scalebar and computer graphics labelling to calculate any thinning or changes.
3.3. Initial Conclusions and Next Steps

It can be concluded that *Sphagnum fimbriatum* and *Mnium hornum* show similar responses with the stimulation of NRA but differences with regards to NO$_3^-$ storage capacity. No large and consistent detectable changes in amino acids and protein concentrations in *S. fimbriatum* or *M. hornum* were found with NRA in vivo and NO$_3^-$ concentration measurements being the best indicators of N-status. The rapid initial induction of NRA, within 15 minutes of the first treatment, was surprising and appears to be the first report of such a short term response in the literature (Stanley *et al*., 2005). The use of mosses with direct application of treatments to foliage which lacks an extensive cuticle and therefore readily takes up the N treatment is a useful tool for such studies on the regulation of NRA. An explanation for the rapid induction of NRA in these mosses could be explained by release of NO$_3^-$ from a storage compartment and the presence or absence of a storage compartment for NO$_3^-$ in both mosses was investigated further using microelectrodes. To further test the original hypothesis that two moss species from contrasting habitats had different metabolic responses to artificial applications of elevated inorganic-N that reflected their differing ecologies biochemical profiling was carried out in the long term in more N-deprived mosses to investigate both the NRA and the NO$_3^-$ storage capacities in both mosses along with further work into the effects of exchange processes on foliar fertilisation and leaching in mosses which underwent longer term N-deprivation.
CHAPTER 4

LONG TERM N-DEPRIVATION IN MOSSES

4.1. Introduction

4.1.1. N-deprivation effects on N status in plant communities

Determining the most effective fertiliser, optimum concentration and best time to
treat a crop and produce good yields continues to be an area of extensive
research and most recently has involved work on specific molecular pathways
and systems in higher plants. Most studies have looked at the partial withdrawal
of nutrients and the re-application of inorganic-N to plants in order to study the
effects on rates of N uptake and plant enzyme activities such as NR (Meharg and
Blatt, 1994; Sueyoshi et al., 1995; Bungard et al., 1999; Man et al., 1999). An
example of a long term N-deprivation study carried out in laboratory controlled
environments on higher plants, to determine the effects of depleted N on the
function of NR and associated metabolites, was a study on barley leaf NRA (Man
et al., 1999), where the plants underwent NO$_3^-$-deficiency regimes for
approximately two weeks, and the basal levels of metabolites were measured
following withdrawal of plants from low-NO$_3^-$ solutions. NO$_3^-$-deficiency
regimes in barley long day, low NO$_3^-$ plants, which underwent 16 d N-dep,
contained approximately 92 $\mu$mol NO$_3^-$ g$^{-1}$ FWt at time 0 h and then declined
steadily to around 0.1 μmol NO$_3^-$ g$^{-1}$ FWt after 8 h. NR$_{\text{max}}$ and NR$_{\text{act}}$ were measured at corresponding time points and showed activity decreasing throughout the course of the experiment. These plants had only about 30% of the NRA$_{\text{max}}$ and NR protein of high-NO$_3^-$ plants, however, the low NRA$_{\text{max}}$ in long day, low NO$_3^-$ plants was slightly compensated by a higher activation state of NR. The phosphorylation state of NR may be a factor controlling NR degradation in these long day, low NO$_3^-$ plants. Artificial activation of NR from dephosphorylation, by such factors as tissue acidification and anoxia, has been shown to result in a higher NRA$_{\text{max}}$ in dark treated leaves compared with untreated control leaves where NR was less activated. The decrease in NRA$_{\text{max}}$ during the second light phase in these long day, low NO$_3^-$ plants occurred in spite of normal high activation state. This may indicate that NR synthesis was strongly decreased once the tissue NO$_3^-$ concentration had become very low in these plants (Man $et$ $al.$, 1999). Experiments that study N-deprivation effects on N status in plants, such as these are rare (Kaiser and Huber, 2001) and an extensive study of long term N deprivation has not been conducted in this way before, to the best of our knowledge. Therefore in $S$. fimbriatum and $M$. hornum NR $in$ $vivo$ was measured, alongside tissue NO$_3^-$ concentration, to investigate N-deprivation effects on N status in mosses during long term N-deprivation.

4.1.2. Foliar N as a measure of N-deprivation status

Alongside the use of fertilisers in agriculture there has been a lot of discussion on the effects of increased anthropogenic inputs in the environment with regards to the identification and use of an effective bioindicator to help determine the N
status of an ecosystem. Foliar N is usually used as a long term marker for plant and ecosystem responses to experimental N addition on lowland and upland heathland communities in the UK. Foliar N concentrations were shown to increase linearly with treatment additions within individual sites and showed a consistent response to N when three sites were combined (Power et al., 2004). In this respect foliar N is a useful parameter for assessing plant and ecosystem community responses to increases in atmospheric N over the course of a year. Foliar N was measured to see if there would be any correlation between NRA induction and NO$_3^-$ increases in order to investigate N-deprivation effects on N status in mosses during long term N-deprivation and to identify the potential for environmental biological markers.

4.1.3. PSII as a measure of N-deprivation status

N deficiency induces modifications of many morphological and physiological parameters: limitations of whole plant growth (biomass), leaf number and leaf area have been reported (Ciompi et al., 1996). A strong correlation has also been found between the photosynthetic capacity of leaves and their N content (Sage and Pearcy, 1986, and Sugiharto et al., 1990). Nitrogen deficiency has been associated with decreases in the fluorescence parameters $F_q/F_{\text{max}}$, $F_q/F_{\text{v}}$ and $F_{\text{v}}/F_{\text{max}}$ in higher plants (Ciompi et al., 1996). A fluorescence imaging system capable of measuring the parameter $F_{\text{v}}/F_{\text{max}}$ was used to assay its suitability in detecting the N-status of the mosses in this study.
Chapter 3 described evidence of the unusual induction of NRA by NH₄⁺ in both mosses and there is some debate over whether this could be due to the operation of a pH stat. Several different biochemical or metabolic 'pH stats' have been proposed as additional mechanisms responsible for the control of pH homeostasis in the cytosol (Davies, 1986 and Raven, 1986). NO₃⁻ assimilation is thought to cause an alkalisation of the cytosol and NH₄⁺ an acidification (Raven and Smith, 1976). Cytosolic pH changes by this mechanism are routinely implicated as a major response to the differential pH stresses proposed to result from variations in plant N nutrition (Marschner, 1995). NO₃⁻ assimilation is considered to be a net proton-consuming process, as the summary reaction through NO₃⁻ and NR suggests:

1) \[ \text{NO}_3^- + 4[NAD(P)H + H^+] + 2H^+ \rightarrow \text{NH}_4^+ + 4[NAD(P)^+] + 3H_2O \]

NH₄⁺ assimilation by contrast is generally viewed as a net proton-producing process, because NH₃, the inorganic substrate for glutamine synthetase, is produced from the deprotonation of NH₄⁺:

2) \[ \text{NH}_4^+ \rightarrow \text{NH}_3 + H^+ \]

Raven and Smith (1976) take into account other processes consuming and generating acidity as other metabolic processes occur that support plant N assimilation, such as PO₄³⁻ and SO₄²⁻ use which can both generate OH⁻. Thus given NO₃⁻ as a sole source of N 0.78 mole of OH⁻ is proposed to be generated.
for 1 mole of NO$_3^-$, conversely 1.22 mole H$^+$ is proposed to be generated for 1 mole NH$_4^+$ when it is a sole N source. Measurements of NRA and tissue NO$_3^-$ concentration in *S. fimbriatum* and *M. hornum* plants treated with inorganic-N and acids, would check to see if NRA was induced by a decrease in pH, after long term N-deprivation. All values were converted to % changes compared to controls to allow a comparison of the data between both species.

### 4.1.5. Chemical Analysis of Total Major and Trace Elements in N-deprived Plants

Soares and Pearson (1995) suggested that cations could be used for short term homoeostasis of cell pH within bryophytes and higher plants. These authors hypothesised that charged particles present in treatment solutions, for example NH$_4^+$ and K$^+$, may cause immediate changes in the exchangeable cation pool, which in turn may influence cell physiology with short term changes in both cations and organic acids possibly providing a mechanism for buffering acidity. The availability of nutrients such as Ca, Mg and K, which carry a positive charge in solution and have basic properties, offer one means of alleviating the effects of excess H$^+$ ion inputs (Soares *et al.*, 1995). They showed a strong positive correlation between the rate of NR activity, base cation content and subsequent buffering capacity against acidity for higher plants. These effects were also considered in *S. fimbriatum* and *M. hornum* mosses by evaluating the interactive effects of NRA and base cation content in this study.
Different plant mineral nutrients have differing roles to play in plant cell metabolism and a deficiency in any one may affect the overall cellular function. Ca is a constituent of the middle lamella of cell walls in higher plants and is required as a cofactor by some enzymes involved in the hydrolysis of ATP and phospholipids and can act as a second messenger in metabolic regulation (Evans and Sorger, 1966; Mengel and Kirkby, 1987; Taiz and Zeiger, 1998). It is also involved in NR activation as a calcium-dependant protein kinase (NR-PK) and has been shown to catalyse the phosphorylation of spinach NR in vitro (Backmann et al., 1995). Changes in photosynthesis upon light/dark transitions are known to rapidly change the steady-state concentration of free cytosolic calcium in the alga *Nitellopsis* (Miller and Sanders, 1987), and such changes in Ca could influence Ca-dependent NR-PK activity and subsequently N assimilation. K is required as a cofactor for more than 40 enzymes including NR. It is the principal cation in establishing cell turgor and maintaining cell electroneutrality and is required for normal protein synthesis and enzyme activation (Walker et al., 1998). P is a component of sugar phosphates, nucleic acids, nucleotides, coenzyme, phospholipids and phytic acid. It also has a role in reactions in which ATP is involved such as the phosphorylation and dephosphorylation of NR (see Figure 3.5). Mg is required in many enzymes involved in P transfer and is a constituent of the chlorophyll molecule. Fe is a constituent of cytochromes and nonheme Fe proteins involved in photosynthesis, N₂ fixation and respiration. Ca, K, P, Mg and Fe were looked at in more detail with regards to N-deprivation in both mosses as indicators of the efficiency of N assimilatory pathways and in photosynthesis and as a means of alleviating the
effects of excess H\(^+\) ion inputs (Soares et al., 1995) and involvement in a pH stat and NRA; as touched on previously.

4.1.6. Aim

The hypothesis was tested that Mnium hornum plants treated with inorganic N after long term N-deprivation would still be able to rapidly induce NR due to the capacity of the moss to store inorganic-NO\(_3^-\). Furthermore, the capacity to store NO\(_3^-\) would also increase in response to the environmental stress of N-deprivation. In contrast perhaps NRA in S. fimbriatum would not be induced in the long term due to the plants decreased capacity to store NO\(_3^-\). Foliar N and PSII efficiency were also measured to assess the effects of long term N-deprivation in both mosses as these parameters are established indicators of N status in higher plant systems (Sage and Pearcy, 1986; Sugiharto et al., 1990; Ciompi et al., 1996; Power et al., 2004). Further investigation into the unusual induction of NRA by NH\(_4^+\) in S. fimbriatum and M. hornum using the NR in vivo method tested the hypothesis that NH\(_4^+\) induces NRA by an acidification of the cytosol. Long term N-deprived M. hornum was treated with inorganic and organic acids to test this hypothesis. NH\(_4^+\) is thought to cause an acidification of the cytosol (Raven, 1985) and weak acids have been shown to activate NR (Kaiser and Brendle-Behnisch, 1995). The effectiveness and role of specific treatments on the uptake of N in the long term also looked at the hypothesis that NRA induction is effected by the cation accompanying NO\(_3^-\) in cell membrane exchange processes. The accompanying cation may have an important role in regulating cellular pH (Soares et al., 1995).
4.2. Results and Discussion

4.2.1. N-deprivation effects on N-status in *Sphagnum fimbriatum*

In Figures 4.1. – 4.2. enzyme activity and NO$_3^-$ concentrations of *S. fimbriatum* declined overall with increased N-deprivation and were sensitive to changes in N supply. NRA showed a steady decline in activity (approximately 2.75 – 0.60 μmol NO$_2^-$ h$^{-1}$ g$^{-1}$ FWt.) over 80 d and NO$_3^-$ concentration also declined (approximately 6.00 – 2.00 μmol NO$_3^-$ g$^{-1}$ FWt.).

![Graph showing NRA over N-deprivation period](image)

**Figure 4.1.** NRA in *S. fimbriatum* misted with dH$_2$O (white circles) over N-deprivation period of 80 days. ± SE bars are shown (n=5).
Figure 4.2. $\text{NO}_3^-$ concentration in \textit{S. fimbriatum} misted with dH$_2$O (white circles) over N-deprivation period of 80 days. ± SE bars are shown (n=5).

Figure 4.3. Foliar N in \textit{S. fimbriatum} misted with dH$_2$O (white circles) over N-deprivation period of 80 days. ± SE bars are shown (n=5). A fitted linear regression line is drawn using Excel (Microsoft).

Figure 4.3 showed that \textit{S. fimbriatum} foliar N levels remained at around 2% and did not change overall when plants were deprived of N over 60 d. This kind of response would suggest that plant metabolism is unaffected by N-deprivation.
After a period of approximately 80 d N-dep it was observed that the plants became necrotic and died. This was usually due to fungal infections, most commonly powdery mildew.

4.2.2. N-deprivation effects on N-status in Mnium hornum

In *M. hornum* there was a decline in plant metabolism overall and most significantly, in contrast to *S. fimbriatum*, this moss species demonstrated a tremendous capacity to sustain itself over long term N-deprivation. Figures 4.4. – 4.5. illustrate a decline in enzyme activity and NO$_3^-$ concentrations overall in *M. hornum* when deprived of N. Following N-deprivation NRA initially declined steadily, as in *S. fimbriatum*, and reached stable NRA post 50 d up to 450 d where activity levelled out at approximately 1.00 μmol NO$_2^-$ h$^{-1}$ g$^{-1}$ FWt. NO$_3^-$ concentration also declined steadily and became more stable post 50 d to 450 d (approximately 33.00 – to 5.00 μmol NO$_3^-$ g$^{-1}$ FWt.).
Figure 4.4. NRA in *M. hornum* misted with dH₂O (white circles) over N-deprivation period of 80 days. ± SE bars are shown (n=5).

Figure 4.5. NO₃⁻ concentration in *M. hornum* misted with dH₂O (white circles) over N-deprivation period of 80 days. ± SE bars are shown (n=5).
Figure 4.6. Foliar N in *M. homunum* misted with dH₂O (white circles) over N-deprivation period of 80 days. ± SE bars are shown (n=5). A fitted linear regression line is drawn using Excel (Microsoft).

Figure 4.6 shows that *M. hornum* foliar N levels declined steadily over 350 days when plants were deprived of N from approximately 2.0 % to approximately 1.0%. With such a large decline in overall N it can be hypothesised that there will be a corresponding decline in plant metabolism. Measuring foliar N was useful as a measure of the N status of the plants. In *S. fimbriatum* there was no overall change in foliar N up to 80 d, but after this it died. *M. hornum* showed a declining foliar N up to 350 days of long term N deprivation and demonstrated a remarkable capability to sustain itself. Foliar N as a measure of N status is not as quick to respond to changes in N supply as enzyme activity or tissue NO₃⁻ concentration changes. Therefore, NRA and tissue NO₃⁻ measurements were used in later research for comparative studies between the mosses.

*S. fimbriatum* and *M. hornum* NRA and NO₃⁻ concentrations were quick to decline when deprived of N and the rate of decline was similar in both species.
(Figures 4.1 – 4.6). *S. fimbriatum* had lower control NRA and basal NO$_3^-$ concentration and less NO$_3^-$ storage capacity than *M. hornum* and this affected the ability of the species to thrive in the long term and *S. fimbriatum* plants died after around 80 d N-dep. There are parallels between this and the differences between the two species in their ability to store NO$_3^-$ when treated with inorganic-NO$_3^-$ in the early stages of N-deprivation (see Chapter 3) and more specifically the capacity for both mosses to store NO$_3^-$ and sense N status was therefore further investigated by treating both species with inorganic-N when plants had been deprived of N in the long term. To obtain a direct comparison of the data the NRA and NO$_3^-$ concentration changes were calculated as a percentage induction or increase from the control value established prior to treatment with inorganic-N at known N-deprivation status.

### 4.2.3. N-deprivation effects on N-status and PSII

*S. fimbriatum* and *M. hornum* PSII photosynthetic efficiency were measured as an indicator of the N-status of the plants. Nitrogen deficiency in *S. fimbriatum* lead to a reduction in photosynthetic efficiency as the $F_v / F_{max}$ ratio declined from 0.20 in N-replete plants compared to 0.10 in N-starved plants over 80 d (see Table 4.1.). Figure 4.7. a) – b) show the visible change in fluorescence in *S. fimbriatum* undergoing N-deprivation, with N-replete plants more green and fluorescing more than plants that were N-starved. Nitrogen deficiency in *M. hornum* seems to indicate that the efficiency of the photochemistry of PSII was not affected by nitrogen deprivation. The $F_v / F_{max}$ ratio fluctuates around 0.15 – 0.19 regardless of N-deprivation of up to approximately 350 d (see Table 4.1).
Figure 4.7. c) – e) show the visible similarities in colour of the fluorescence in *M. hornum*.

**Figure 4.7.** Representative fluorescence photographs taken with a CCD camera of plants at various N-deprivation stages. a) *S. fimbriatum* N-replete status (0 d N-dep), b) *S. fimbriatum* N-starved status (80 d N-dep), c) *M. hornum* N-replete status (0 N-dep), d) *M. hornum* N-deprived status (150 d N-dep), e) *M. hornum* N-starved status (350 d N-dep). PSII operating efficiency quantified by calculating mean overall fluorescence values with ImageJ software. Images in red equate to PSII at high efficiency, yellow at medium and blue minimum efficiency. Black central dots used for calibration purposes.
Table 4-1. Photosynthetic efficiency PSII values (Fv / Fmax) of mosses undergoing N-deprivation.

These ratios are very low compared to higher plant systems which have typical Fv / Fmax values of around 0.80. Ratios in this region are considered normal for healthy higher plants and are visible in the red and yellow range in photographic images (Ciompi et al., 1996). However both species of moss are found in environments with low light intensities such as under tree canopies so low fluorescence is not unexpected. These observations may provide some explanations for the capacity of the moss species to thrive in N-deprived environments. Although foliar N did not show any decline in S. fimbriatum undergoing N-deprivation, a decline in PSII efficiency was observed and this suggests that N-deprived S. fimbriatum is compromised by a lack of N.

4.2.4. Inorganic-N application long term and percentage increases in NRA and NO₃⁻ in Sphagnum fimbriatum

S. fimbriatum responded to KNO₃, Ca(NO₃)₂ and NH₄Cl treatments with a rapid induction of NRA up to approximately 35 d N-dep (Figure 4.8.). Ca(NO₃)₂ had the largest induction capacity with NRA induction at 854 % and 786 % between
30 – 35 d. However for both inorganic-NO$_3^-$ treatments the capacity of the plant to be induced decreased after 35 d. These responses could be explained by _S. fimbriatum_’s decreased capacity to store NO$_3^-$ in both the short and long term (see Chapter 3). NH$_4$Cl had the least inductive effect overall with NRA induction at 38 d of only 67 %. The continuation of NRA induction for example when treated with NH$_4$Cl, is difficult to explain and could be either due to regulation of intracellular pH and / or stress response to try to utilize available N in the environment for protein production in times of nutrient deficiency.

![Figure 4.8](image_url)

**Figure 4.8.** Maximal NRA % induction compared to controls in _S. fimbriatum_ misted with 0.5 mM KNO$_3$ (black bar), 0.25 mM Ca(NO$_3$)$_2$ (grey bar) and 0.5 mM NH$_4$Cl (hatched bar) over N-dep (left hand y axis). Control NRA in _S. fimbriatum_ misted with dH$_2$O (white circles) (right hand y axis). Plotted against N-dep with line drawn to join the mean values. Data points are an average of 5 replicates. SE bars are shown.
*S. fimbriatum* did respond to KNO$_3$ and Ca(NO$_3$)$_2$ treatments with an increase in NO$_3^-$ storage (Figure 4.9). KNO$_3$ had the largest NO$_3^-$ increase at 589 % at 29 d, compared to controls. However for both inorganic-NO$_3^-$ treatments the capacity of the plant to store NO$_3^-$ decreased after 35 d and was low overall. There were no changes in tissue NO$_3^-$ for NH$_4$Cl with 5.5% (SE 19.9 %) at 33 d and negative values at 48 d. *S. fimbriatum*’s reduced capacity to store NO$_3^-$ in both the short and long term could affect this species’ ability to sustain itself when undergoing N-deprivation. Lower levels of leaf NO$_3^-$ assimilation in higher plants are often associated with slow growing climax species which only exhibit very small constituent levels of foliar NO$_3^-$ assimilation (Soares *et al.*, 1995). *S. fimbriatum* moss metabolism is more like a climax species in this respect.
Figure 4.9. Maximal NO$_3^-$ % increase compared to controls in *S. fimbriatum* misted with 0.5 mM KNO$_3$ (black bar), 0.25 mM Ca(NO$_3$)$_2$ (grey bar) and 0.5 mM NH$_4$Cl (hatched bar) over N-dep (left hand y axis). Control NO$_3^-$ concentration in *S. fimbriatum* misted with dH$_2$O (white circles) (right hand y axis). Plotted against N-dep with line drawn to join the mean values. Data points are an average of 5 replicates. SE bars are shown.

4.2.5. Inorganic-N application long term and percentage increases in NRA and NO$_3^-$ in *Mnium hornum*

In the case of *M. hornum* this also responded to KNO$_3$, Ca(NO$_3$)$_2$ and NH$_4$Cl treatments with a rapid induction of NRA during long term N-deprivation up to approximately 450 d (Figure 4.10). KNO$_3$ had the largest and most consistent induction capacity with NRA induction at 438 % at 13 d, 348 % at 71 d and 339
at 437 d. Ca(NO₃)₂ induced NRA less, at approximately 125% at 30 d but NRA induction increased to 347% at 323 d. The presence of a cation effect was investigated further by looking at the tissue content of cations and heavy metals when plants underwent long term N-deprivation in both species. Treatment with NH₄Cl induced NRA up to 450 d but the changes were not significantly different as can be seen by the overlapping error bars (Figure 4.10).

Figure 4.10. Maximal NRA % induction compared to controls in M. homum misted with 0.5 mM KNO₃ (black bar), 0.25 mM Ca(NO₃)₂ (grey bar) and 0.5 mM NH₄Cl (hatched bar) over N-dep (left hand y axis). Control NRA in M. hornum misted with dH₂O (white circles) (right hand y axis). Plotted against N-dep with line drawn to join the mean values. Data points are an average of 5 replicates. SE bars are shown.
M. hornum responded to KNO$_3$ and Ca(NO$_3$)$_2$ treatments with an increase in tissue NO$_3^-$ during long term N-deprivation up to approximately 450 d (Figure 4.11). KNO$_3$ had the largest tissue NO$_3^-$ increase at 1741 % at 13 d and then decreased to 920 % at 71 d and 168 % at 437 d. Ca(NO$_3$)$_2$ followed a similar pattern but at lower concentrations, similar to those observed up to 60 d in S. fimbriatum. NO$_3^-$ increased to 470 % at 29 d and then decreased to 306 % at 30 d and 193 % at 323 d. In M. hornum there was no NO$_3^-$ increase in tissues for the NH$_4$Cl treatment, the change being only 8.2 % (SE 30.6 %) at 32 d and negative values at 324 d. The induction of NRA in M. hornum by inorganic-N and storage capacity of NO$_3^-$ both short and long term, enables the species to sustain itself when undergoing N-deprivation. Higher levels of leaf NO$_3^-$ assimilation in higher plants are commonly associated with faster growing pioneer species which can readily induce NRA in their leaves to accommodate increased availability of NO$_3^-$ from the environment and are more physiologically active (Soares et al., 1995). In this regard M. hornum moss could be regarded as a pioneer species in mosses.

The induction of NRA by NH$_4$Cl in the absence of additional NO$_3^-$ occurs in the long term in M. hornum. This effect in both mosses will be investigated further using inorganic and organic acids to see if a decrease in cell pH also stimulates NR.
Figure 4.11. Maximal NO$_3^-$ % increase compared to controls in *M. hornum* misted with 0.5 mM KNO$_3$ (black bar), 0.25 mM Ca(NO$_3$)$_2$ (grey bar) and 0.5 mM NH$_4$Cl (hatched bar) over N-dep (left hand y axis). Control NO$_3^-$ concentration in *M. hornum* misted with dH$_2$O (white circles) (right hand y axis). Plotted against N-dep with line drawn to join the mean values. Data points are an average of 5 replicates. SE bars are shown.

4.2.6. pH and other salt application long term and percentage increases in NRA and NO$_3^-$ in *Mniium hornum*

*M. hornum* responded to H$_2$SO$_4$, K$_2$SO$_4$ and butyric acid with induction of NRA during long term N-deprivation up to approximately 450 d (Figure 4.12. and Table 4.2). The induction of NRA by these other treatments in *M. hornum* occurred in the absence of any additional N-containing ions (see Table 4.3).
Figure 4.12. Maximal NRA % induction compared to controls in *M. hornum* misted with pH 3 H$_2$SO$_4$ (black bar), 10 mM K$_2$SO$_4$ (hatched bar), 1 mM CaSO$_4$ (cross-hatched bar) and 10 mM butyric acid (grey bar) over N-dep (left hand axis). Control NRA in *M. hornum* misted with dH$_2$O (white circles) (right hand y axis). Plotted against N-dep with line drawn to join the mean values. Data points are an average of 5 replicates. SE bars are shown.

H$_2$SO$_4$, K$_2$SO$_4$ and butyric acid all showed quite marked NRA induction with a range between 113 % to 52 % from 23 to 46 d N-dep. The acids appear to have an effect on NRA and support the theory that NRA is induced by a drop in cellular pH (Kaiser and Huber, 2001.). This goes some way to support the idea that when plants are treated with NH$_4$Cl NRA may be induced as part of a pH-stat to nullify proton production as NH$_4^+$ is incorporated into amino form.
Treatm ent N-dep NRA % induction response % SE

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N-dep</th>
<th>NRA % induction response</th>
<th>% SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 3 H₂SO₄</td>
<td>32</td>
<td>81</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>46</td>
<td>70</td>
<td>34</td>
</tr>
<tr>
<td></td>
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<td>110</td>
</tr>
<tr>
<td>10 mM K₂SO₄</td>
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<td>28</td>
</tr>
<tr>
<td></td>
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<td>52</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>326</td>
<td>54</td>
<td>52</td>
</tr>
<tr>
<td>1 mM CaSO₄</td>
<td>32</td>
<td>-10</td>
<td>12</td>
</tr>
<tr>
<td>10 mM butyric acid</td>
<td>33</td>
<td>89</td>
<td>9</td>
</tr>
</tbody>
</table>

Table 4-2. NRA % induction in *M. hornum* misted with pH 3 H₂SO₄, 10 mM K₂SO₄, 1 mM CaSO₄ and 10 mM butyric acid treated at different stages of N-deprivation compared to controls misted with dH₂O.

Interestingly NRA seemed to be more inducible by H₂SO₄ when deprived of N long term (266 % after 325 d) and further work is required to understand this effect. To examine this phenomenon the effect of cations was considered as the more N-deprived plants the more deficient they are in many other nutrients and elements; for example K. This could reduce any buffering capacity affects and make the cytosol even more acidic, in turn resulting in a larger induction of NRA due to greater acidity (Kaiser and Brendle-Behnisch, 1995). Reasons for acidity causing increased NRA are unclear but this could be due to an increase in dephosphorylated NR enzyme due to lack of available Pi⁺, the denaturing of NIP or perhaps the increased activation of NR kinase. The most likely cause, as proposed by Kaiser and Brendle-Behnisch (1995) was that acid-induced activation was mediated by protein dephosphorylation. A decrease in available phosphorous as a result of a decrease in overall nutrient status in *M. hornum* moss that is N-deprived could provide a further explanation. The induction of
NRA by K$_2$SO$_4$ is unexpected as the salt solution has a neutral pH however the provision of these ions to an N-deprived plant may provide an explanation for preferential uptake and utilisation. The inability of CaSO$_4$ to induce NRA may have been due to the lower molarity of the solution used, 1 mM rather than 10 mM, or it could indicate that specific cations cause different reactions.

Figure 4.13. Maximal NO$_3^-$ % increase compared to controls in *M. homum* misted with pH 3 H$_2$SO$_4$ (black bar), 10 mM K$_2$SO$_4$ (hatched bar), 1 mM CaSO$_4$ (cross-hatched bar) and 10 mM butyric acid (grey bar) over N-dep (left hand axis). Control NO$_3^-$ concentration in *M. homum* misted with dH$_2$O (white circles) (right hand y axis). Plotted against N-dep with line drawn to join the mean values. Data points are an average of 5 replicates. SE bars are shown.
Table 4-3. NO$_3^-$ % increase in *M. hornum* misted with pH 3 H$_2$SO$_4$, 10 mM K$_2$SO$_4$, 1 mM CaSO$_4$ and 10 mM butyric acid treated at different stages of N-deprivation compared to controls misted with dH$_2$O.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N-dep</th>
<th>NO$_3^-$ % increase response</th>
<th>% SE</th>
</tr>
</thead>
<tbody>
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<td>pH 3 H$_2$SO$_4$</td>
<td>32</td>
<td>-7</td>
<td>29</td>
</tr>
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<td></td>
<td>46</td>
<td>-16</td>
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</tr>
<tr>
<td></td>
<td>325</td>
<td>-27</td>
<td>17</td>
</tr>
<tr>
<td>10 mM K$_2$SO$_4$</td>
<td>23</td>
<td>-10</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>33</td>
<td>-83</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>326</td>
<td>7</td>
<td>15</td>
</tr>
<tr>
<td>1 mM CaSO$_4$</td>
<td>32</td>
<td>20</td>
<td>69</td>
</tr>
<tr>
<td>10 mM butyric acid</td>
<td>33</td>
<td>-6</td>
<td>9</td>
</tr>
</tbody>
</table>

Recent research carried out by Britto and Kronzucker (2005) has called into question established theory on the classic biochemical pH-stat model of cytosolic pH regulation in plants. They say that some of the models key assumptions are fundamentally problematic, particularly in the context of the effects on cellular pH of N source differences. They state that the current model fails to account for proton transport accompanying inorganic-N transport which if considered, renders the H$^+$ production of combined transporter and assimilation (although not the accumulation) to be equal for NO$_3^-$ and NH$_4^+$ as externally provided N sources. They hypothesise that NO$_3^-$ is cotransported overall with one H$^+$ and this is the chemical equivalent of nitric acid, a strong acid, in the cytosol. NH$_4^+$ would be countertransported with one H$^+$ and this is the chemical equivalent of NH$_3$, a weak base, in the cytosol following the transport and subsequent membrane repolarisation across the plasma membrane. These pH changes are hypothesised to be only transient as subsequent assimilation rapidly alters the
amounts of the acid and base pools and overall they explain that when NO$_3^-$ is assimilated the net proton introduced to the cytosol in the NO$_3^-$ transport step is effectively neutralised. Importantly the antiport or symport of protons with ions such as K$^+$, Na$^+$ or Cl$^-$ across the plasma membrane may have been underestimated and could play a role in pH changes in the cytosol (Britto and Kronzucker, 2005) and provide an explanation for the induction of NRA by some of the inorganic acids, organic acids and neutral salts. In light of the induction of NRA with acids and even some neutral salts this theory required further investigation and electrophysiological measurements were carried out in $M. hornum$ to investigate the effect on membrane polarity, transporter activity and pH regulation (see Chapter 6).

4.2.7. Cation and heavy metal analysis long term in $Sphagnum fimbriatum$ and $Mnium hornum$

The presence of a cation effect in the NRA induction of $S. fimbriatum$ and $M. hornum$ was investigated further by looking at the tissue content of cations and heavy metals when plants underwent long term N-deprivation and five major elements were selected. Concentrations of Mg and Fe remained similar for both mosses overall with quite a lot of variability regardless of the N-dep of the plant or the species type. Ca and K did show some variation.
Table 4-4. Element concentrations in *S. fimbriatum* at varying N-dep following N-starvation.

<table>
<thead>
<tr>
<th>Concentration (ppm)</th>
<th>Ca</th>
<th>K</th>
<th>P</th>
<th>Mg</th>
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<tr>
<td>mean</td>
<td>2,111</td>
<td>19,013</td>
<td>1,552</td>
<td>1,702</td>
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<td>338</td>
<td>503</td>
<td>37</td>
<td>189</td>
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<tr>
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<td>600</td>
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<tr>
<td>mean</td>
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<tr>
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<td>940</td>
<td>1,807</td>
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<td>225</td>
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<td>126</td>
<td>331</td>
<td>28</td>
<td>84</td>
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</table>

The concentrations at 0 d N-dep in both mosses are comparative to studies carried out on the lichen *Paramecia sulcata* in healthy tissue from Burnham Beeches (Purvis *et al.*, 2005). In *S. fimbriatum* NRA induction was larger in the long term when treated with Ca(NO₃)₂ than KNO₃ and in *M. hornum* NRA induction was larger in the long term when treated with KNO₃ than Ca(NO₃)₂.

When concentrations of either cation are compared at 0 d N-dep on average Ca is twice as low and K is twice as high in *S. fimbriatum*, with the converse situation in *M. hornum*. This could demonstrate a Ca-deprivation effect in the long term in *S. fimbriatum* and K-deprivation effect in *M. hornum* in the long term as well as an N-deprivation effect in both species in the long term. It can be suggested that the moss is most responsive to the treatment solution containing both an anion (NO₃⁻) and cation (Ca in the case of *S. fimbriatum* and K in the case of *M. hornum*) when there is a combined deficit.
<table>
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<tr>
<th>Concentration (ppm)</th>
<th>Ca</th>
<th>K</th>
<th>P</th>
<th>Mg</th>
<th>Fe</th>
</tr>
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<td>mean</td>
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<td>2,188</td>
<td>1,471</td>
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<tr>
<td>mean</td>
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<td>8,304</td>
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<td>97</td>
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<td>277</td>
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<td>64</td>
<td>58</td>
<td>14</td>
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</table>

Table 4-5. Element concentrations in *M. hornum* at varying N-dep following N-starvation.

From the findings in Chapter 3 it was suggested that there may be a K-deprivation effect in both species in the short term in conjunction with an N-deprivation effect, with the combined supply of inorganic-KNO$_3$ containing both K and N having a larger stimulatory effect on moss metabolism overall (including NRA, NO$_3^-$, amino acid and protein concentration); in part due to the role K plays in protein synthesis and enzyme activation. However in light of these results it can be concluded that in the long term *S. fimbriatum* is more likely to be Ca-deprived as supported by the larger increase in NRA when treated with inorganic-Ca(NO$_3$)$_2$. For *M. hornum* in the long term the plants are more...
likely to be K-deprived, as supported by the larger increase in NRA when treated with inorganic-KNO$_3$.

An important observation that can be made from observing cations could be that changes in cations may provide a mechanism for buffering acidity with pH changes in the cytosol involving the antiport or symport of protons with ions across all cellular membranes (Britto and Kronzucker, 2005). For example K$^+$ deficiency has been shown to lead to an acidification of the cytoplasm in work carried out using triple-barrelled microelectrodes (Walker et al., 1996). It has been suggested that the relationship must indicate a long term effect of K$^+$ status on cytosolic pH. Three mechanisms contribute to the maintenance of cytosolic pH in plant cells (Smith and Raven, 1979): physicochemical buffering (Roos and Boron, 1981), the biochemical pH-stat (Davies, 1986 and Raven, 1986) and H$^+$ export from the cytosol to the external medium or the vacuole (Smith and Raven, 1979). Presumably, effects on one or several of these contribute to the change in cytosolic pH in K$^+$ starved cells. The first defence against a fall in plant cell pH after addition of an acid load is the buffering of H$^+$ by available bases, including K$^+$ in processes such as physicochemical buffering. This then acts to regulate the intracellular pH-stat by substituting itself for H$^+$ ions and exporting them from the cytosol to the external medium or vacuole. Hence a decrease in available K$^+$, a deficiency, causes cells to lose their physicochemical buffering capabilities, H$^+$ accumulates and there is an increase in acidity in the cell. Therefore cations acting as buffers to changes in cytosolic pH could be an important mechanism in preventing damage to metabolic process in the cytosol. \textit{S. fimbriatum} and \textit{M. hornum} have similar cation concentrations up to 60 d then availability of such
cations could provide a means of creating a stabilised pH gradient across plant cell membranes by stabilising charges. The ability to do this would not to be compromised in either species due to the ability of both species to maintain their cation concentration whilst undergoing N-deprivation.

*S. fimbriatum* does not show an overall decline in cations or heavy metals over the course of N-dep in comparison to *M. hornum*. This is in contrast to findings in higher plants where plants that exhibit more climax species characteristics have lower overall nutrient content in the form of a decline in N, Ca, K etc. (Soares *et al.*, 1995). In a study on cation content in the semi-aquatic bryophyte *Philonotis fontana* by Soares and Pearson (1995) these plants actually had the highest overall cation content of the three species studied, the others being terrestrial species. They concluded that this most likely reflected its semi-aquatic habitat and the availability of nutrients in run-off water, rather than the inorganic-N treatments that were applied in the experiments. Cations and heavy metals were measured during a time course in *S. fimbriatum* and *M. hornum* (0 h – 8 h) however no large changes were seen in the overall concentration of elements after treatment with inorganic-N or inorganic or organic acids (data not shown). In *M. hornum* there is a tendency for some of the cations to show a decline in concentration over 437 d N-dep. The changes observed in *M. hornum* over 437 d N-dep are slow and somewhat variable. From the variability in Ca, K and P over N-dep we cannot draw firm conclusions about the contribution of a growth dilution effect due to the cation concentrations being inconsistent over time. Further experimentation would be required to prove conclusively that there was any decline in the elements studied in light of the fluctuations in
concentrations for the duration of the study. Ions, particularly Ca$^{2+}$, Mg$^{2+}$ and K$^+$, are readily lost from the surfaces of all wet leaves by cation exchange processes. This loss would show as a decrease in cation concentration over time. However this was not observed in *M. hornum* under controlled experimental conditions with dH$_2$O misting. An explanation for the variability could be that these ions are lost from the moss and washed across the entire canopy being reabsorbed according to availability and N-status of the moss at the time. To test this hypothesis in the next chapter field trials (Chapter 5) are described where cation and heavy metal concentrations were measured in the *M. hornum* tissue and field site wet deposition (rainwater) to see if there would be an increase in these elements in plants placed back into a natural environment.

4.3. Initial Conclusions and Next Steps

The hypothesis was tested that NH$_4^+$ may cause an acidification of the cytosol (Raven, 1985) and this in turn would induce NR (Kaiser and Brendle-Behnisch, 1995) and it was shown that NH$_4^+$ induced NRA both in *S. fimbriatum* and *M. hornum* during long term N-deprivation. Further discussion of these conclusions are outlined in Chapter 7 and to test that an acidification of cytosol could occur upon addition of NH$_4$Cl intracellular pH changes were measured using microelectrodes (see Chapter 6).

Cations and heavy metals were measured during N-deprivation in *S. fimbriatum* and *M. hornum* and were shown to have similar cation concentrations up to 60 d. It can be suggested that the ability of both species to stabilise this gradient with
available cations would not be compromised in either species due to the ability of both species to maintain their cation concentration whilst undergoing N-deprivation. So to further investigate the cation effect electrophysiological measurements were carried out in *M. hornum* on membrane polarity, transporter activity and pH regulation (see Chapter 6) and the findings are discussed in Chapter 7.

The hypothesis was partially proven that *M. hornum* plants treated with inorganic N after long term N-deprivation were still able to rapidly induce NRA. However the capacity to store NO$_3^-$ decreased over 450 d. *S. fimbriatum* had lower basal NRA and NO$_3^-$ concentrations and less NO$_3^-$ storage capacity than *M. hornum* primarily to do with proposed differences in stoargae compartments between species. Interestingly, *M. hornum* had a tremendous capacity to sustain itself whilst undergoing N-starvation. Due to *M. hornum*’s tremendous capacity to sustain itself long term this moss was used to test a hypothesis that *M. hornum* would be able to sense changes in atmospheric N deposition.
CHAPTER 5

*MNium Hornum and its response to atmospheric N inputs*

5.1. Introduction

5.1.1. Atmospheric N inputs

Three likely sources of nutrients for terrestrial mosses in nature are; the substratum, wet deposition, such as precipitation including leachates from any plant or other surfaces over which it flows, and dry deposition such as dust and gases in the forms of NH$_3$, SO$_2$ and NO$_2$ (Bates, 2000). Mosses may utilise several of these sources to obtain essential elements, including cations and heavy metals. Analysing moss tissue and rainwater precipitation can therefore provide a useful measure of the atmospheric inputs applied to mosses.

In a study of NRA *in vivo* in *Sphagnum fuscum*, an ombrotrophic peat-moss at an unpolluted site in northern Sweden, activity remained low during dry periods but was rapidly induced during natural precipitation containing dilute NO$_3^-$ (Woodin *et al.*, 1985). Values for NRA ranged from approximately 0.50 – 2.50 µmol NO$_2^-$ h$^{-1}$ g$^{-1}$ FWt which are values in the same region as those observed in *Sphagnum fimbriatum* in this study. In a separate study by Press & Lee (1982)
on *Sphagnum fuscum* there was a similar close relationship between the atmospheric supply of NO$_3^-$ ions in wet deposition and the utilisation of this N source by NR. The study also demonstrated the susceptibility of *Sphagnum* species to perturbation by atmospheric gaseous pollutants or their solution products (Press and Lee, 1982).

A study on the mineral nutrients in rainwater determined the major ions experimentally from the water dripping from *Calliergonella cuspidata* plants and in this species of moss it was concluded that NH$_4^+$ was the only ion that appeared to be absorbed by the plants in significant amounts from the natural wet and dry deposition received (van Tooren *et al.*, 1990). However, work by Soares & Pearson (1997) on three moss species showed that they were all capable of taking up and utilising NO$_3^-$, although a slight preference was shown for NH$_4^+$ uptake.

5.1.2. Foliar N and $^{15}$N atom analysis in mosses and higher plants in the field

The N content of some plants has been shown to indicate atmospheric inputs of N (Baddeley *et al.*, 1994; Pitcairn *et al.*, 1995, 1998; Bobbink *et al.*, 1998) alongside observable changes in species composition in the environment. In recent studies of plant community responses to atmospheric N deposition critical loads have been established to assess the N status and health of the vegetation. Total N content and $\delta^{15}$N signatures of plant tissue have both been posed as candidates as biological tracers (Skinner *et al.*, 2004; Marsh *et al.*, 2004; Power *et al.*, 2004). An effective biological tracer would enable information to be obtained quickly and accurately and be transferable to the effects of widespread
N deposition on vegetation and can also be related to critical loads. The application of relating critical loads to foliar N in relation to atmospheric N inputs and their impact on a woodland forest community was outlined in a recent study by Pitcairn *et al.*, (2001). Foliar N was used to define the impacts of NH₃ deposition from nearby livestock buildings on species composition of woodland ground flora, using a woodland site close to a major poultry complex in the UK. Tissue N concentrations in trees, herbs and mosses were all large, reflecting the substantial NH₄⁺ emissions at this site. Tissue N content of ectohydric mosses ranged from approximately 4 % at 30 m downwind to 1.6 % at 650 m downwind. It was established that a critical foliar N content for these mosses was the most useful method for providing spatial information which could be of value to policy developers and planners with regards to assessing the impacts of increased N inputs in the environment. A critical foliar N concentration had been set at 2 % for ectohydric mosses in a study by Pitcairn *et al.*, (1998) prior to this study that correlated with critical loads of 15 – 20 kg N ha⁻¹ year⁻¹ which had also been set previously for the protection of woodland ground flora. Foliar N greater that 2 % resulted in considerable changes in woodland ground flora being observed with a bias towards more N-tolerant species such as *Deschampsia flexuosa*, *Holcus lanatus*, *Rubus idaeus* and *Urtica dioica* near the livestock units and N-sensitive species including many mosses being absent (Pitcairn *et al.*, 1998). Foliar N concentration being quick and easy to measure was therefore proposed as a bio-indicator of both atmospheric inputs of N and resulting impacts on the surrounding environment.
δ-15N values have also been used in systems as environmental tracers. δ-15N and NRA at the Mardley Heath site were shown to be indicators of uptake and assimilation of N in *Crataegus monogyna* foliage (Marsh *et al.*, 2004). δ-15N became less negative closer to the motorway due to oxidised N from the A1M motorway site being taken up and incorporated into the plant tissues. The Mardley Heath site for the field experimentation of *M. hornum* has been established as a source of high levels of atmospheric N oxides from the A1M motorway traffic and it is hypothesized that the *M. hornum* plants close to the motorway site will show a similar relationship to that discovered in trees in the woodland. This hypothesis is strengthened by evidence based on mosses exposed to heavy traffic in London, in contrast to rural areas, which found that there was a very good correlation between traffic exposure and tissue δ-15N (Pearson *et al.*, 2000). NOx was shown to have a positive and NHx a negative δ-15N signature with the average δ-15N of shoots from busy roadsides in London having values of +3.66 %o and samples from farm buildings – 7.8 %o.

The hypothesis that changes in δ-15N signal from an NH3 emission source would be observable in nearby vegetation was also tested on *Calluna vulgaris* by Skinner *et al.*, (2004). Analysis of shoots showed a significant linear correlation between δ-15N and foliar N in plant material in controlled conditions of open top chambers and at a NH3 release site, with the potential for the use of this technique in N deposition biomonitoring.
5.1.3. Tissue cation and heavy metal analysis of mosses placed in the field

The cell walls of most plants possess a net negative charge owing to the ionization of weak acids built into their structure. For example primary cell walls (PCW) of bryophytes, including mosses, contain uronic acid which is not found in higher plants having further implications in the evolutionary events associated with changes in the PCW composition (Popper and Fry, 2002). Cations usually saturate these sites when they are in plentiful supply. Binding affinities of cations are affected by higher concentrations, larger hydrated atomic radii or higher valencies (Bates, 2000). Metals and other cations permeating the cell wall could easily become relatively firmly held by the negative charges with the external medium receiving the displaced protons, sometimes leading to its acidification. Experiments by Rodenkirchen (1992) and Kooijam et al., (1994) have demonstrated the relative mobility of cations and high exchange capacities in mosses and this may be of benefit in alleviating the effects of acidic pollutants. These kinds of responses occur in environments with small fluctuations in acidity however, with some moss species not being able to adapt to more extreme changes in pH or N regimes. The mosses that are most tolerant to changes in these factors could be those with larger cation exchange capacities. Cations and heavy metals were measured during N-deprivation in _S. fimbriatum_ and _M. hornum_ and were shown to have similar cation concentrations up to 60 d with no observable changes in the short term following treatments with KNO₃ or Ca(NO₃)₂. There was also no loss of cation concentration in _M. hornum_ following long term deprivation, which would usually be expected in a nutrient deprived environment. Cation concentrations were measured in _M. hornum_.
tissue and field site wet deposition (rainwater) to see if there would be an increase in these elements when plants were placed back into a natural environment. Some studies of moss species treated with inorganic-N have shown variable responses of base cation content with regard to the type of treatment used (Soares and Pearson, 1995). These studies found that Ca was the most mobile cation overall with a tendency to increase with NO$_3^-$ treatments in some mosses. These changes in Ca and to some extent K were also similar to those found by Bates (1994) for *Brachythecium rutabulum* and *Pseudoscleropodium purum* after short term N applications but these effects were not observed in lab controlled conditions in *S. fimbriatum* and *M. hornum* and it remained to be seen if changes in cations occur in *M. hornum* placed into the field.

### 5.1.4. Rainwater pH effects on plant nutrient uptake

Pollutant gases and particles may be transferred large distances before being deposited in precipitation and without these pollutants rain would be acidic due to the presence of carbonic acid from dissolved atmospheric CO$_2$ (Ashenden, 2002). Acid rain is taken to be at a lower pH than 5.6. The acidity of rainwater is important when considering the effects of foliar fertilisation and leaching. In a study on *Phaseolus vulgaris* sulphate entered the plant’s leaves faster at pH 2.7 than at pH 5.7 whereas tritiated water (T$_2$O) entered foliage at similar rates for all pHs indicating that absorption of materials by leaf surfaces is a selective process (Evans *et al.*, 1981). Theoretically similar processes may occur with N so the pH and comprehensive chemical composition of the rainwater at Mardley Heath was
measured in the field by collecting rainwater in gauges to account for any such effects. Losses of cations due to cation exchange processes such as Ca$^{2+}$, Mg$^{2+}$ and K$^+$, from wet leaves are more frequent and rates are also enhanced by increases in acidities of rainfall. Large increases in Ca especially, but also in Mg and K in droplets collected from leaves of *Artemisia tilesii*, suggested that cations played an important role in increasing the pH of acidic droplets (Adams and Hutchinson, 1984). Cation losses from the mosses will be measured from tissue cation and heavy metal analysis to see if there are any declines in tissue cation content during the time of the field experimentation and also if this may correlate with any shifts in rainwater pH. Importantly the precipitation supplying the plants with nutrients will also be analysed for cation content which should contain any losses from surrounding vegetation.

5.1.5. Ion analysis and quantity of ion supplied in rainwater to *Mnium hornum* experimental sites in the field

The exposure of vegetation to air pollutants has been expressed in a variety of ways using a variety of methods but for the purposes of measuring localised N inputs simple techniques were used to collect rainwater and analyse the contents. Accounting for N inputs onto the *M. hornum* trays would provide information on any changes to the plant metabolism. Hypothesised changes in N metabolism in *M. hornum* may provide a measure of atmospheric N inputs and a biological tracer may be identified. The ion concentrations in rainwater were converted by accounting for variables such as the volume of rainwater collected, catchment surface area of the gauge used and the tray surface area. This gave accurate
localised information on the quantity of ions specifically precipitated onto the area where the plants had been placed. *M. hornum*, at a previously established metabolic status, was placed into the field to measure the level of N pollution at that site.

5.1.6. Aim

From the results in Chapters 3 and 4 the hypothesis was posed that N-starved *M. hornum* could be placed into the field to measure the level of N pollution at that site by assessing the rate of tissue N increase. This method could provide a measure of atmospheric N inputs and perhaps *M. hornum* could thereby serve as a reliable bioindicator. In addition metabolic changes in NRA and tissue NO$_3^-$ concentration were also measured. NRA and tissue NO$_3^-$ concentration changes were established as good indicators of N status in *M. hornum* throughout the course of N-deprivation and induction trials with rapid responses to the application of inorganic-N.
5.2. Results and Discussion

5.2.1. NRA, Tissue NO$_3^-$, Foliar N and $^{15}$N atom in _Mnium hornum_ experimental sites in the field

Trays of _M. hornum_ at 151 d N-dep with low basal NRA were placed at two field sites; 20 m from the A1M motorway and 700 m away in adjacent woodland. They showed an increase in NRA after just 7 d from less than 1.0 $\mu$mol NO$_2^-$ h$^{-1}$ g$^{-1}$ FWt up to 7.4 $\mu$mol NO$_2^-$ h$^{-1}$ g$^{-1}$ FWt at the woodland site and 7.5 $\mu$mol NO$_2^-$ h$^{-1}$ g$^{-1}$ FWt at the A1M (Figure 5.1). Although there was some variability in NRA over the course of the trial, with some weeks the A1M having maximal activity and others the woodland, NRA remained around these levels of induction over the course of the month with 7.7 $\mu$mol NO$_2^-$ h$^{-1}$ g$^{-1}$ FWt at the A1M and 7.5 $\mu$mol NO$_2^-$ h$^{-1}$ g$^{-1}$ FWt at the woodland site at 27 d. _M. hornum_ NRA was induced rapidly when placed back into the field experimental site and activities reached those found in _M. hornum_ moss collected from the field after just 7 d.
Figure 5.1. NRA response of *Mnium hornum* placed into the field following 151 d N-dep (time post N-dep 0 d) under controlled experimental conditions (nitrogen starved). Plants were sampled approximately every week and received ambient rainfall. A1M site (black circles) samples, woodland site (white circles) samples. Data points are an average of 5 replicates. SE bars are shown.

Tissue NO$_3^-$ concentrations showed steady increases in concentration over 27 d. N-deprived moss at 151 d N-dep had concentrations of 4.0 μmol NO$_3^-$ g$^{-1}$ FWt and 6.8 μmol NO$_3^-$ g$^{-1}$ FWt when placed at the A1M and woodland site respectively (Figure 5.2). This increased up to 21 d when maximum concentrations were 31.0 μmol NO$_3^-$ g$^{-1}$ FWt and 34.3 μmol NO$_3^-$ g$^{-1}$ FWt. There was a decline in tissue NO$_3^-$ concentration after 27 d but this did not appear to effect NRA. *M. hornum* tissue NO$_3^-$ concentrations increased when plants were placed back into the experimental field site with activities reaching those found in *M. hornum* moss collected from the field site after 21 d. These results
supported the hypothesis that N-starved *M. hornum* placed into the field experimental site can give a measurable recovery of tissue N status.

**Figure 5.2.** Tissue NO$_3^-$ concentration changes in *Mnium hornum* placed into the field following 151 d N-dep (time post N-dep 0 d) under controlled experimental conditions (N starved). Plants were sampled approximately every week and received ambient rainfall. A1M site (black circles) samples, woodland site (white circles) samples. Data points are an average of 5 replicates. SE bars are shown.

Foliar N showed no increase over 21 d with N-deprived moss at 151 d N-dep between 1.45 % - 1.50 % when placed at the A1M and woodland site respectively (Figure 5.3). Foliar N did increase after 21 d in the field with levels reaching 1.99 % at the A1M and 2.30 % at the woodland sites after almost 2 months, 56 d, in the field. Original foliar N values from material collected from the sites were 1.87 - 1.94 % at the A1M and 1.78 - 1.79 % at the woodland so
with *M. hornum* placed at the experimental field site foliar N exceeded levels found naturally in the field. These foliar N values are just below those set for critical loads of N with levels actually exceeding critical loads in the woodland at 56 d. Analysis of rainwater provided useful data in explaining these trends.

![Figure 5.3](image.jpg)

**Figure 5.3.** Foliar N changes in *Mnium hornum* placed into the field following 151 d N-dep (time post N-dep 0 d) under controlled experimental conditions (N starved). Plants were rain watered during the course of the trial and sampled approximately every week in the first week and fortnightly thereafter. AIM site (black circles) samples. Woodland site (white circles) samples. Data points are an average of 2 replicates.

δ-15N measurements revealed no variations over time or between sites with values staying around the natural abundance of 15N = 0.3663 atom %. This could be due to the type of N deposition onto the moss canopy as δ-15N signatures tend to show trends with dry deposition. The three forms of N deposition, wet, dry
and particulate, may all contribute different δ-15N signatures (Pitcairn et al., 1995).

5.2.2. Tissue cation and heavy metal analysis of *Mnium hornum* placed in the experimental field site

There were some interesting trends in cation concentration in *M. hornum* after placement into the field (Tables 5.1 – 5.2). For Ca, A1M field collected moss, at 0 d N-dep 4,037 ppm declined to 3,252 ppm after 151 d N-dep and once placed into the field declined further to 2,777 ppm before increasing steadily up to 6,699 ppm after two months. At the woodland site field collected moss at 4,623 ppm declined to 3,252 ppm after 151 d N-dep and once placed into the field declined further to 3,046 ppm before increasing more slowly up to 4,423 after 56 d.

<table>
<thead>
<tr>
<th>A1M Site</th>
<th>Concentration (ppm)</th>
<th>Ca</th>
<th>K</th>
<th>P</th>
<th>Mg</th>
<th>Fe</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mnium</strong> Field Collected</td>
<td>N-dep (d) 0 (n=2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1M Site mean</td>
<td></td>
<td>4,037</td>
<td>5,775</td>
<td>1,533</td>
<td>1,411</td>
<td>1,023</td>
</tr>
<tr>
<td>N-saturated SE</td>
<td>152</td>
<td>318</td>
<td>36</td>
<td>96</td>
<td>104</td>
<td></td>
</tr>
<tr>
<td><strong>Mnium</strong> Moss Trays Laboratory Cultured</td>
<td>N-dep (d) 151 (n=2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>3,252</td>
<td>8,956</td>
<td>2,466</td>
<td>3,129</td>
<td>231</td>
<td></td>
</tr>
<tr>
<td>N-deprived SE</td>
<td>64</td>
<td>2,158</td>
<td>86</td>
<td>2,158</td>
<td>91</td>
<td></td>
</tr>
<tr>
<td><strong>Mnium</strong> Moss Trays Transplanted A1M Post N-dep (d) (n=1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time Post N-dep</td>
<td>7</td>
<td>2,777</td>
<td>8,241</td>
<td>2,095</td>
<td>2,414</td>
<td>1,120</td>
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<tr>
<td></td>
<td>14</td>
<td>3,473</td>
<td>6,260</td>
<td>2,134</td>
<td>2,603</td>
<td>722</td>
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<tr>
<td></td>
<td>21</td>
<td>3,827</td>
<td>5,522</td>
<td>1,873</td>
<td>2,388</td>
<td>2,196</td>
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<tr>
<td></td>
<td>27</td>
<td>4,343</td>
<td>4,961</td>
<td>1,920</td>
<td>2,296</td>
<td>1,522</td>
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<tr>
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<td>41</td>
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<td>5,282</td>
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<td>56</td>
<td>6,699</td>
<td>6,802</td>
<td>2,483</td>
<td>2,917</td>
<td>1,510</td>
</tr>
</tbody>
</table>

**Table 5-1.** Five element concentrations in *Mnium hornum* measured at intervals during field work at the A1M site.
Woodland Site

<table>
<thead>
<tr>
<th>Concentration (ppm)</th>
<th>Ca</th>
<th>K</th>
<th>P</th>
<th>Mg</th>
<th>Fe</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mni um Field Collected</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Woodland Site</td>
<td>4,623</td>
<td>9,830</td>
<td>2,188</td>
<td>1,471</td>
<td>333</td>
</tr>
<tr>
<td>N-saturated</td>
<td>2</td>
<td>31</td>
<td>40</td>
<td>26</td>
<td>21</td>
</tr>
<tr>
<td><strong>Mni um Moss Trays</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laboratory Cultured</td>
<td>3,252</td>
<td>8,956</td>
<td>2,466</td>
<td>3,129</td>
<td>231</td>
</tr>
<tr>
<td>N-deprived</td>
<td>64</td>
<td>2,158</td>
<td>86</td>
<td>2,158</td>
<td>91</td>
</tr>
<tr>
<td><strong>Mni um Moss Trays</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transplanted Woodland Post N-dep (n=1)</td>
<td>3,046</td>
<td>6,899</td>
<td>1,891</td>
<td>2,614</td>
<td>283</td>
</tr>
<tr>
<td>7</td>
<td>3,354</td>
<td>5,301</td>
<td>1,890</td>
<td>2,549</td>
<td>202</td>
</tr>
<tr>
<td>14</td>
<td>3,604</td>
<td>4,471</td>
<td>1,871</td>
<td>2,501</td>
<td>269</td>
</tr>
<tr>
<td>21</td>
<td>3,860</td>
<td>4,866</td>
<td>2,077</td>
<td>2,428</td>
<td>276</td>
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<tr>
<td>27</td>
<td>4,095</td>
<td>5,302</td>
<td>2,280</td>
<td>2,539</td>
<td>377</td>
</tr>
<tr>
<td>41</td>
<td>4,423</td>
<td>7,355</td>
<td>2,681</td>
<td>2,586</td>
<td>426</td>
</tr>
<tr>
<td>56</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5-2. Five element concentrations in *Mni um hornum* measured at intervals during field work at the woodland site.

K in *M. hornum* declined overall at the A1M and woodland after collection from the field. K at 8,956 ppm after 151 d N-dep declined further after placement back into the field and continued to decline until 21 – 27 d, at 4,471 – 4,961 ppm, when the plants began to increase in K back up to levels found in the original field material to 6,802 – 7,355 ppm. The initial decline in Ca, before the increase in accumulation in the tissue, could be due to the increased demand on the plant to take up N; utilising existing sources in uptake and assimilation before taking up external Ca. The variability between sites was unclear and further analysis of cation deposition in rainwater may provide some explanation for the differences. K decline was slower overall than for Ca but recovery was more rapid and the response similar at both sites. The decline and then increase in K at a different rate to Ca could indicate that the metabolic systems in operation are distinct from those of Ca, although they are both involved in increases in metabolism overall.
To determine the pathways cations were being used in would require further and extensive study. For example Ca is known to be involved in NR activation but the levels of change observed here are too large for this to be the only causative effect.

P and Mg did not show any noticeable changes following N-deprivation and placement into the field site. Fe at the A1M site was around five-fold higher than at the woodland site with values in the range of 1,023 – 2,196 ppm at the motorway site and 202 – 426 at the woodland site. Values following 151 d N-dep fell to 231 ppm and recovery at the motorway site took 7 days post-placement at the experimental field site. This is evidence of rapid heavy metal accumulation experienced by mosses at roadsides. Evidence of higher Pb and Ti accumulation was also observed at the motorway site than the woodland site (data not shown). Tissue concentrations of Pb have been shown to have a strong positive correlation with traffic exposure (Pearson et al., 2000).
5.2.3. Rainwater volume and weather conditions at *Mnium hornum* experimental field sites

Data on weather conditions collected at Rothamsted Research showed rainfall in March to be 43.2 mm with 17 days having 0.2 mm or more of rain, but only 2 having more than 5.0 mm. There were 99.3 hrs of sunshine. The maximum and minimum temperatures were 10.0 °C (+0.52) and 3.2 °C (+0.87) respectively, the highest maximum temperature was 17.9 °C on 16th and the lowest minimum was -4.5 °C on the night of 4th.

![Figure 5.4. Rainfall volume (mm) at the field site during March and April 2005.](image)

Rain was collected weekly from the four rain gauges on site during the field work. A1M site gauge Mo1 (dark blue bar), Mo2 (light blue bar) and woodland site gauge W1 (dark green bar) and W2 (light green bar).

In April rainfall was 65.6 mm, with 21 days with 0.2 mm or more of rain, but only three having more than 5.0 mm. There were 149.5 hrs of sunshine. The
maximum and minimum temperatures were 13.1 °C (+1.14) and 4.7 °C (+1.10). The warmest day was 19.8 °C on the 30th. The months of March and April were selected to try to carry out field trials that simulated the wet deposition conditions in the laboratory controlled environments and treatments used in the time course assays. From this information it can be seen that March and April had high rainfall, temperatures a little below those used in the CE cabinets with more variability and quite consistent hours of sunshine. FWt: DWt ratios remained consistent throughout the course of field experimentation (data not shown).

Rainfall data collected at the field site (Figure 5.4) was variable throughout March and April from week to week and site to site. Considerably less rainfall volume was collected at the Mardley Heath site compared to that collected at Rothamsted Research. This was due to the sites not having clear access to the sky due to the canopy cover in the forest. Rainfall volume collected at Mardley Heath does however provide an accurate measure of the amount of precipitation that fell on the moss tray and surrounding ground flora, along with the run-off from the tree canopy and dissolved nutrients, which are discussed later. In the moss placed in the experimental field site, NRA was induced at both sites after just 7 d and values remained high throughout the month along with high levels of wet deposition overall. Highest NRAs were observed on 07/03/2005 and 27/03/2005 at both sites. On these dates the lowest average rainfall and highest average rainfall were measured in all rain gauges respectively so there appears to be no correlation between NRA and rainfall volume. As rainfall volume showed no correlation with NRA and therefore N uptake, the rainfall volumes were used
to calculate the quantity of N supplied in the rainwater to provide a useful measure of the N inputs in the precipitation falling onto the moss canopy (see Section 5.2.6.).

5.2.4. pH of rainwater at *Mnium hornum* experimental field sites

The pH of rainfall at all field sites was not considered to be in the range to constitute the term ‘acid rain’ as the pH’s at the sites were above the pH 5.6 threshold (Ashenden, 2002). The only gauge that contained rainwater in the acidic range was on 27/03/2005 in the W1 gauge when pH was at 5.24.

![Rainfall pH at field site during March and April 2005](image)

**Figure 5.5.** Rainfall pH at the field site during March and April 2005. Rain was collected weekly from the four rain gauges on site. A1M site gauge Mo1 (dark blue bar), Mo2 (light blue bar) and woodland site gauge W1 (dark green bar) and W2 (light green bar).
However at the same woodland site the W2 gauge contained pH 6.20 with an average value for the two sites equalling pH 5.72, less acidic than acid rain. These results demonstrated that the rainwater precipitate over the two months at Mardley Heath was only slightly acidic and an average pH for all sites over the two month period was 6.34. If the pH had been in the acidic range there could have been complications in interpreting the data based on the induction of NRA upon acid treatment in *M. hornum*. So the effects on NRA can be largely attributed to changes in N content in the rainwater in this case due to the lack of acidic pH measured in rainfall.

5.2.5. Total N and δ-15N in rainwater at *M. hornum* experimental sites

Total N and δ-15N were both analysed in rainfall collected in the rain gauges at Mardley Heath to determine inputs at the sites and look for any effects these inputs may have on the field moss.

Total N in rainfall was found to range from 0.0127 - 0.0191 % and these values can be discounted as contributing sufficient N to the plants.

It was not surprising, considering the lack of a δ-15N signature in the moss tissue itself, that the δ-15N measurements in rainwater revealed no variations with values staying around the natural abundance of δ-15N = 0.3663 atom %. This data suggests that N inputs to the moss tissue in the field were primarily from wet deposition of ions in rainwater rather than from dry deposition effects.
5.2.6. Ion analysis and quantity of ion supplied in rainwater to *Mnium hornum* experimental field sites

![Graphs showing ion quantity supplied in rainwater](image)

Figure 5.6. Quantity of ion supplied in rainwater collected weekly from gauges at *M. hornum* tray experimental field sites: AIM site gauge a) Mo1, b) Mo2, and woodland site gauge c) W1, d) W2. Ions NO$_3^-$ (black bar) and NH$_4^+$ (hatched bar). Data points are an average of 2 replicates. SE bars too low to be seen.
Ion analysis of rainwater from the rain gauges was given in concentrations of ppm which were useful for the purposes of comparing varying concentrations between sites and over time and also to establish the contribution of the various ions. However for the purposes of comparability the values were converted to quantity of ion supplied in rainwater in order to establish the natural levels of N inputs to the *M. hornum*. Considering the variability in rainwater volume values were converted to mmol amounts supplied to the tray. These calculations gave a more accurate picture of the N inputs to the localised environment. It is important to note here that NO$_2^-$-N ion measurements were not shown as they were in trace amounts.

Inorganic-N applications in time courses on N-deprived *M. hornum* involved the application of a known concentration per litre of 0.50 mM KNO$_3$, 0.25 mM Ca(NO$_3$)$_2$ (of which there are two NO$_3^-$ ions per Ca$^{2+}$ so the amount of NO$_3^-$ applied is equivalent to 0.50 mM KNO$_3$) or 0.50 mM NH$_4$Cl to the surface area of the moss trays. To simply calculate the actual amount applied to the tray in mmol this concentration used in mM is multiplied by the volume used:

$$0.50 \text{ mM KNO}_3 \times 0.2 \text{ (l)} = 0.10 \text{ mmol}$$

So the amount of N supplied to the *M. hornum* tray was 0.10 mmol per treatment.

To convert ppm concentrations into mM concentrations a standard conversion factor was used:

$$1 \text{ ppm 'Ion'-N} = 71 \mu\text{M 'Ion'} = 0.071 \text{ mM 'Ion'}$$

The mM concentration of the rainwater in the gauge was then known for separate ions however the mmol amount supplied to the tray was dependant on the
volume of rainwater collected in the gauge, the catchment area of the gauge and the surface area of the tray. Subsequent calculations revealed that the catchment area of the gauge was 7,854 mm\(^2\) and the surface area of the tray 35,582 mm\(^2\) with the catchment area of the gauge fitting into the tray 4.53 times. Therefore the following calculation was applied:

\[ \text{mmol} = \text{mM} \times \left[ \left( \text{volume (ml)} \times 4.53 \right) / 1000 \right] \]

Artificial applications of N were applied to *M. hornum* in the laboratory during time courses at 0.10 mmol amounts per treatment. \(\text{NO}_3^-\) inputs reached 0.07 mmol at the W2 site on 27 d (see Figure 5.6. d) with a rainfall volume of 33 ml and a rainwater concentration of 0.48 mM which is comparative to mM concentrations applied in the NRA induction time courses (0.50 mM \(\text{KNO}_3\) for example). These values were amongst the highest concentrations of ion inputs over the course of the two months but demonstrated the large amounts of \(\text{NO}_3^-\) that were present in the rainwater, in this case at a woodland site. Rain gauges could have collected both dry and wet deposition over the course of the week and this may provide an explanation for some of the higher N inputs measured.

Using molarity as a measure of N pollution inputs of \(\text{NO}_3^-\) concentrations of 0.322 mM were found to have no detrimental effects to *Sphagnum magellanicum* growth and metabolism with \(\text{NH}_4^+\) concentrations having a threshold of 0.225 mM before there was a reduction in NRA and photosynthesis in a study by Rudolph and Voigt (1986). The only incidence of a high concentrations of \(\text{NO}_3^-\) at Mardley Heath was outlined in the previous example on 27 d at W2, however with a threshold of 0.225 mM \(\text{NH}_4^+\) the W2 site exceeded this from 14 d – 41 d (0.551 – 1.614 mM), although its sister site W1 only exceeded it once on 36 d
(0.298 mM). The Mo1 site (0.688 mM) and Mo2 site (0.7964 mM) also exceeded the NH$_4^+$ threshold at 50 d. If NRA and tissue NO$_3^-$ concentration measurements had been continued throughout April NRA activity may have been shown to have declined according to this study due to detrimental effects of prolonged high N inputs as demonstrated by Rudolph and Voigt (1986). However the effects of combined NO$_3^-$ and NH$_4^+$ application in *M. hornum* remain to be understood as NH$_4^+$ has been shown to induce NRA in the absence of additional NO$_3^-$ (see Chapters 3 and 4).

The maximum NRA and tissue NO$_3^-$ concentrations were reached within 7 d for NRA and 21 d for NO$_3^-$ and values were within those established from prior experimentation on existing N-saturated field tissue.

Importantly there are good parallels in trends between NRA and tissue NO$_3^-$ concentrations of experimental field site *M. hornum* and laboratory time course trial *M. hornum* (see Figures 5.1 – 5.2). NRA values in field site *M. hornum* after 7 d reached 8 μmol NO$_3^-$ h$^{-1}$ g$^{-1}$ FWt and in time course trial *M. hornum* NRA after ‘initial induction’ of 15 minutes values were between 4 – 10 NO$_3^-$ h$^{-1}$ g$^{-1}$ FWt, depending on the source of inorganic-N applied so activities were comparative. Similarly for tissue NO$_3^-$ concentrations for experimental field site *M. hornum* after 7 d NO$_3^-$ was around 15 – 20 μmol NO$_3^-$ g$^{-1}$ FWt and inorganic-NO$_3^-$ treated plants after ‘initial induction’ of 15 minutes were between 10 – 20 μmol NO$_3^-$ g$^{-1}$ FWt. Comparing N inputs in the field and in the laboratory after 7 d ion amounts were approximately 0.01 mmol in the field (as a combined supply of NO$_3^-$ and NH$_4^+$ at both sites) whereas inorganic-N treatments in the laboratory
were 10 fold higher at 0.10 mmol. This implied that 0.01 mmol N inputs were enough to induce a rapid response in N uptake in the moss and *M. hornum* is very sensitive to changes in atmospheric N deposition. When drawing these comparisons it should be noted that NRA induction and tissue NO$_3^-$ increases were shown to be largely unaffected by N-deprivation status up to 151 d as shown in Section 4.2.5.

Foliar N concentration increased after 21 d with levels reaching 1.99 % at the A1M and 2.30 % at the woodland sites over the course of April. Although there was some variation after 27 d the ion analysis showed high concentrations of combined NO$_3^-$ and NH$_4^+$ at 27 d followed by predominantly NH$_4^+$ inputs at both sites in April with a decline in NO$_3^-$ concentration in the rainwater (see above for mM values and rational on NH$_4^+$ mM threshold values). The woodland site showed the largest increases in foliar N and this could be due to the higher concentrations of NH$_4^+$ at the woodland site. The foliar N values were just below those set for critical loads of N with levels actually exceeding critical loads in the woodland. The increases in NH$_4^+$ could have been from wash off from trees in the form of urea degradation from bird waste but further analysis with cations, specifically Na, did not show changes in tissue Na concentration and increases in NH$_4^+$ therefore suggesting this did not come from such a localised source.

In March, NO$_3^-$ and NH$_4^+$ were seen in quite equal combined ion quantities with no apparent inhibition of NRA or NO$_3^-$ accumulation overall. Throughout experimentation on N-deprived *M. hornum* it has been observed that the plants
can take up N in the form of NH$_4^+$ and induce NRA in the absence of additional NO$_3^-$. Treatments using combined NH$_4$NO$_3$ compounds were not applied in a controlled environment to determine the effects. This field trial has demonstrated no detrimental effects to the metabolism of the moss when both ions are in solution. On 14 d and 21 d there were some differences in NRA with a slight reduction in overall activity at both sites and higher NRA at the A1M site on 14 d and higher NRA at the woodland site on 21 d but looking at the mmol amounts at both sites during this period there were no apparent differences in ion composition or amount between sites. It could be suggested that NH$_4^+$ had an inhibitory effect on the metabolism of NRA at either site and explain some of the shifts in activity but further experimentation would be required to say this with any confidence.

5.2.7. Cation and heavy metal analysis of rainwater at *Mnium hornum* experimental field sites

At both the A1M site and woodland site the individual cation concentrations were variable throughout the two months and showed no correlation with rainfall volume, pH of rain or ion concentrations of rainwater (Table 5.3 – 5.4). The cations collected in wet deposition were also within the same individual concentration ranges regardless of site location.
Table 5-3. Five element concentrations in rainwater measured at intervals during field work at the A1M experimental field site.

Ca and K were found in the highest concentrations in the rainwater. Ca and K tissue cation concentrations did show some variability over the course of the field trial however this was more likely to be due to the metabolism of the plants when placed back into the field rather than the availability of cations collected in rainwater. This was due to the variability in the data, with no observable differences between the tissue cation concentration and the rainwater cation concentration. Tissue Fe at the A1M site was shown to be around five-fold higher than at the woodland site however the heavy metals were not effectively captured in the rainwater gauges. As heavy metal deposition is usually
associated with dry deposition. Na was present in low concentrations and there was no correlation between Na and increases in NH$_4^+$ therefore increases in NH$_4^+$ were more likely to be due to widespread changes in atmospheric N rather than local inputs such as urea degradation from bird waste.

<table>
<thead>
<tr>
<th>Woodland Site</th>
<th>Concentration (ppm)</th>
<th>Ca</th>
<th>K</th>
<th>P</th>
<th>Mg</th>
<th>Fe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Post N-dep (d) 7</td>
<td>2.1</td>
<td>3.1</td>
<td>0.7</td>
<td>0.6</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Post N-dep (d) 14 (n=2)</td>
<td>mean</td>
<td>3.2</td>
<td>7.1</td>
<td>0.6</td>
<td>0.9</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>1.8</td>
<td>4.0</td>
<td>0.5</td>
<td>0.5</td>
<td>0.1</td>
</tr>
<tr>
<td>Post N-dep (d) 21 (n=2)</td>
<td>mean</td>
<td>2.2</td>
<td>14.2</td>
<td>1.1</td>
<td>0.8</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>1.0</td>
<td>5.6</td>
<td>0.9</td>
<td>0.5</td>
<td>0.0</td>
</tr>
<tr>
<td>Post N-dep (d) 27 (n=2)</td>
<td>mean</td>
<td>3.3</td>
<td>11.2</td>
<td>0.7</td>
<td>1.1</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>1.4</td>
<td>4.0</td>
<td>0.5</td>
<td>0.6</td>
<td>0.0</td>
</tr>
<tr>
<td>Post N-dep (d) 36</td>
<td>mean</td>
<td>5.3</td>
<td>36.4</td>
<td>4.8</td>
<td>1.5</td>
<td>0.4</td>
</tr>
<tr>
<td>Post N-dep (d) 41</td>
<td>mean</td>
<td>4.0</td>
<td>59.1</td>
<td>7.9</td>
<td>3.5</td>
<td>0.3</td>
</tr>
<tr>
<td>Post N-dep (d) 50</td>
<td>mean</td>
<td>7.4</td>
<td>135.6</td>
<td>13.7</td>
<td>7.2</td>
<td>0.3</td>
</tr>
<tr>
<td>Post N-dep (d) 56 (n=2)</td>
<td>mean</td>
<td>6.1</td>
<td>55.7</td>
<td>5.5</td>
<td>3.1</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>SE</td>
<td>2.4</td>
<td>20.6</td>
<td>3.5</td>
<td>1.3</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Table 5-4. Five element concentrations in rainwater measured at intervals during field work at the Woodland experimental field site.

5.3. Initial Conclusions and Next Steps

Overall, after one month of fieldwork, *M. hornum* NRA and tissue NO$_3^-$ concentrations recovered to levels found in the field at Mardley Heath. Time taken to recover for NRA was 7 d and for tissue NO$_3^-$ concentration was 21 d.
Inorganic-N inputs, measured in the rainwater, correlated well with the increase in NRA and tissue NO$_3^-$ concentration measurements. It can be concluded that *M. hornum* could be used as a sensitive bioindicator of N inputs in the environment which would prove useful in monitoring N pollution.
CHAPTER 6

MEASURING N STATUS IN MOSSES USING MICROELECTRODES

6.1. Introduction

Microelectrodes were used to measure transport of N across cell membranes in plants. Single barrelled microelectrodes measure voltage and when inserted into cells measure the membrane potential, in mV, between the inside and outside of the cell. In addition ion-selective microelectrodes can also be used to measure the ion gradients across membranes (see Chapter 2). Published information on the transport mechanisms involved in N uptake in mosses is somewhat limited. Work on a related moss species Mnium cuspidatum, into the effect of cyanide on the membrane potential of cells, distinguished two distinct compartments which were suggested to be the cytoplasm and the vacuole (Fischer et al., 1976). Fischer noted advantages of using moss leaves in electrophysiology, for example the one cell layer thickness as compared to more complex higher plant structure which enables the recording of rapid responses from the cells when treated with inhibitors in a Perspex chamber.
6.1.1. N and ion uptake mechanisms in bryophytes

Various transport processes and specific mechanisms of cellular ion uptake in bryophytes have been studied by Raven et al., 1998 (see Figure 6.1.). The electrically negative cell interior is in part due to active efflux of H⁺, probably catalysed by a ‘P’ type ATPase which expends one mole of ATP per mole of H⁺ pumped out (Bates, 2000) with this pump probably regulating cytoplasmic pH in bryophytes (see Figure 6.2). The proton pump activity also provides the driving force for a number of specific symporter proteins allowing passage through the plasma membrane and against a concentration gradient. For K⁺ and NH₄⁺ the negative membrane potential can provide energy for uptake but for anions cotransport with protons for the active entry of NO₃⁻, SO₄²⁻ and H₂PO₄⁻ and for efflux of Ca²⁺ is the likely mechanism although none of these have yet been unequivocally demonstrated in bryophytes (Bates, 2000).
Figure 6.1. Transport processes at the plasma membrane and tonoplast of an embryophyte cell, with those characterised for bryophytes indicated by an asterisk (reproduced from Raven et al., 1998 with kind permission of the British Bryological Society).
6.1.2. Microelectrode use in establishing theory on N uptake transport mechanisms with implications for a pH stat

In higher plants active transport for NO\textsubscript{3}\textsuperscript{-} uptake is required at the plasma membrane and the tonoplast of epidermal cells and in both maize and barley roots (Miller and Smith, 1996). It was demonstrated that to maintain the measured intracellular concentrations of NO\textsubscript{3}\textsuperscript{-} active transport is necessary and this can use the proton electrochemical gradient across both the tonoplast and the plasma membrane to provide the energy for this transport (Miller and Smith, 1996). Active NO\textsubscript{3}\textsuperscript{-} transport at the plasma membrane is thought to occur by symport with protons (see Figure 6.2.) and measurements in maize and barley of the NO\textsubscript{3}\textsuperscript{-} which elicited changes in electrical potential difference across the plasma membrane support this model (McClure et al., 1990; Glass et al., 1992). The usual effect of NO\textsubscript{3}\textsuperscript{-} on membrane potential is to give a transient depolarisation (less negative potential) followed by a relatively slower hyperpolarisation (more negative potential) and an overall alkalisation of the cytosol, in accordance with the classic pH-stat mechanisms (Davies, 1986 and Raven, 1986). An initial transient depolarisation of the membrane potential is thought to be caused by a H\textsuperscript{+}:NO\textsubscript{3}\textsuperscript{-} stoichiometry greater than one, as 1:1 would be electrically neutral and would not cause depolarisation of membrane potential, with the subsequent net hyperpolarisation that usually follows this occurring due to stimulated proton pumping by the plasma membrane H\textsuperscript{+}-ATPase (McClure et al., 1990).
Figure 6.2. The role of proton transport in inorganic N transport with transport for NO$_3^-$ and NH$_4^+$ across the plasma membrane charge-balanced by proton pumping membrane ATPase.

Studies of the energetics of NO$_3^-$ transport suggest that a 2:1 stoichiometry is required for the cotransport (Miller and Smith 1996). Studies in higher plant systems suggested that changes in NO$_3^-$ concentration and electrical changes were related to the activity of the NO$_3^-$ transport system and it was assumed that the initial depolarisation involved the actual H$^+$/NO$_3^-$ cotransport step and the later hyperpolarisation resulted from stimulation of the plasma membrane proton pump due to the cytosolic acidification associated with the activity of the NO$_3^-$
transporter (Miller et al., 2001). Once the plasma membrane proton pump was fully operational an overall alkalisation of the cytosol would occur with a homeostatic restoration of cytosolic pH to the pH of the cell before the NO₃⁻ treatment was applied. Overall, upon the influx of NO₃⁻ there is a depolarisation of the membrane potential, which corresponds to the NO₃⁻ transporter and results in the short term acidification of the cytosol, which in turn stimulates the plasma proton pump which is usually evident as a hyperpolarisation of the membrane potential, with a corresponding overall alkalisation of the cytosol due to an H⁺ efflux. Additionally Britto and Kronzucker (2005) highlighted that when NO₃⁻ is cotransported overall with one H⁺ this produces the chemical equivalent of nitric acid, a strong acid, in the cytosol which would support an initial acidification during active NO₃⁻ transportation. This phenomenon would be only transient as subsequent assimilation would rapidly alter the sizes of the acid and base pools and there would be an overall alkalisation of the cytosol before returning to the original compartmental pH.

In the roots of intact barley and tomato plants the addition of NH₄⁺ to the medium bathing the roots caused an almost immediate depolarisation of the membrane potential which was greater at higher concentrations of NH₄⁺ (Ayling, 1993). Addition of NH₄Cl also induced a rapid depolarisation of membrane potential of rice epidermal cortical cells (Wang et al., 1994). At equivalent concentrations to those applied in our research NH₄⁺ influx appeared to be an active process in roots of rice plants and a possible mechanism for this active uptake via a high affinity transport system (HATS) was an H⁺:NH₄⁺ symport (see Figure 6.2) with evidence for a proton motive force (Wang et al., 1994). This
would result in an overall acidification of the cytosol and electrophysiological measurements in this chapter look into this hypothesised transportation of NH$_4^+$. Britto and Kronzucker (2005) highlighted that when NH$_4^+$ is countertransported with one H$^+$ this is the chemical equivalent of NH$_3$, a weak base, in the cytosol following the transport and subsequent membrane repolarisation events across the plasma membrane which would support an initial alkalisation during active NH$_4^+$ transportation. This phenomenon would also only be transient as subsequent assimilation would rapidly alter the sizes of the acid and base pools and there would be an overall acidification of the cytosol before returning to the original compartmental pH.

It is known that higher plants have evolved regulated energy dependant systems for the uptake of NO$_3^-$ using both high and low affinity transporters but less is known about the transporters involved in NH$_4^+$ uptake. Genes that encode representatives of each class of transport system for NO$_3^-$ have been identified in higher plant systems and fall into two families: NRT1 and NRT2 (Williams and Miller, 2001). Members of these families are induced in response to NO$_3^-$ in the environment and are regulated by internal signals including N metabolites and shoot demand from N. The evidence to date indicates that the NRT2 transporter contributes specifically to the NO$_3^-$ inducible high affinity NO$_3^-$ uptake system (HATS) while the NRT1 transporter contributes more broadly to N uptake and shows both inducible and constitutive expression (LATS) (Crawford and Glass, 1998). Much less is known about the specific details of transporters in bryophytes and still less about any physiological differences that may exist between species with vacuoles and those lacking them.
6.1.3. Cation use and pH buffering capacity during N uptake

It has been suggested that depolarisation events and cationic exchanges could be used in short-term regulation and homeostasis of cell pH within bryophytes and higher plants (Soares and Pearson, 1995). Concentration changes of nutrient ions in higher plants, to compensate for proton fluxes, have been shown to be readily tolerable by the plant, given that baseline proton concentrations are many orders of magnitude smaller than the concentration of most nutrient ions (Britto and Kronzucker, 2005). An alternative model has been proposed where the uptake of \( \text{NO}_3^- \) and \( \text{NH}_4^+ \) is neutral and the antiport or symport of protons with ions such as \( K^+ \), \( Na^+ \) or \( Cl^- \) across the plasma membrane could play a role in pH changes in the cytosol (Britto and Kronzucker, 2005) providing an explanation for the induction of NRA by some of the inorganic acids, organic acids and neutral salts. It can be hypothesised that the availability of such cations could provide a means to balance \( H^+ \) ion movements to stabilise the pH gradient across plant cell membranes. It can be suggested that the ability of both species to stabilise the transmembrane pH gradients with available cations would not be compromised in either species with a surplus of available nutrients. However with depleted cation concentrations then the ability of the mosses to balance protons could become compromised. With this considered \( M. hornum \) was the most suitable moss species to use to investigate these effects due to its ability to sustain itself whilst undergoing long term N-deprivation (Chapter 4) and also in light of its ability to maintain its cation concentration during this process. Electrophysiological measurements were therefore carried out in \( M. hornum \) on membrane polarity, transporter activity and pH regulation.
6.1.4. Moss physiological structure and NO$_3^-$ storage capacity

Little is known about the physiological differences that may exist between moss species especially with regards to vacuoles as potential storage compartments for mineral nutrition. Observations using microscopy and staining techniques will provide more information on the structure and storage compartmental volumes of *S. fimbriatum* and *M. hornum*. In higher plants the vacuole is important for long term N storage. NO$_3^-$ is usually transported from the roots to the leaves but it is stored in the vacuoles of both roots and leaves, where it participates as a general osmoticant or serves as a reservoir to sustain the growth process when the external N supply becomes limiting (McIntyre 1997; der Leij et al., 1998). The role of the vacuole in moss N storage is also as important aspect of this research.

6.1.5. Aim

Investigations into the uptake of N has developed theories on transporter activity and plant N storage capacity in higher plants which has been achieved, in part, by using single barrelled microelectrodes, intracellular NO$_3^-$-selective microelectrodes and pH-selective microelectrodes (Ullrich and Novacky, 1990; McClure et al., 1990; Glass et al., 1992; Wang et al., 1994; Miller and Smith, 1996; Miller et al., 2001). Electrophysiological measurements in mosses, used alongside observations of cellular structure in both species of moss using microscopy, will look at the similarities and differences between these two species and higher plants to understand and compare their N uptake and storage capacities.
From Chapters 3 and 4, a hypothesis was developed and this is tested in this chapter, namely that an acidification of cytosol could occur upon addition of NH$_4$Cl potentially providing a reason for the stimulus of NRA induction in mosses. Measuring the intracellular pH changes upon application of inorganic-N and other compounds may provide information on the N uptake and assimilatory mechanisms in mosses and this will be examined further throughout this chapter.

It has been hypothesised in this thesis that considerable structural differences between _S. fimbriatum_ and _M. hornum_ may provide an explanation for the differences in NO$_3^-$ storage capacity in both mosses with _M. hornum_ having similar structure to that observed in higher plants; perhaps including a central storage vacuole. There was also the possibility that the two mosses may have different mechanisms of N uptake demonstrated by the inability of _S. fimbriatum_ to store NO$_3^-$ both in the short and long term (see Chapters 3 and 4). Using NO$_3^-$- selective microelectrodes to measure NO$_3^-$ pools and looking at microscopy images may help to prove or disprove this hypothesis. Further theories on the homoeostasis of cell pH within bryophytes and higher plants (Soares and Pearson, 1995) have suggested that cations could be used in short-term regulation of intracellular pH. The hypothesis that charged particles present in treatment solutions may cause immediate changes in the exchangeable cation pool, which in turn may influence cell physiology with short term changes in both cations and organic acids possibly providing a mechanism for buffering acidity, was tested using electrophysiology which enabled measurements to be recorded specifically and rapidly in real time.
6.2. Results and Discussion

Ion-selective microelectrode data can give information on both intracellular activities and electrical gradients across the plasma membrane and tonoplast and this information on the intracellular electrochemical gradients can be used to determine the likely mechanisms of transport across cell membranes (Miller and Wells, 2005). pH and NO₃⁻-selective microelectrode measurements have been used to determine the thermodynamics of NO₃⁻ transport systems across the plasma membrane (Miller and Smith 1996) and tonoplast (Miller and Smith 1992). Also measuring transporter activity, using single barrelled microelectrode recordings of cell membrane potential can be used to establish the similarities and differences between the two moss species.

6.2.1. Single barrelled microelectrode measurements in mosses

Single barrelled microelectrode measurements in *M. hornum* were relatively easy to obtain and the membrane potential differences shown here are representative of a typical recording in a *M. hornum* cell.
Figure 6.3. Single barrelled microelectrode measurement in *M. hornum* at N-dep 0. Prior to penetrating the cell the external solution reads 0 mV. The final resting potential difference when the microelectrode tip enters the cell is around -80 mV and is achieved after about 3 minutes. When the electrode tip was withdrawn from the cell the measured voltage returns to 0 mV in solution.

Figure 6.3 shows a recording in a compartment of a *M. hornum* moss cell. It was difficult to establish in which compartment the microelectrode tip was located therefore several measurements were taken and data examined to determine if different clusters of mV potential values could be found. If these data cluster into different groups this will help to identify if the tip was located in the cytosol, the vacuole or even in the cell wall (see Figure 6.8 and 6.10). The membrane potential recorded from the cytoplasm is more negative when compared with that recorded in the vacuole (Miller *et al.*, 2001). Typically recordings in the cytosol of an embryophytic cell range from -100 mV to -200 mV and in the vacuole from embryophytic cell -85 mV to -185 mV (Raven *et al.*, 1998). Recordings in the two moss species ranged from -48 mV to -80 mV in *M. hornum* and -44 mV to -136 mV in *S. fimbriatum*. The less negative values around -40 mV were
assumed to be in the space outside the cell presumed to be the apoplast and/or cell wall.

6.2.2. Single barrelled microelectrode measurements in *M. hornum* – response to treatment with inorganic N

*M. hornum* plants were treated with different inorganic-N sources to investigate the membrane voltage changes associated with the uptake of N in mosses.

![Graph showing membrane voltage depolarisation](image)

**Figure 6.4.** Membrane voltage depolarisation during a series of 0.25 mM Ca(NO$_3$)$_2$ treatments recorded from a *M. hornum* leaf cell (N-dep 41 d) (Stanley *et al.*, 2005). V1 = maximal amplitude of depolarisation (mV) and T1 = duration of the response (s).

When NO$_3^-$ was supplied outside a moss cell the membrane potential changes were an indicator of the activity of the symporter responsible for NO$_3^-$ uptake. In
*M. hornum* treatment with NO$_3^-$ gave a change in the membrane potential showing that the anion was readily transported (Figure 6.4). The maximal amplitude of the NO$_3^-$-elicited depolarisation (V1 mV) and duration of the response (T1 s) depended on the type of treatment applied and the N-status (N-dep) of a plant (Stanley et al., 2005). It should be noted that the value of T1 depends on the fact that the duration of the NO$_3^-$ treatment is always the same.

### 6.2.3. Single barrelled microelectrode responses in *M. hornum*: V1 and T1 analysis

![Depolarisation amplitude (V1) and N-deprivation (N-dep) graph](image)

**Figure 6.5.** V1 for N-dep *M. hornum* - V1 following 0.5 mM KNO$_3$ (black circles and triangles), 0.25 mM Ca(NO$_3$)$_2$ (grey circles and triangles) and 0.5 mM NH$_4$Cl (black circles and triangles) treatments plotted against N-dep time.
*M. hornum* was perfused with known amounts of inorganic-N when a single barrelled microelectrode tip was recording from a compartment within the moss cell. Responses showed a depolarisation and recovery to the original resting membrane potential of the cell following treatment with inorganic-N and the V1 and T1 values were plotted according to the N-dep status of the *M. hornum* tissue.

![Graph showing V1 and T1 values](image)

**Figure 6.6.** T1 for N-dep *M. hornum* - T1 following 0.5 mM KNO₃ (black circles and triangles), 0.25 mM Ca(NO₃)₂ (grey circles and triangles) and 0.5 mM NH₄Cl (black circles and triangles) treatments plotted against N-dep time.

The V1 values were more variable when plants were less N-deprived but overall the amplitude of the responses was similar with inorganic-N treatments. T1 again showed more variability at less N-dep status and the values were similar regardless of the type of inorganic-N supplied. There does appear to be a
tendency for Ca(NO₃)₂ to have larger T₁ values than KNO₃ however and this could be due to the different effects the cations have on electrophysiological measurements in the cells. At first KNO₃ and Ca(NO₃)₂ were used as inorganic-N treatments as they had been used in the mass tissue trials, however the K⁺ cation has been shown to interfere with the overall mV change and cannot be used quantitatively for the measurement of NO₃⁻ in the cytosol. Resting membrane potential is very responsive to changes in K⁺ (Hirsch et al., 1998) with membrane potential measurements suggesting that an potassium channel (the AKT1 channel) mediates K⁺ uptake from solutions that contain as little as 10 μM K⁺. So with KNO₃ treatments the measurements are complicated by the additive effects of the sensitive response of the potassium channel to K⁺, illiciting an electrical response, combined with the nitrate channel response to NO₃⁻. This is less of a problem when Ca(NO₃)₂ is used because the membrane potential does not respond to changes in Ca²⁺ outside. Therefore to prevent any complications with interpretation of electrophysiological recordings Ca(NO₃)₂ was used for further NO₃⁻ supply measurements. For the purposes of consistency it should be noted that further electrophysiological measurements in *M. hornum* and *S. fimbriatum* used plants at a more N-replete stage up to approximately 20 days N-dep.

6.2.4. Tissue Compartments in *M. hornum*

Two techniques were used in order to establish in which cellular compartment the microelectrode tip was located. Figure 6.7 shows an example of a typical membrane potential trace in *M. hornum* following sequential penetration through
tissue in the same recording. This trace gave values that were typical of the cytosolic (-80 mV to -100 mV), vacuolar (-60 mV to -80 mV) and cell wall ranges (-40 mV or less) from membrane potential values. To confirm and establish with more confidence this compartmental distribution of electrical potentials in *M. hornum* double barrelled pH microelectrodes were used.

**Figure 6.7.** An example of a typical membrane potential trace for a single electrode tip penetrating several different compartments in *M. hornum* leaf tissue. Hypothesised compartmental distributions according to the membrane potential values recorded: a= cytosolic range, b= vacuolar range, c= cell wall range.

Figure 6.8 shows scatter plots of the pH versus membrane potential of measurements made in *M. hornum* leaves. These results represented measurements in the vacuole, cytoplasm or cell wall of a leaf cell. Figure 6.8 shows that the data points have resolved into three clusters. The most negative membrane potential values and more neutral pH clusters were ascribed to the cytosol (a) with the negative membrane potential and more acidic pH cluster the vacuole (b). The least negative membrane potentials with acidic pH were identified as penetrations in the cell wall (c). Figure 6.7 gave a good indication
of the compartments the microelectrodes were located in; however the use of pH microelectrodes provided a more rigorous method for determining the location of the tip for the purposes of determining the thermodynamics of NO$_3^-$ transport systems across plant cell membranes.

![Figure 6.8. Scatter plots of membrane potential values in *M. hornum* and corresponding pH values. Hypothesised compartments according to pH values: a= cytosol, b= vacuole, c= cell wall.](image)

6.2.5. Tissue Compartments in *S. fimbriatum*

Figure 6.9 shows an example of a membrane potential trace in *S. fimbriatum* following sequential compartmental penetration in the same recording. Unfortunately electrophysiology proved to be more difficult in *S. fimbriatum* leaves and therefore this trace does not show all of the compartments that were observed during the recordings. A compartment was penetrated in the -140 mV
range (a) and this recording can be seen in Figure 6.13. Values that were typical of the cytosolic (-140 mV), vacuolar (-70 mV - -80 mV) and cell wall ranges (-40 mV) from membrane potential values were recorded. This range of values is less negative than those values typically observed in higher plants but the differences in compartments imply that they are in the correct ranges. To establish with more confidence the compartments in *S. fimbriatum* double barrelled pH microelectrodes were used.

![Graph](image)

**Figure 6.9.** An example of a membrane potential trace penetrating several different compartments in *S. fimbriatum*. Hypothesised compartments according to membrane potential values: a= cytosol (see Figure 6.13 for supporting graph to illustrate this hypothesised compartment), b= vacuole, c= cell wall.

Attributing compartments using membrane potentials alone was a useful exercise. However the combined use of membrane potentials and pH recordings provided some very useful data to establish the compartments that the microelectrodes were more commonly penetrating.
Figure 6.10. Scatter plots of membrane potential values in *S. fimbriatum* and corresponding pH values. Hypothesised compartments according to pH values:

- **a**: potential vacuole 1 (acidic compartment),
- **b**: potential vacuole 2 (acidic compartment),
- **c**: cell wall.

Figure 6.10 shows scatter plots of the membrane potential versus pH of measurements made in *S. fimbriatum* leaves. These results represent measurements in an acidic compartment or cell wall of a leaf cell. Figure 6.10 shows the 11 data points have resolved into three clusters. The more negative membrane potential (-140 mV) and acidic pH (2.5) value could be ascribed to a potential vacuole 1 (a) according to work carried out in higher plants with the less negative membrane potential (-60 mV), and acidic pH (3.0) cluster also a potential vacuole 2 (b). The least negative membrane potential (-40 mV) with less acidic pH (4.0) were identified as penetrations in the cell wall (c) and this compartment could be identified with more confidence. Obtaining recordings with *S. fimbriatum* did prove to be more difficult than in *M. hornum* and the
smaller numbers of values represented in Figure 6.10 are evidence of the
difficulties encountered when trying to record cytosolic and vacuolar pH using *S.
fimbriatum* moss leaves intact. Using Figure 6.9 it could be hypothesized that
the single value (a) was in the cytosolic range for membrane potential but Figure
6.10 shows that the compartment is acidic and therefore not cytosolic and more
likely to be a potential vacuole. Due to the differences in structure between *S.
fimbriatum* and higher plants it can be hypothesized that many of the recordings
obtained using electrophysiology are actually located in the hyaline cells as they
dominate the structure of the moss leaves. Therefore the potential vacuoles are
more likely to be acidic storage compartments and will subsequently be referred
to as such from this point in the chapter. It has been suggested that the hyaline
cells are dead at maturity with the potential to act as storage compartments for
such compounds as NO$_3^-$ (Clymo, 1963) as they are known to be involved in
water absorption and storage of other compounds. *S. fimbriatum* leaves are only
one cell thick, without a midrib with a leaf structure unlike that of any other plant
(Mozingo *et al.*, 1969). The large colourless water-filled cells, or hyaline cells,
have spiral wall thickenings and alternate with smaller living green cells.
Targeting these smaller living green cells proved to be difficult as using higher
magnifications and shorter working distances with larger lenses to see the tip
location caused major obstructions for impaling cells. This is one of the
limitations of using this set up but the core advantage is minimal disruption to the
plant when undergoing penetration. Tissue remains intact and in a largely
natural state. Alternatives such as patch clamping, used alongside the technique
used here, may have provided further evidence as to the location of the tip and
responses of the moss, however due to time limitations and perhaps the potential
for some of the tissue preparation methods to alter the chemistry and structural integrity of the plant causing the plants to be in more altered and in a less natural state, this was not carried out and is an area of further work. Further work was carried out in *M. hornum* and *S. fimbriatum* using pH electrodes and inorganic-N treatments to establish the responses of the cells and determine cellular compartmentation location. Microscopy was also used to investigate the structure of *M. hornum* and *S. fimbriatum* to try to provide an explanation for the electrophysiological and biochemical responses in both mosses whilst sensing N status.

6.2.6. pH penetration in *M. hornum* – cytosol and vacuole

A typical example of a membrane potential voltage at -80 mV with corresponding pH of 6.0 in *M. hornum* leaf cell is shown in Figure 6.11 and this recording suggests that the tip is located in the cytosolic compartment. The membrane potential value and the pH suggest that the tip is most likely located in the cytosol (Miller *et al.*, 2001).
Figure 6.11. A typical example of a recording from a pH-selective microelectrode impaling the cytosol of a leaf cell in *M. hornum*. N-free rain solution perfused throughout.

Figure 6.12 shows an example of a typical recording in the vacuole of a *M. hornum* cell. The membrane potential voltage was less negative than in some recordings however the compartment was so acidic (pH 3.0) that the tip was determined to be in a vacuolar compartment. It should be noted that in the following figures the time of treatment application was determined as the point of switching between treatment type and N-free rain solution and due to fluid dynamics may have a slight delay in delivery to the chamber and subsequently the plant and its membrane potential response. Another notable feature may be the presence of electrical spiking, caused by surges in the circuit, which are an artefact on the trace evident as a black vertical line that returns to the original
membrane potential rapidly. Spiking can take longer to return to the original values that were observed prior to the surge with the use of ion or pH-selective microelectrodes due to the increased resistance present with a selective 'plug' at the end of the microelectrode.

Figure 6.12. A typical example of a recording from a pH-selective microelectrode in the vacuole of a leaf cell in *M. hornum*. 0.25 mM CaCl₂ applied – see treatment bar.

6.2.7. pH penetration in *S. fimbriatum* – acidic compartment

Due to the difficulties in identifying the location of the tip in the *S. fimbriatum* leaf cell all penetrations were referred to as being located in acidic
compartments. Figure 6.13 gave an example of a typical recording in one of these acidic compartments in *S. fimbriatum*. This very negative membrane potential voltage (-140 mV) related to compartment (a) as outlined in Section 6.2.5, with a corresponding acidic compartment (pH 2.0) which was responsive to Ca(NO$_3$)$_2$. The effect of treatments is discussed in detail in Sections 6.2.8 – 6.2.10.

**Figure 6.13.** A typical example of a recording from a pH-selective microelectrode in an acidic compartment of a leaf cell in *S. fimbriatum*. 0.25 mM Ca(NO$_3$)$_2$ applied – see treatment bar.
6.2.8. Responses to treatments in *M. hornum* -cytosol and vacuole

The aim of the HCl treatment was to reproduce the acid misting conditions described in Chapter 4. Figure 6.14. clearly shows a rapid depolarisation and return to resting membrane potential in *M. hornum* with a corresponding acidification and the inability to return to the original cytosolic pH when treated with pH 3 HCl.

![Figure 6.14. A typical example of a recording from a pH-selective microelectrode in the cytosol of a leaf cell in *M. hornum*. pH 3 HCl applied – see treatment bar.](image)

Figure 6.14. A typical example of a recording from a pH-selective microelectrode in the cytosol of a leaf cell in *M. hornum*. pH 3 HCl applied – see treatment bar.
Figure 6.15. A typical example of a recording from a pH-selective microelectrode in a cytosol of a *M. hornum* leaf cell. 0.5 mM NH₄Cl and 0.25 mM Ca(NO₃)₂ applied – see treatment bar.

Figure 6.15. shows a response of a cytosolic compartment in a *M. hornum* cell to treatment with NH₄Cl and Ca(NO₃)₂. There was an overall depolarisation and return to the resting membrane potential on addition of NH₄Cl and a corresponding overall acidification of the cytosol before returning to the original cytosolic pH. Importantly, when *M. hornum* was treated with NH₄Cl there was also an induction of NRA, which was unexpected, and a hypothesis was posed that this could be due to the acidification of the cytosol and decrease in pH. These recordings are evidence to support such a theory and the induction of NRA with NH₄Cl addition in *M. hornum* could be attributed to the entry of NH₄⁺ in the cytosol and corresponding acidification in the cytosol. Similarly, on addition of
Ca(NO$_3$)$_2$ there was a depolarisation before returning to the original resting membrane potential but in contrast there was an overall alkalisation of the cytosol before the pH was restored to the previous value. This response is typical of those observed in _Limnobium_ root hairs (Ullrich and Novacky, 1990) and supports the 2:1 H$^+$/NO$_3^-$ co-transport model of NO$_3^-$ entry across the plasma membrane into the cytosol. In _Limnobium_ root hairs the effect of NO$_3^-$ on the membrane potential gave only a transient depolarisation, and no hyperpolarisation, but there was a corresponding alkalisation of the cytosol (Ullrich and Novacky, 1990). In this situation it is thought that the 2:1 H$^+$/NO$_3^-$ cotransporter mechanism is operating but there is no evidence of a rapid early pre-acidification of the cytosol upon NO$_3^-$ application or a corresponding pre-hyperpolarisation of the membrane potential. It is therefore assumed that this effect is caused by rapid NO$_3^-$ reduction rates with an overall alkalisation of the cytosol which ties in with the homeostasis of cytosolic pH. An interesting observation here is that prior to the overall acidification on addition of NH$_4$Cl there appears to be a slight alkalisation effect in the pH trace and conversely on addition of Ca(NO$_3$)$_2$ a slight acidification before an overall alkalisation. The phenomenon of cytosolic acidification, due to the activity of the NO$_3^-$ transporter co-transporting H$^+$ into the cytosol, on addition of NO$_3^-$ has been proposed by McClure _et al._, (1990) and Miller _et al._, (2001). This initial acidification is thought to stimulate the plasma membrane proton pump which then leads to an overall alkalisation of the cytosol which is observed here. The converse situation could occur with NH$_4^+$ treatments in view of the responses observed in Figure 6.15 whereby there could be an initial alkalisation and then an overall acidification of the cytosol as NH$_4^+$ is countertransported with one H$^+$ and this is
the chemical equivalent of uptake of NH$_3$, a weak base, in the cytosol. Following the transport and subsequent membrane repolarisation events across the plasma membrane which would support an initial alkalinisation during active NH$_4^+$ transportation. An overall acidification of the cytosol before returning to the original compartmental pH could be caused by the subsequent assimilation of NH$_4^+$ which would rapidly alter the sizes of the acid and base pools (Britto and Kronzucker, 2005). However care should be taken when interpreting this data due to the slow response time of these pH electrodes which can require several minutes to reach stable pH values and these pre-alkalisations and acidifications could be artefacts.

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<td>62</td>
<td>7.00</td>
<td>-3.20</td>
</tr>
<tr>
<td>14</td>
<td>light response</td>
<td>-79</td>
<td>-2</td>
<td>6.33</td>
<td>-0.04</td>
</tr>
</tbody>
</table>

**Table 6-1.** Responses of recordings from the cytosol in *M. hornum* to treatment with inorganic-N, cations and acids.

The cytosol of *M. hornum* has been shown to be acidified on addition of HCl and NH$_4$Cl and alkalinised on addition of Ca(NO$_3$)$_2$ and in Table 6.1. several of these responses are summarised. When plotted graphically there appears to be no
correlation between the N-dep status of the plant and the amplitude of the responses (data not shown). All plants used for the purposes of electrophysiological data were at no more than 20 days N-dep and should have been replete in most nutrients minimising any complicating nutritional deprivation responses. On addition of CaCl₂ outside the cell there was an alkalisation of the cytosol and this is difficult to explain (Table 6.1). It should be noted that some of the values in Table 6.1 for the resting pH are more acidic than usual for the cytosol (which is typically in the range of pH 7.0 to 8.0) and more work is required to support these pH changes in the cytosol but based on supporting data obtained from vacuolar recordings in M. hornum (see Table 6.2) theories can be expanded upon with some confidence. One theory for changes in pH with salt application would support the pH-stat as proposed by Davies (1986) and Raven (1986) whereby the alkalisation was due to an excess of anion influx, due to Cl⁻ addition causing an increase in cytosolic pH. Another theory that Ca²⁺ cations individually cross the plasma membrane into the cytosol in a counter-exchange with a resultant quantity of protons corresponding to the charge of the ion, potentially causing an imbalance and alkalisation of the cytosol. As Ca²⁺ enters the cell through channels there is a very large Ca²⁺ gradient driving the cation in to the cell (Ca²⁺ is 100 nM inside and 1 mM outside). When these channels are open Ca²⁺ floods into the cytosol (personal communication Tony Miller, CPI division, Rothamsted Research). Further work would need to be done to evaluate the potential mechanism in operation here.
Figure 6.16. A typical example of a recording from a pH-selective microelectrode in the vacuolar compartment of a leaf cell in *M. hornum*. pH 3 \( \text{H}_2\text{SO}_4 \) applied – see treatment bar.

Figure 6.16. shows a rapid depolarisation and no return to the resting membrane potential in *M. hornum* with a corresponding acidification and a slow return to the original vacuolar pH when treated with pH 3 \( \text{H}_2\text{SO}_4 \). From Table 6.2, vacuolar recordings in *M. hornum* generally showed an acidification on addition of \( \text{H}_2\text{SO}_4 \) and \( \text{NH}_4\text{Cl} \) and alkalisation on addition of \( \text{Ca(No}_3\text{)}_2 \) and \( \text{CaCl}_2 \). Again there appears to be no correlation between the N-dep status of the plant and the amplitude of the responses and the patterns of response are similar to those observed in the cytosol.
An intracellular decrease in pH was recorded by pH-selective microelectrodes upon addition of acids in the cytosol and the vacuole of *M. hornum* which could provide an explanation for the stimulus of NRA induction in *M. hornum* as demonstrated in this study and reported previously in higher plants (Kaiser and Brendle-Behnisch, 1995). Most interestingly, with regards to uptake of alternative inorganic-N sources by leaf tissue, NRA had been induced in both mosses upon addition of NH$_4$Cl (see Chapter 4) and a corresponding depolarisation of the membrane potential and acidification of compartments in both mosses was recorded by pH-selective microelectrodes. These recordings are evidence to support the theory on the induction of NRA with NH$_4$Cl in these mosses and could be attributed to the specific transport mechanisms operating in these plants during the assimilation of N, regulation of cytosolic pH and enzyme activity.

<table>
<thead>
<tr>
<th>N-dep (d)</th>
<th>Treatment (Concentration)</th>
<th>Resting Membrane Potential Voltage (mV)</th>
<th>mV change (mV)</th>
<th>resting pH (pH)</th>
<th>pH change (pH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0.25 mM Ca(NO$_3$)$_2$</td>
<td>-50</td>
<td>13</td>
<td>4.30</td>
<td>0.40</td>
</tr>
<tr>
<td>6</td>
<td>0.25 mM Ca(NO$_3$)$_2$</td>
<td>-81</td>
<td>43</td>
<td>3.82</td>
<td>0.63</td>
</tr>
<tr>
<td>6</td>
<td>0.25 mM Ca(NO$_3$)$_2$</td>
<td>-52</td>
<td>23</td>
<td>3.80</td>
<td>0.20</td>
</tr>
<tr>
<td>6</td>
<td>0.25 mM Ca(NO$_3$)$_2$</td>
<td>-50</td>
<td>20</td>
<td>3.90</td>
<td>0.10</td>
</tr>
<tr>
<td>6</td>
<td>0.25 mM Ca(NO$_3$)$_2$</td>
<td>-77</td>
<td>18</td>
<td>3.95</td>
<td>0.06</td>
</tr>
<tr>
<td>6</td>
<td>0.25 mM Ca(NO$_3$)$_2$</td>
<td>-85</td>
<td>27</td>
<td>3.78</td>
<td>0.04</td>
</tr>
<tr>
<td>14</td>
<td>0.25 mM Ca(NO$_3$)$_2$</td>
<td>-80</td>
<td>20</td>
<td>2.50</td>
<td>0.25</td>
</tr>
<tr>
<td>3</td>
<td>0.25 mM CaCl$_2$</td>
<td>-52</td>
<td>19</td>
<td>3.44</td>
<td>0.91</td>
</tr>
<tr>
<td>14</td>
<td>0.5 mM NH$_4$Cl</td>
<td>-50</td>
<td>32</td>
<td>4.00</td>
<td>-0.11</td>
</tr>
<tr>
<td>18</td>
<td>0.5 mM NH$_4$Cl</td>
<td>-50</td>
<td>3</td>
<td>4.19</td>
<td>-0.72</td>
</tr>
<tr>
<td>12</td>
<td>pH 3 H$_2$SO$_4$</td>
<td>-80</td>
<td>60</td>
<td>3.00</td>
<td>-1.00</td>
</tr>
<tr>
<td>13</td>
<td>pH 3 H$_2$SO$_4$</td>
<td>-60</td>
<td>52</td>
<td>3.00</td>
<td>-1.00</td>
</tr>
</tbody>
</table>

Table 6-2. Response of recordings from the vacuole in *M. hornum* to treatment of cells with inorganic-N, cations and acids.
6.2.9. Responses in to treatments in *S. fimbriatum* – acidic compartment

Figure 6.17 shows a typical response of an acidic compartment in *S. fimbriatum* to treatment with Ca(NO$_3$)$_2$ and NH$_4$Cl. On addition of Ca(NO$_3$)$_2$ there was a depolarisation before returning to the original resting membrane potential and corresponding overall alkalisation of the compartment before a return to the original compartmental pH. The changes in pH were very small however in this particular trace (see Table 6.3). There was an overall depolarisation and return to the resting membrane potential on addition of NH$_4$Cl and a corresponding overall acidification of the compartment before returning to the original compartmental pH.

![Figure 6.17](image)

**Figure 6.17.** A typical example of a recording from a pH-selective microelectrode in an acidic compartment of a leaf cell in *S. fimbriatum*. a = 0.25 mM Ca(NO$_3$)$_2$, b = 0.5 mM NH$_4$Cl - see treatment bar.
Table 6.3 shows more of these responses in the acidic compartments of *S. fimbriatum*.

<table>
<thead>
<tr>
<th>Treatment (Concentration)</th>
<th>Resting Membrane Potential Voltage (mV)</th>
<th>mV change (mV)</th>
<th>resting pH (pH)</th>
<th>pH change (pH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25 mM Ca(NO$_3$)$_2$</td>
<td>-49</td>
<td>34</td>
<td>3.90</td>
<td>0.01</td>
</tr>
<tr>
<td>0.25 mM Ca(NO$_3$)$_2$</td>
<td>-49</td>
<td>31</td>
<td>3.92</td>
<td>0.05</td>
</tr>
<tr>
<td>0.25 mM Ca(NO$_3$)$_2$</td>
<td>-53</td>
<td>24</td>
<td>3.32</td>
<td>0.02</td>
</tr>
<tr>
<td>0.25 mM Ca(NO$_3$)$_2$</td>
<td>-56</td>
<td>22</td>
<td>3.28</td>
<td>0.05</td>
</tr>
<tr>
<td>0.25 mM Ca(NO$_3$)$_2$</td>
<td>-63</td>
<td>26</td>
<td>3.08</td>
<td>0.09</td>
</tr>
<tr>
<td>0.25 mM Ca(NO$_3$)$_2$</td>
<td>-136</td>
<td>43</td>
<td>2.50</td>
<td>0.53</td>
</tr>
<tr>
<td>0.5 mM NH$_4$Cl</td>
<td>-44</td>
<td>22</td>
<td>4.09</td>
<td>-0.04</td>
</tr>
<tr>
<td>0.5 mM NH$_4$Cl</td>
<td>-49</td>
<td>26</td>
<td>4.05</td>
<td>-0.09</td>
</tr>
<tr>
<td>0.5 mM NH$_4$Cl</td>
<td>-59</td>
<td>9</td>
<td>3.08</td>
<td>-0.28</td>
</tr>
<tr>
<td>0.5 mM NH$_4$Cl</td>
<td>-60</td>
<td>18</td>
<td>3.25</td>
<td>-0.34</td>
</tr>
</tbody>
</table>

Table 6-3. Response of acidic compartments in *S. fimbriatum* to treatment with inorganic-N, cations and acids.

It appears that the overall uptake and assimilation of inorganic-N in both mosses support the theory that the assimilation of NH$_4^+$ is proton producing and assimilation of NO$_3^-$ is proton consuming process and the mechanisms for this uptake in moss mirror one another.

Measuring the compartmental pH in moss cells of *M. hornum* and *S. fimbriatum* proved useful in determining if the mobility of cations were of benefit in regulating intracellular pH and preventing cell death and damage to the plants. In both mosses the addition of treatments resulted in membrane potential changes and pH changes that were reversible and cells demonstrated the ability to...
regulate intracellular process. This is important as it demonstrates the ability of both species to regulate cellular pH, in turn preventing damage to organelles and it can be suggested that this could be due in part to the remobilisation of protons and cations to buffer acidity in the plants. Both *M. hornum* and *S. fimbriatum* N uptake mechanisms support the classical pH-stat theory and current thinking on plant N transporter mechanisms.

6.2.10. Light response in *M. hornum*

The membrane potential in the vacuole changed in response to light-dark transitions and Figure 6.18. was typical of responses observed in the vacuole of *M. hornum* leaf cells in terms of magnitude, duration and shape of response. There was a slow and gradual depolarisation followed by a 10 minute stable period and hyperpolarisation following the dark-light transition. Unfortunately the tip popped out of the cell shortly after this transition so no further information on the membrane potential response is available. Interestingly this response is typical of those observed in wild-type epidermal vacuoles and cytosol of *Arabidopsis* (Cookson et al., 2005). There also appears to be an alkalisation of the compartment in response to the light-dark transition and this is in contrast to the light-induced changes in the cytosol reported in the coccal green alga *Eremosphaera* (Bethmann et al., 1998).
Figure 6.18. A typical example of a recording from a pH-selective microelectrode in the vacuole of a leaf cell in *M. hornum*. Light on and light off - see treatment bar.

In *Eremosphaera*, after darkening, a transient hyperpolarisation was released and a rapid return to the resting membrane potential observed after just 5 minutes. A corresponding immediate acidification occurred after darkening which was reversed after just 1.5 min. This alga appears to give the opposite response to that observed in *M. hornum* and higher plants. This suggests that *M. hornum* plants have similar photosynthetic apparatus, thylakoid membranes and stroma, to that present in higher plants. Darkening of green plant cells induces a transient acidification and illumination causes a transient alkalisation of pH in higher plants and these responses have been shown to be caused by light-dependent proton translocation across the thylakoid membrane.
across the chloroplast envelope propagate the pH deviations from the chloroplast stroma to the cytosol (Thaler et al., 1992; Hansen et al., 1993). Unfortunately minimal data was obtained with regards to a photosynthetic response in S. fimbriatum but this can be largely attributed to the tips only penetrating acidic compartments that are unlikely to have photosynthetic capabilities.

6.2.11. Disruption of the cells and compartmentation in M. hornum and S. fimbriatum

As many of the recordings in M. hornum moss were not as negative as those typically observed in higher plant electrophysiological measurements (Miller et al., 2001) further verification was required as to which compartments the tips were penetrating. Therefore 10 % SDS was used for a minimum of 24 hrs to disrupt the membrane and cell structure (Biswal and Mohanty, 1976). Figure 6.19. showed that once plants were treated with SDS microelectrode penetrations were all above approximately -40 mV, the membrane potential value that was previously attributed to the cell wall. The parameters previously used to define microelectrode impalements of the cell wall in M. hornum could therefore be used with confidence. To confirm that the cell structure was disrupted by SDS treatment moss tissue was stained with neutral red and viewed under light microscopy. This work imaging M. hornum and S. fimbriatum revealed some interesting structural features in the two mosses. Neutral red dye stains acidic compartments, such as vacuoles and so this treatment can be used to show the amount of vacuolation present in the tissue (Marschall et al., 1998).
Figure 6.19. A typical example of a recording from a pH-selective microelectrode in SDS treated *M. hornum*. c= cell wall.

The same concentration of dye was applied in Figures 6.20. and 6.21. In Figure 6.20a untreated *M. hornum* cell walls were stained red and there were distinct green chloroplasts visible in individual cells with vacuoles present as concentrated dark red dots that had accumulated dye. However in Figure 6.20b *M. hornum* treated with SDS had cell walls stained red but the internal structure was considerably disrupted and the contents were a homogenised compartment with no distinct structures. In Figure 6.21. a) in untreated *S. fimbriatum*, cell walls were stained red with hyaline cells visible as caterpillar-like large interconnecting spaces to the living green cells that were visible with their distinct green chloroplasts. Vacuoles were not visible under the light microscope.
Figure 6.20. Light microscope pictures of *M. hornum* leaf stained for 12 hours with 0.02% (w/v) neutral red stain. a) untreated moss b) 10 % SDS treated. *M. hornum* cell walls stained red in a) and b). Distinct green chloroplasts visible in a) however the internal structure disrupted in b). Vacuoles accumulated dye visible as concentrated dark red dots a) and absent in b). At 200 X Magnification.
In Figure 6.21. b) S. fimbriatum treated with SDS, the cell walls were stained red but the internal structure of the living green cells had also been disrupted and the structure looked quite transparent and lacking cellular structural content. These observations also supported the disruptive ability of the SDS treatments but most importantly these images have demonstrated the differences between M. hornum and S. fimbriatum structure. The presence or absence of cell walls, vacuoles and cytosol were clearly visible with these images. In S. fimbriatum the large hyaline cells dominated the leaf space with small living green cells being considerably smaller than the dense living green cells seen in M. hornum. From these observations a better understanding of the tissue compartmentation in S. fimbriatum and M. hornum was obtained. These observations, combined with the range of electrophysiological measurements obtained, made it possible to conclude that M. hornum had both cytosolic and vacuolar compartments whereas S. fimbriatum had predominantly acidic compartments which were almost certainly hyaline cells. It is possible that S. fimbriatum has lower NRA and NO$_3^-$ storage capacity because of differences in plant structure when compared with M. hornum and higher plants due to the lack of a distinct vacuole for storage of NO$_3^-$.

There is a school of thought that the hyaline cells in Sphagnum mosses may act as storage compartments for NO$_3^-$ (Clymo, 1963) however this remains to be elucidated. It can be concluded that in M. hornum NRA is rapidly induced and NO$_3^-$ is stored at concentrations comparable with higher plants, perhaps due to the vacuoles observed with this light microscopy and also its similarity in structure to these higher plants.
Figure 6.21. Light microscope pictures of *S. fimbriatum* leaf stained for 12 hours with 0.02% (w/v) neutral red stain. a) untreated moss and b) 10 % SDS treated *S. fimbriatum*. *S. fimbriatum* cell walls stained red in a) and b). Hyaline cells visible as caterpillar-like large interconnecting spaces a) and b). Vacuoles not visible. Living green cells with distinct green chloroplasts visible in a) however the internal structure disrupted in b). At 200 X magnification.
Electron micrographs of *S. fimbriatum* show that some cells may contain vacuoles that are very small discrete vesicles (Ligrone and Duckett, 1998) but the capacity of these compartments to store NO$_3^-$ would probably be much decreased. The capacity for *M. hornum* and *S. fimbriatum* to store NO$_3^-$ was investigated using NO$_3^-$-selective microelectrodes.

6.2.12. NO$_3^-$ concentration in the cytosol of *M. hornum*

It was difficult to measure the compartmental cytosolic or vacuolar concentrations of NO$_3^-$ in *M. hornum* and *S. fimbriatum* and the difficulties were probably due to optimising the manufacture of the NO$_3^-$-selective microelectrode tips for use in both mosses. More measurements were obtained with *M. hornum* although the compartmental location of the tip was difficult to determine. Compartmental analysis and cell fractionation techniques may measure the amount of NO$_3^-$ in the cytoplasm as a whole (including organelles and the vacuole); by contrast NO$_3^-$-selective microelectrodes and NRA estimate only the cytosolic NO$_3^-$ and hence may result in lower estimates for NO$_3^-$ concentration measurements (Siddiqi and Glass, 2002) and this should be considered when analysing electrophysiological data.
Figure 6.22. A typical example of a recording from a \( \text{NO}_3^- \)-selective microelectrode in a cytosolic compartment of a leaf cell in \textit{M. hornum}. \textit{M. hornum} N-dep 151. \( a=0.25 \text{ mM Ca(NO}_3^-)_2 \) - see treatment bar.

Several experiments in \textit{M. hornum} gave responses like these shown in Figure 6.22 and all showed an increase in the cellular \( \text{NO}_3^- \) concentration after sequential \( \text{Ca(NO}_3^-)_2 \) perfusion treatments. Values of mM \( \text{NO}_3^- \) concentration are low and only just within the detection limit of the microelectrodes. Similar patterns have been observed in \textit{Chara corallina} cells cultured under low \( \text{NO}_3^- \) conditions showing measurements which were actually lower than the detection limit of the electrodes (Miller and Zhen, 1991). Tissue \( \text{NO}_3^- \) concentration in biochemical profiling studies at 151 d N-dep in \textit{M. hornum} typically showed a low baseline level of around 4 µmol \( \text{NO}_3^- \) g\(^{-1}\) FWt. Figure 6.22 could therefore be considered representative of the cytosolic compartment of the cell with the location of the \( \text{NO}_3^- \)-selective microelectrode tip in the cytosol. The known \( \text{NO}_3^- \) concentration of the tissue, measured using the tissue \( \text{NO}_3^- \) assay, was 4 µmol.
NO$_3^-$ g$^{-1}$ FWt so NO$_3^-$ was present in the tissue but not measurable in the compartment. It could therefore be deduced that the NO$_3^-$ must be located in the vacuole where the tip is not located to measure it. It could therefore be concluded, from observing Figure 6.22 and using known concentration measurements of tissue NO$_3^-$, that higher NO$_3^-$ concentration measurements with the electrodes would represent vacuolar NO$_3^-$ concentrations and lower values would represent the cytosolic concentrations. More work is required to quantify the NO$_3^-$ storage capacities of *M. hornum* and *S. fimbriatum* using electrophysiological techniques and this could be achieved in part by perfecting the manufacture of NO$_3^-$-selective microelectrodes for use in these mosses. With more successful NO$_3^-$-selective microelectrodes measurements could be achieved in all compartments identified to be present in both mosses including the cytosol, vacuoles and acidic compartments to further understand the NO$_3^-$ storage capacities of both mosses.

6.3. Initial Conclusions and Next Steps

It was found that considerable structural differences between *S. fimbriatum* and *M. hornum* may provide an explanation for the differences in NO$_3^-$ storage capacity in both mosses with *M. hornum* having similar structure to that observed in higher plants; perhaps including a central storage vacuole. In *M. hornum* NO$_3^-$ is stored at concentrations comparable with higher plants, perhaps due to the vacuoles observed with light microscopy and its similarity in structure to higher plants. Measurements using pH-selective microelectrodes demonstrated that there was a pH change generated in the cytoplasm and vacuoles in *M. hornum*
and there was certainly the potential for the response to act as a mechanism for buffering acidity but further work would be required to evaluate the potential mechanisms in operation in these mosses. An intracellular decrease in pH was recorded by pH-selective microelectrodes upon addition of acids in all compartments of _M. hornum_ which could provide an explanation for the stimulus of NRA induction in _M. hornum_ as demonstrated in this study (Kaiser and Brendle-Behnisch, 1995). Both _M. hornum_ and _S. fimbriatum_ N uptake mechanisms support the classical pH-stat model and current thinking on plant N transporter mechanisms and all these findings are discussed further in Chapter 7.
CHAPTER 7

GENERAL DISCUSSION

The induction of NRA, supposedly a substrate inducible enzyme and established indicator of the uptake and assimilation of \( \text{NO}_3^- \) (Campbell, 1996), was used to measure the N status of the plant in the short term and in the long term (Stanley et al., 2005). The short term induction of NRA with \( \text{NO}_3^- \) was therefore expected, however the induction of NRA with \( \text{NH}_4^+ \), acids and other ions was not. There was a rapid initial induction of NRA in both *M. hornum* and *S. fimbriatum* by spraying the plants with a mist of various different treatments, including applications of inorganic-N, after just 15 minutes and most interestingly NRA *in vivo* was induced by the application of \( \text{NH}_4^+ \) in leaves of both species (Stanley et al., 2005).

‘Is NR Truly Substrate Inducible?’

A hypothesis to explain the response of the induction of NRA with \( \text{NH}_4^+ \) treatment was that NRA may be involved in maintaining intracellular pH. The direct uptake and assimilation of \( \text{NH}_4^+ \) would produce \( \text{H}^+ \) with NR being induced in order to regulate cellular pH (Kaiser & Brendle-Behnisch, 1995; Raven, 1988). Further investigation into cellular changes upon addition of \( \text{NH}_4^+ \) showed an intracellular decrease in pH, which was recorded by pH-selective microelectrodes upon addition of these treatments, in compartments of both mosses.
It could therefore be proposed that in the short term NRA is induced by NH$_4^+$ in *S. fimbriatum* and *M. hornum* and NR is not substrate inducible in this case and is in fact regulated by pH. This study has also shown that NRA was induced in the short term by acids and other ions in *M. hornum* along with a corresponding change in intracellular pH using pH-selective microelectrodes.

Reasoning behind these responses in the plants could be attributed to the N-deprivation regimes that they underwent prior to experimentation, and the rapidity or the NR assay work. A decrease in available nutrients and ions, which may be used to buffer intracellular acidity, could make the cytosol more acidic and in turn result in a larger induction of NRA due to greater acidity (Kaiser and Brendle-Behnisch, 1995). An increase in dephosphorylated NR enzyme due to lack of available Pi$^+$, the denaturing of NIP or perhaps the increased activation of NR kinase could all be caused by increased acidity in the cell resulting in a subsequent induction of NRA; but further work is still required to establish whether this is a valid hypothesis. The most likely cause, as proposed by Kaiser and Brendle-Behnisch (1995) is that acid-induced activation is mediated by protein dephosphorylation. The NRA inductions in this study are indicative of rapid short term responses and in these cases the unusual induction of NR may be regulated by the active and inactive forms of enzyme due to changes in intracellular acidity. Longer term *de novo* synthesis of NR protein may be the effect that is most commonly observed in higher plant studies whereby NO$_3^-$ is observed as the primary signal for the induction of NR (Gowri *et al.*, 1992, Long *et al.*, 1992).
Both mosses demonstrated different susceptibilities to increased N deposition and changes in external pH and this could be attributed to structural differences between \textit{S. fimbriatum} and \textit{M. hornum}. Differences in the NO$_3^-$ storage capacities of both mosses were initially postulated in response to sequential treatments with inorganic-NO$_3^-$. The rapid initial induction of NRA and corresponding increase in NO$_3^-$ storage pools in \textit{M. hornum} indicated that \textit{M. hornum} was a nitrophilous species; whereas \textit{S. fimbriatum}, even though NRA was rapidly induced, stored less NO$_3^-$ (Stanley \textit{et al.}, 2005). \textit{M. hornum} was more like a higher plant in its pattern of NO$_3^-$ storage when compared to experiments carried out in barley (Sueyoshi \textit{et al.}, 1995). \textit{M. hornum} plants treated with inorganic N after long term N-deprivation were still able to rapidly induce NRA. However the capacity to store NO$_3^-$ did decrease after 450 d. The storage of NO$_3^-$ in the vacuole may require a specific transporter in this membrane (Miller and Smith 1992) and under longer term N-deprivation the ability to produce this protein may have been lost resulting in such a decline.

\textit{S. fimbriatum} had lower basal NRA and NO$_3^-$ concentrations and less NO$_3^-$ storage capacity than \textit{M. hornum} and this affected the ability of the species to thrive in the long term and \textit{S. fimbriatum} plants died after just 80 d N-deprivation. It can be suggested from electrophysiological measurements and microscopy that \textit{S. fimbriatum} had a lower NO$_3^-$ storage capacity; due to the lack of a distinct vacuole or storage compartment for NO$_3^-$. \textit{M. hornum} had both cytosolic and vacuolar compartments and was more similar in structure to a higher plant than \textit{S. fimbriatum} which was shown to be dominated by hyaline cells. It is unlikely that hyaline cells act as a storage compartment for NO$_3^-$ as
only low tissue nitrate concentrations have been measured in *S. fimbriatum* whereas in *M. hornum* NO$_3^-$ was stored at concentrations comparable with higher plants and this could perhaps be due to accumulation of NO$_3^-$ in the vacuoles of *M. hornum* cells.

*M. hornum* had a tremendous capacity to sustain itself with NRA being induced and NO$_3^-$ stored long term even whilst undergoing N starvation. This moss was then used to test a hypothesis that *M. hornum* would be able to sense changes in atmospheric N deposition, perhaps serving as a useful bioindicator to measure changes in atmospheric N. This type of experiment had not been conducted before and after one month of fieldwork, *M. hornum* NRA and tissue NO$_3^-$ concentrations recovered to levels found in the field at Mardley Heath. Inputs from rainwater data revealed that critical loads for N were almost reached on several occasions on the site during that month based upon critical load values established by Rudolph and Voigt (1986) on *Sphagnum magellanicum*. However no detrimental effects to NRA or tissue NO$_3^-$ concentration accumulation were observed on the N-starved mosses. It can be concluded that *M. hornum* can serve as a good bioindicator of atmospheric N pollution if NRA *in vivo* and tissue NO$_3^-$ concentrations are measured in conjunction with one another. Measuring NRA singularly may not be so accurate due to the potential for acid and other ions to stimulate NRA. Living bioindicators also have the advantage of detecting interactions between environmental pollutants in this way, compared to established systems for monitoring increases in atmospheric N such as dried moss resin bags. The combined effects of atmospheric N inputs and acid may be much worse than separate doses of each of these pollutants and being able to
measure the combined effects in mosses enables the more accurate interpretation of results.

Observations using SEM showed that the application of acidic treatments to both mosses effected the epicuticular wax layer and thin cuticles; possibly making the exchange of gases, water vapour, nutrients and pollutants, from the external environment, difficult to regulate. Mosses take up N readily and are very sensitive to changes in atmospheric N supply. This is in part due to their single celled structure and lack of an extensive cuticle. *S. fimbriatum* was shown to be more sensitive to changes in inorganic-N supplied and this supported work carried out in the field to date which predicts a decline in *Sphagnum* bog and other sensitive moss habitats due to increased anthropogenic N (Soares and Pearson, 1997; Lee and Caporn, 1998; Aldous, 2000; Bridgham, 2000; Pearce and van der Wal, 2002). In this respect *S. fimbriatum* was not such a good candidate species to monitor changes in atmospheric N at Mardley Heath as its response to changes in N did not demonstrate such reliable trends in data. However, the sensitivity of mosses to changes in atmospheric N and pH make them good candidates for studies into pollution. This study has shown that mosses are good systems to use for biochemical studies and microelectrodes lend themselves well to investigations to into why mosses are sensitive to increases in atmospheric N.

Attributing cell compartments using membrane potentials alongside intracellular pH measurements proved useful in determining the location of the microelectrode tip and the cell transporter processes in operation. Using pH-
selective microelectrodes the compartments being penetrated were established and the use of microscopy and staining techniques helped to determine the presence of cell walls, vacuoles and cytosol. In *S. fimбриatum* large hyaline cells dominated the leaf space whereas in *M. hornum* green chlorophyll containing cells were obvious with distinct cytoplasmic and vacuolar compartments. Measuring pH changes in both *M. hornum* and *S. fimбриatum* using pH-selective microelectrodes demonstrated that both mosses appear to have the same inorganic-N transport mechanisms to those common in higher plants for regulating intracellular pH. To the best of our knowledge this is the first time that these inorganic-N transporters have been shown to exist unequivocally in mosses.

Directly measuring the compartmental cytosolic or vacuolar concentrations of NO$_3^-$ with microelectrodes in *M. hornum* and *S. fimбриatum* was difficult. More success was achieved with *M. hornum* with several experiments showing a qualitative trend towards NO$_3^-$ uptake after sequential Ca(NO$_3$)$_2$ perfusion and with starting levels of NO$_3^-$ just within the detection limit of the NO$_3^-$-selective microelectrodes. More work is required to quantify the NO$_3^-$ storage capacities of *M. hornum* and *S. fimбриatum* using electrophysiological techniques. Working with plants in a more N-replete state may overcome some of the problems associated with the detection level starting activities of NO$_3^-$. Most interestingly, with regard to uptake of alternative inorganic-N sources by leaf tissue, NRA had been induced in both mosses upon addition of NH$_4$Cl and a corresponding depolarisation of the membrane potential and acidification of compartments in both mosses was recorded by pH-selective microelectrodes. These recordings
were evidence to support the theory on the induction of NRA with NH$_4$Cl in these mosses where it appears that the uptake of inorganic-N in both mosses supports the theory that the overall assimilation of NH$_4^+$ is a proton producing process and an increase in acidification of the cytosol may be responsible for the increase in NRA. Assimilation of NO$_3^-$ is a proton consuming process as there is evidence for this idea from using pH-selective microelectrodes in both mosses. From measurements obtained using pH-electrodes it was conclude that both *M. hornum* and *S. fimbriatum* N uptake mechanisms support the classical pH-stat model and current thinking on plant N transporter mechanisms in higher plants.

The aim of this research was to assess and compare how these two mosses may sense their N status and this project brought together the biochemistry, physiology and localised changes in metabolite pools and pH to explain why two moss species behaved so differently to increases in N deposition. Some of the major findings of this research were that mosses can be sensitive, but also robust, to changes in their external environment with the potential to provide information on such far reaching topics as evolution, global climate change and environmental policy.

The research reported in this thesis was aimed at investigating the following hypotheses:

1. Two moss species (*S. fimbriatum* and *M. hornum*) from contrasting habitats may have differing metabolic responses to artificial applications of elevated inorganic-N and these differences may be used to assess the N status of the moss.
2. Mosses show a range of differing sensitivities to atmospheric N pollution and the use of moss as a bioindicator could prove useful in monitoring climate change and pollution.

3. Microelectrode measurements in moss, like higher plants, can be used to measure moss N status including membrane transporter activity and N storage capacity.

The first hypothesis was partially proven as both *S. fimbriatum* and *M. hornum* showed similar responses with stimulation of NRA but differed in NO$_3^-$ storage capacity. Following on from this the second hypothesis was proven that *M. hornum* was sensitive to changes in atmospheric N and robust enough to be used as a bioindicator in the field to monitor atmospheric N inputs by measuring NR in vivo activities and tissue NO$_3^-$ concentrations after undergoing long term N-deprivation. *S. fimbriatum* was shown to be more sensitive to changes in inorganic-N supply and not a good candidate for use as a bioindicator due to the inability of the plants to sustain themselves in the long term following N-deprivation. Finally microelectrode measurements in moss, like higher plants, can be used to measure moss N status including membrane transporter activity and N storage capacity. The two mosses used in this study *M. hornum* and *S. fimbriatum* demonstrated from measurements obtained using pH-electrodes that both *M. hornum* and *S. fimbriatum* N uptake mechanisms support the classical pH-stat model and current thinking on plant N transporter mechanisms in higher plants, which goes a long way to understanding how mosses sense N status, especially with respect to NR.
Future work

Further work is now needed to show how the phosphorylation status of the NR in both mosses may be changing during treatments with inorganic-N, acids, other ions and also alkali solutions. It would also be interesting to carry out work using alkali solutions as well. Developing measuring NR in vitro in both mosses could provide further information on the NR activation status and the role of phosphorylation in the induction of NR under N-deprived conditions. NR has an important role in pH regulation and species susceptibility to atmospheric N deposition. Developing the NR in vitro assay for mosses undergoing inorganic-N treatments (along with other NR inducers) could provide further information on the NR activation status and the role of phosphorylation in the induction of NR under N-deprived conditions. The objective would be to compare NR in vivo and in vitro to see if NR induction in these experiments was due to post-translational modification of enzyme. We would hypothesise that NR phosphorylation state is dependent on NO$_3^-$ storage and intrinsically its N-deprivation status and this has an important role in the cell’s ability to maintain pH homeostasis.

Further work using pH-selective microelectrodes in mosses is required to show how the pH in compartments changes in relation to treatments with inorganic-N, acids, other ions and alkali solutions. This would then be related back to NRA in vivo and in vitro along with tissue NO$_3^-$ concentration measurements to give a more detailed understanding of how mosses sense N status. The techniques used in electrophysiology enable compartments to be penetrated and pH to be measured directly in real-time in order to try to understand the mechanisms
involved in N uptake in order to elucidate how mosses sense N status. More work is also required to quantify the NO$_3^-$ storage capacities of *M. hornum* and *S. fimbriatum* using electrophysiological techniques. Working with plants in a more N-replete state may overcome some of the problems associated with the detection level starting activities of NO$_3^-$.

*M. hornum*, at known N-deprivation status, has the potential to be used as a bioindicator for use in monitoring global climate change or pollution by measuring key metabolites and this is a very exciting prospect for future work. For example *M. hornum* could be used to monitor the effects of increased traffic flow on a road scheme. In this way adverse effects on the plant metabolism could be picked up, having the potential to give an early warning if there was an increase in atmospheric N exceeding critical loads. In turn this would provide an early warning to prevent long term detrimental effects to the environment.

In the search for answers as to how mosses, and plants, sense N the picture is a complex one and there is still much scope for further work.
A.1 Theory of ISEs (From Miller and Wells 2006)

The theoretical background has already been described by many authors and will only be outlined here. The properties of an ion-selective microelectrode are defined by several characteristics:

- Detection limit
- Selectivity
- Slope
- Response time

The ideal relationship between electrode output (mV) and the activity \((a_i)\) of the ion of interest \((i)\) is log-linear and is described mathematically by the Nernst equation. Calibration of the electrode against a range of standard solutions should ideally yield a slope \((s)\) of 59 mV (at 25 C) per decade change in the activity of a monovalent ion. In practice, however, the situation is more complicated than this because no ion-selective electrode (ISE) has ideal selectivity for one particular ion and under most conditions there is more than one ion present in the sample solution. Hence contributions to the overall electro-motive force (EMF) made by each interfering ion, \(j\), must be taken into account. In this situation, the Nicolsky-Eisenman equation, a modified Nernst equation, describes the EMF:

\[
EMF = E + s \cdot \log \left( a_i + K_j^{\text{pot}} (a_j)^{v_j/s_j} \right)
\]  

(1)
where $K_{ij}^{\text{pot}}$ is the selectivity coefficient of the electrode for the ion $i$ with respect to ion $j$. This term expresses, on a molar basis, the relative contribution of ions $i$ and $j$ to the measured potential.

The parameters $s$ and $K_{ij}^{\text{pot}}$ are the two main characteristics defining any type of ion-selective electrode. The slope should be a near ideal Nernstian response when an electrode is calibrated against ion activity, but $s$ is temperature sensitive. The selectivity coefficient measures the preference of the sensor for the detected ion $i$ relative to the interfering ion, $j$. It can be determined by the separate solution method, the fixed interference method or the fixed primary ion method. For ideally-selective membranes, or for samples containing no other ions with the same net charge as the ion in question, $K_{ij}^{\text{pot}}$ must be zero. A log selectivity coefficient $<1$ indicates a preference for the measuring ion $i$ relative to the interfering ion $j$, and vice versa for a selectivity coefficient $>1$. The $K_{ij}^{\text{pot}}$ values should not be considered to be constant parameters that characterize membrane selectivity under all conditions; the values are dependent on both the method used for determination, and on the conditions under which the calibrations are made. The fixed interference method is most commonly used to calculate the selectivity coefficient and it is the method recommended by the International Union of Pure and Applied Chemistry (Inczédy et al. 1998). Whichever type of method is chosen the one used should always be quoted.

A schematic representation showing an ideal ion-selective microelectrode calibration curve is given in Figure A.1. The slope $s$, is the change in EMF per decade change in activity of a monovalent anion $i$, which is equivalent to 59.2
mV at 25 °C; the limit of detection is defined as described in the text and is also indicated.

Another important parameter of an ion-selective microelectrode is the detection limit, which is the lowest ion activity that can be detected with confidence and is defined by the intercept of the two asymptotes of the Nicolsky response curve (see Figure A.1.). In practice, the detection limit seems to depend on the tip geometry and composition of the microelectrode's ion-selective membrane. Finer or smaller diameter tips have higher detection limits; while composition affects detection in ways that can only be determined experimentally. The presence of interfering ions alters the detection limit (e.g. chloride for nitrate-selective microelectrodes, see Miller and Zhen 1991). Electrodes provide no useful information below their detection limits and for maximum benefit should be used in the linear portion of their calibration curves. The response time of ISEs can be important when measuring changes in ion activities. This microelectrode parameter is dependent on many factors, including tip geometry, membrane composition and resistance. Response time can be measured during the calibration as the time taken for the voltage to adjust when ion activity at the tip is changed.
Figure A.1. A schematic representation showing an ideal ion-selective microelectrode calibration curve. The slope $s$, is the change in EMF per decade change in activity of a monovalent anion $i$, which is equivalent to 59.2 mV at 25°C; the limit of detection is defined as described in the text and is also indicated.
APPENDIX 2

PUBLICATIONS

The following paper is based on work carried out for this thesis.

REFERENCES


GANE Programme NERC (2005) http://www.nerc.ac.uk/funding/therematics/gane