Physiological Responses and Susceptibility of Plants to Atmospheric Ammonia Pollution

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Abstract

A range of physiological responses of higher plants and mosses to atmospheric ammonia (NH₃) and ammonium (NH₄⁺) (collectively NHₓ), were investigated.

Foliar uptake of NHₓ caused an induction of glutamine synthetase and NADP malic enzyme, whereas nitrate reductase activity was inhibited. The concentration of organic acids also declined, particularly malate and citrate. Net photosynthesis and stomatal conductance were also stimulated following uptake of NHₓ. Physiological responses were the same for both NH₃ and NH₄⁺ applications for a number of species. The ability of plants to store nitrogen (N), coupled with NHₓ assimilation leading to the production of acidity, suggests that pH is an important factor that plants have to control, in the presence of excess NHₓ.

Multivariate assessment of physiological characteristics of higher plants and mosses, provided a means of assessing plant susceptibility to atmospheric NHₓ and acidity. Plants that are capable of foliar nitrate (NO₃⁻) assimilation have higher buffering capacities against acidity. Plants which assimilate most of their NO₃⁻ in the leaf, tend to have high base cation contents, which may also contribute towards overall buffering ability.

The total N content of mosses increased after exposure to N containing pollution. In addition, δ¹⁵N values varied consistently between mosses collected in rural and urban areas, with δ¹⁵N being more negative and more positive respectively. δ¹⁵N values reflected the predominant N compound present in each area, as well as the level of N pollution. The results suggest that N compounds in the atmosphere, particularly NHₓ and NOₓ, have individual ¹⁵N signatures, that can be traced in biological material.
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Live long and prosper

Adie
Publications from this thesis

The following articles are published, accepted for publication or submitted for publication, using original data presented in this thesis:-


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CHAPTER 1

Ammonia as a nutrient and a pollutant of plants

1.1 Ammonia as a nutrient

1.1.1 The nitrogen cycle

Ammonia (NH₃) and the aqueous ion ammonium (NH₄⁺) (collectively referred to as NHₓ) are both major sources of nitrogen (N), utilised directly or indirectly by virtually all living organisms. As a result, NHₓ forms an integral part of the global N cycle, a fundamental biological and ecological process. To understand the importance of NHₓ as a plant nutrient, it is important to consider the various mechanisms of the N cycle that lead to the availability of NHₓ as a nutrient source.

The N cycle consists of a complex series of inter-conversions of N, from one form to another, by both physical and biological processes (Figure 1.1). The N cycle is a relatively balanced system with inputs and outputs to the system, being small in relation to the amounts of N transfer and recycling between soil and plants (Tamm, 1991). The influence of human activities has altered the balance dramatically and thus the availability of N compounds to plants; this point will be considered later. The availability of N compounds from natural sources will firstly be considered.

The 3 main inorganic compounds of N available for plant nutrition are di-nitrogen gas (N₂), nitrate (NO₃⁻) plus other oxides of N and NHₓ. The assimilation of N₂ or N fixation as the process is known consists of the reduction of N₂ to form NH₄⁺. This process is almost exclusive to prokaryotic micro-organisms. Plants take advantage of this source of N by establishing symbiotic relationships with N-fixing bacteria in the form of root nodules, for example some members of the
family Leguminosae. Plants can also take advantage of fixed nitrogen from free living soil bacteria that release NH$_4^+$ into the soil medium, which is then taken up by roots. Despite the availability of N$_2$ (78% of the Earth’s atmosphere), it is estimated that only 2% of total global N assimilation by plants is carried out by N fixation (Raven et al., 1993). Primary assimilation of oxides of N, predominantly NO$_3^-$, forms a

Figure 1.1 The global nitrogen cycle, with the main biological processes shown in squares.
larger proportion (between 27 - 50 %) of total global primary N assimilation (Raven et al., 1993). NO$_3^-$ is made available to plants from a variety of natural sources including the process of nitrification; the conversion of NH$_4^+$ to NO$_3^-$ by soil-borne bacteria. Atmospheric sources of NO$_3^-$, formed by the combination of N$_2$ and oxygen, are also important, as they are deposited along with rain water into the soil. In comparison to N$_2$ and NO$_3^-$, the most abundant form of primary N assimilation is fuelled by NH$_x$, with estimates ranging from 45 - 70 % of total global N assimilation (Raven et al., 1993).

Why plants may favour one N source as opposed to another is the subject of much debate in the literature. The presence of N compounds in the environment is not considered to be limiting, although certain habitats may have an abundance of one source of N over another. The largest available source of N is N$_2$, yet not all plants have the ability to form root nodules and take advantage of N$_2$. The conversion of N$_2$ into organic N compounds is energy consuming and this may explain why N$_2$ fixation is not universally established throughout plants (Mohr, 1994). However, most plants have the ability to assimilate NH$_4^+$ and NO$_3^-$ to some extent. Therefore, the distinction between NH$_x$ and NO$_3^-$ as N sources for plants is not fully clear. The above estimates put forward by Raven et al., (1993) are not mutually exclusive and overlap to some extent. Pearson and Stewart (1993) categorise plants into three roughly divided groups :- firstly, plants that assimilate mostly NH$_x$; secondly, plants that assimilate mostly NO$_3^-$ and thirdly, plants that assimilate both NH$_x$ and NO$_3^-$.

The preference plants may show towards different N sources is dependant on several factors, most probably, the availability of individual N sources at any one time. Recent work by Pearson and Stewart (1993), Soares et al., (1995) and Pearson and Soares (1995) have shown that the ecology of a plant plus physiological characteristics have some influence on which N
compounds plants utilise. In the case of NH₃, Raven et al., (1992a) has demonstrated that NH₃ is a preferred N source for some plants, where both NH₃ and NO₃⁻ are available in equal quantities. The same author states that higher specific growth rates can be achieved by plants utilising NH₃ instead of NO₃⁻, with NH₃ assimilation requiring less energy than NO₃⁻ assimilation. Despite this, there are still plants that demonstrate lower growth rates with NH₃ nutrition than NO₃⁻ (Raven et al., 1992a).

In order for NH₃ to account for a high proportion of global N assimilation, NH₃ must be available in large quantities. The production of NH₃ by microbial N fixation or NO₃⁻ conversion via ammonification are not sufficient to account for the estimates of global plant N assimilation fuelled by NH₃. Likewise, NH₃ production from natural sources such as volcanic activity and weathering of minerals, only provide a small proportion of globally available NH₃, despite estimates that N content of igneous rocks is 3-15 times greater than the atmosphere (Mohr, 1994). Possibly the largest source of NH₃ present in the N cycle, originates from the biological degradation of plant residues and animal wastes (Wellburn, 1994). The breakdown of biological waste by soil microbes provides the major N component of the soil. Mineralisation of dead organic matter by soil microbes leads to the release of NH₄⁺ and NO₃⁻, which are then taken up by roots. NH₃ is also released into the atmosphere initially in the form of NH₃ by a process known as volatilisation. The volatilisation of NH₃ is considered to provide the largest source of atmospheric NH₃ (Wellburn, 1994). Volatilisation takes place predominantly at or near the soil surface, where plant and animal waste accumulates and is subsequently degraded. The released NH₃ has a high affinity for H₂O, leading to the rapid production of NH₄⁺ ions. NH₄⁺ directly from mineralisation or indirectly from volatilisation, is quickly combined with other ions to
form ammonium salts, for example: ammonium sulphate \((\text{NH}_4)_2\text{SO}_4\), ammonium carbonate \((\text{NH}_4)_2\text{CO}_3\) and ammonium nitrate \((\text{NH}_4\text{NO}_3\)), which are easily diffused through the soil and subsequently taken up by plants. The formation of \(\text{NH}_4^+\) salts can also take place in the atmosphere as \(\text{NH}_3\) is carried upwards and interacts with \(\text{NO}_x\), carbon dioxide \((\text{CO}_2)\), sulphur dioxide \((\text{SO}_2)\), ozone \((\text{O}_3)\) and \(\text{H}_2\text{O}\). Atmospheric sources are subsequently deposited back to the soil as \(\text{NH}_4^+\) salts in rain water. The rate at which \(\text{NH}_3\) volatilisation occurs is highly dependant on the characteristics of the soil, for example \(\text{H}_2\text{O}\) content, \(\text{pH}\) and other environmental variables (Wellburn, 1994). Nevertheless, volatilisation is a process which takes place in virtually all habitats and provides the most abundant source of atmospheric \(\text{NH}_3\). The process of volatilisation is not only confined to decaying plant material; emissions of \(\text{NH}_3\) have been measured from photosynthesising leaves of \textit{Hordeum vulgare} (Schjoerring \textit{et al.}, 1993a) and \textit{Brassica napus} (Olsen \textit{et al.}, 1995) under conditions of high \(\text{N}\) supply. Emissions of \(\text{NH}_3\) from leaves are thought to occur predominantly in agricultural areas, although at present, little is known about the magnitude of \(\text{NH}_3\) losses from any natural vegetation types. Schjoerring \textit{et al.}, (1993a) reported a loss of 0.5-1.5 kg ha\(^{-1}\) y\(^{-1}\) from \textit{Hordeum vulgare}, Harper \textit{et al.}, (1987) found a loss of 15 kg ha\(^{-1}\) y\(^{-1}\) from \textit{Triticum aestivum}. Emissions of \(\text{NH}_3\) from plants are variable and dependant on environmental conditions, the \(\text{N}\) status of the plant, the age of leaves and the activities of \(\text{NH}_x\) assimilating enzymes (Schjoerring \textit{et al.}, 1993b). The contribution that \(\text{NH}_3\) emissions from plants makes to atmospheric levels of \(\text{NH}_3\) and \(\text{NH}_x\) availability in the \(\text{N}\) cycle is at present uncertain.

\(\text{N}_2\), \(\text{NH}_x\) and \(\text{NO}_3^-\) form the major inorganic forms of \(\text{N}\) in the nitrogen cycle. However, some plants have the opportunity to utilise organic \(\text{N}\) sources as well. Plants growing in infertile soils often have
mycorrhizal fungi associated with their roots. These mycorrhizae compete efficiently with free living soil microbes for \( \text{NH}_4^+ \) and possibly \( \text{NO}_3^- \), producing organic N compounds such as amino acids, which are then taken up by the plant roots. In addition, mycorrhizae can breakdown organic matter, thus competing for organic waste material as well and making it available to plants. A recent paper by Northup et al., (1995) has not only suggested that organic sources of N may provide a substantial portion of available N to plants but also, that plants may be able to influence the availability of dissolved organic N to themselves. Northup demonstrated how \textit{Pinus muricata} can influence the release of dissolved organic N in soils, through the production of polyphenols in leaf litter. The polyphenols are bound to organic N, thus plants that can metabolise phenol-bound N, may take rapid advantage of this type of N source and not have to compete with other plants as actively for inorganic or organic N sources. Although this mechanism for organic N acquisition needs further clarification, a previously unregarded route by which plants can bypass the need for mineralisation of dead organic matter by free living microbes, may have been discovered (Chapin, 1995). For the time being, the release of \( \text{NH}_4^+ \) and \( \text{NO}_3^- \) by mineralisation and \( \text{NH}_x \) by volatilisation from decaying organic material, are probably the most influential sources of N to plants. However, the potential influence of organic N uptake should not be underestimated.

1.1.2 The uptake of \( \text{NH}_x \) by plants

The availability of \( \text{NH}_x \) to plants is established as part of the N cycle. The next process is for a plant to incorporate \( \text{NH}_x \) into organic form. The first stage of this process involves the uptake of \( \text{NH}_x \) from soil and atmospheric sources. Recent reviews on this subject have been compiled by Raven \textit{et al.}, (1992a), Pearson and Stewart (1993) and
Fangmeier *et al.*, (1994). These studies have considered the mechanisms of root uptake of NH$_x$, the influence of soil type on root uptake of NH$_x$ and the effects that NH$_x$ uptake have on the rhizosphere. A more generalised account of NH$_x$ uptake from soil by plant roots can also be found in Marschener (1995). The above reviews also consider the uptake of NH$_x$ directly from the atmosphere by shoots. As a consequence, these subjects will only be dealt with briefly here.

NH$_x$ found in the soil is predominantly in the form of NH$_4^+$ as any NH$_3$ present is rapidly combined with H$_2$O to form NH$_4^+$, or lost to the atmosphere. NH$_4^+$ transport in soil is rapid by diffusion and is increased by higher H$_2$O and NH$_4^+$ content (Wellburn, 1994). The uptake of NH$_4^+$ by plant roots requires that a balancing ion be excreted at the root surface into the soil. The ion often used is H', leading to a net production of acidity in the soil. This can be countered by the soils buffering potential, which includes the release of OH' ions from the conversion of NH$_3$ to NH$_4^+$ by soil microbes, coupled with the presence of alkylating compounds in the soil such as HCO$_3$.

Lithophytic plants such as mosses, have no root attachment to the soil and therefore, rely almost solely on above ground uptake of nutrients. Foliar uptake of NH$_x$ is also significant in environments where high concentrations of atmospheric NH$_x$ exist; Pearson and Stewart, (1993) have reviewed several experiments where foliar applied NH$_x$ was found to account for between 0.5 - 77 % of total plant N in a range of plants. In higher plants, foliar uptake of NH$_x$ from the atmosphere, occurs essentially via stomata, with a small proportion of foliar uptake ( approx. 3%) estimated to occur directly via cuticular absorption (Pearson and Stewart, 1993). A concentration gradient between atmospheric NH$_3$ and mesophyll NH$_3$ is thought to provide the driving force behind foliar uptake (Fangmeier *et al.*, 1994). NH$_3$ entering the stomata is quickly dissolved in the water film surrounding
mesophyll cells to form $\text{NH}_4^+$ (van Hove et al., 1988) or in water vapour contained in the sub-stomatal cavity. Wet deposited $\text{NH}_4^+$, or $\text{NH}_4^+$ that has formed on the leaf surface can also enter via the stomata. This process is thought to be accompanied by the release of cations such as calcium, magnesium and potassium, although the mechanism by which $\text{NH}_4^+$ enters the leaves is not yet fully understood (Fangmeier et al., 1994). Whatever the form of $\text{NH}_4$ entering the stomata, uptake depends on the level of stomatal conductance, which in turn is influenced by climatic conditions, water availability, internal $\text{CO}_2$ concentration and irradiation. The concentration gradient mentioned above facilitating $\text{NH}_3$ uptake, can also be altered where mesophyll concentrations of $\text{NH}_3$ exceed external $\text{NH}_3$ concentrations, or increases in cell alkalinity occur. In both cases, plants themselves can release $\text{NH}_3$ into the atmosphere. This situation is thought to be common in agricultural areas, where internal plant levels of $\text{N}$ are high (Schjoerring et al., 1993b). Root uptake of $\text{NH}_4^+$ appears to be easier than foliar uptake of $\text{NH}_4^+$ and conversely, foliar uptake of $\text{NH}_3$ is more significant than root uptake. The soil can also provide a relatively stable reservoir of $\text{NH}_4^+$, in comparison to atmospheric fluctuations. Despite this, direct foliar uptake of $\text{NH}_4^+$ may be important in environments that receive a high proportion of rainfall and are far away from gaseous sources of $\text{NH}_3$. Habitats such as heathlands and moorlands, have slow cycling processes, often due to climatic conditions, thus $\text{NH}_3$ release from volatilisation may be low in comparison to the wet deposition of $\text{NH}_4^+$. Foliar uptake may at present be less understood than root uptake, even so, foliar uptake still provides a major pathway for the acquisition of nutrients for many plants.
1.1.3 The assimilation of NH₃ by plants

Since plants can take in NH₃ by their roots or shoots, it is reasonable to assume that assimilation of NH₃ can occur in both tissues. This is in fact the case, as there is little evidence of transport of NH₄⁺ from roots to shoots or vice-versa. NH₃ is also known to be phytotoxic to plants at high concentrations (Pearson and Stewart, 1993), with no evidence of a significant accumulation of NH₄⁺ under normal conditions. Thus NH₃ is generally assimilated rapidly and close to the point of uptake. The assimilation of NH₃ by plants takes place via the GS / GOGAT cycle (Figure 1.2). NH₃ entering the plant is combined with glutamate to form glutamine, in the presence of ATP. This stage of the cycle is carried out by glutamine synthetase (GS) (EC 6.3.1.2). The resultant molecule of glutamine, in conjunction with 2-oxoglutarate, is converted into 2 molecules of

\[
\begin{align*}
\text{ADP} & \quad \text{H}^+ & \quad \text{Pi} \\
\text{ATP} & \quad \text{NH}_3 & \quad \text{GS} & \quad \text{GOGAT} & \quad \text{2-oxoglutarate} \\
\text{glutamate} & \quad \text{amino acids} & \quad \text{protein metabolism} \\
\text{glutamate} & \quad \text{glutamine}
\end{align*}
\]

Figure 1.2 The assimilation of NH₃ via the GS / GOGAT cycle.

glutamate, by glutamate synthase (GOGAT) (EC 1.4.7.1). One glutamate molecule enters back into the GS / GOGAT cycle forming the precursor molecule for glutamine formation via GS; the other molecule of glutamate is used for the biosynthesis of amino acids and proteins. The partitioning of glutamate between the GS / GOGAT
cycle and protein synthesis, is not always stoichiometric and can depend on the activity of GOGAT and the demand for glutamate or glutamine in other aspects of metabolism. GS represents the first step in the primary assimilation of NH₃ by plants and probably the most important step. This is due to the fact that glutamine is not considered to be toxic and can be stored for later use or transported inside the plant. The assimilation of NH₃ leads to the release of H⁺ ions (Raven, 1988). It is probable that background levels of NH₃ assimilation by GS do not exceed the buffering capacity of plant cells to H⁺, as the resultant acidity may be harmful.

Since the GS / GOGAT cycle was first discovered by Lea and Miflin (1974), much work has been carried out on the characteristics of GS. GS is considered to be universally present in the leaves and roots of higher plants. It is often present at a relatively high level and generally, with more GS in the leaves than in roots (Pearson and Stewart, 1993). The high activities in the shoots are thought to enable the rapid re-assimilation of NH₃ produced from photorespiration. There are 2 main isoforms of GS, a cytoplasmic form and a chloroplastic form. Plants contain varying proportions of the 2 isoforms; Chloroplastic GS is thought to occur in virtually all plants, accounting for much of the assimilation of photorespired NH₃ (Pearson and Ji, 1994); Cytoplasmic GS on the other hand, is thought to occur in only some species (Fangmeier et al., 1994). The relative proportions of both isoforms in plants under different conditions are uncertain at present; Woodall et al., (1996) reviews a large assortment of contradictory reports. Whether different plants contain higher proportions of cytoplasmic or chloroplastic GS or equal amounts of both, does not detract from the fundamental role this enzyme plays in NH₃ assimilation.
The enzyme glutamate dehydrogenase (EC 1.4.4.3) (GDH) has been thought to be involved in the assimilation of NH$_4^+$ in plants. However, the precise role GDH undertakes, has generated much controversy and conflicting opinion. GDH catalyses the reductive amination of 2-oxoglutarate to glutamine, in the presence of NH$_4^+$ and NADH. The reaction is reversible resulting in the oxidative deamination of glutamate to reform 2-oxoglutarate and release NH$_4^+$.

\[
\begin{align*}
\text{NH}_4^+ + \text{NADH} & \quad \text{NAD}^+ + \text{H}_2\text{O} \\
\text{2-oxoglutarate} & \quad \text{glutamate} \\
\end{align*}
\]

**Figure 1.3** The GDH pathway in higher plants

GDH is widely distributed throughout plants and could provide a further means of NH$_4^+$ assimilation. However, nearly all experimental work on GDH has failed so far to find a definite role for GDH in NH$_4^+$ assimilation (Pearson and Soares, 1996). Schlee et al., (1994) suggest a role for GDH as a NH$_4^+$ assimilating enzyme in *Pinus sylvestris*, exposed to high levels of atmospheric pollution. In contrast, Pérez-Soba et al., (1994) found no such role for GDH in the same species. The oxidative deamination of glutamate by GDH is currently considered by most workers to be the predominant role for GDH (Robinson et al., 1991; Schjoerring et al., 1993b; Stewart et al., 1995b). It would not be appropriate at this time to totally discount GDH from an NH$_4^+$ assimilatory role in plants, as more research is required to further clarify its role. However, the general inference gained following several decades of research on the assimilation of NH$_4^+$ by plants, still suggests a minimal role for GDH in comparison to GS.
1.2 NH\textsubscript{x} as an atmospheric pollutant

The importance of NH\textsubscript{x} as a major plant nutrient, the almost universal ability of plants to assimilate NH\textsubscript{x}, its role in the N cycle and its abundance in the environment have been established in the previous section. Factors such as these often make the identity of NH\textsubscript{x} as an atmospheric pollutant, causing detrimental effects to plants, hard to imagine. Since the advent of large scale industrialisation and substantial increases in the world population, many natural ecological processes have been greatly affected. The N cycle is one such process that has not escaped the advances of society.

1.2.1 Increased emissions and deposition of atmospheric NH\textsubscript{3}

Scientists have noticed increases in N in the environment over many years, most notably NO\textsubscript{x} and NH\textsubscript{x}. The largest increases in NH\textsubscript{3} emissions in the environment, originate from agricultural practices. Of the estimated total NH\textsubscript{3} emissions for Europe in 1990 of 6956 ktonnes N yr\textsuperscript{-1}, 98% came from agricultural sources, with the remainder originating from industrial sources (Anon, 1990). Emissions of NH\textsubscript{3} from agriculture has risen dramatically in recent years, with a conservative estimate of 50% increase in Europe, between 1950 and 1980 (Anon, 1990). The increases in NH\textsubscript{3} are not uniform across Europe, as countries such as The Netherlands, Belgium and Denmark, which have some of the most intensive farming in the world, have demonstrated much higher increases in NH\textsubscript{3} emissions, than the UK. As a result, estimates of the average NH\textsubscript{3} emissions for the UK are approximately 18 kg ha yr\textsuperscript{-1} (Kruse \textit{et al.}, 1989). In contrast, an extreme emission range of 300-700 kg ha yr\textsuperscript{-1} total N, of which a high proportion is NH\textsubscript{3}, has been quoted for countries such as The
Netherlands, Belgium and Denmark (Wellburn, 1994). Although an average emission range of approximately 60-100 kg ha\(^{-1}\) yr\(^{-1}\) total N is more common for The Netherlands (van der Eerden, 1992).

Agricultural emissions arise essentially from two sources, firstly livestock waste which contributes 76% of agricultural emissions and secondly, the application of N-containing fertilisers. Livestock waste in particular pig, cattle and poultry slurry has a high N content, with NH\(_4^+\) accounting for well over 50% of the total N present (Pearson and Stewart, 1993). The pH of the slurry is often slightly alkaline, which promotes the release of NH\(_3\) by volatilisation into the atmosphere (Lockyer et al., 1989). Animal slurry is spread back onto the land to act as an N fertiliser which can lead to the rapid release of NH\(_3\) by volatilisation, especially in high sunlight. Chemical fertilisers applied to agricultural grasslands, also contain a high proportion of NH\(_4^+\) which has provided an extra source of NH\(_3\) in the environment. The incorrect application of NO\(_3^-\) containing fertilisers can also lead to NH\(_3\) release by volatilisation. Atmospheric concentrations of NH\(_3\) can vary dramatically from area to area, as emission sources tend to be very localised. Fangmeier et al., (1994) summarises a range of atmospheric concentrations of NH\(_3\) from several European countries, ranging from 0.2 µg m\(^{-3}\) NH\(_3\) in background air samples, to 47,000 µg m\(^{-3}\) NH\(_3\) in pig pens. Atmospheric concentrations of NH\(_3\) at any one time, are also influenced by environmental factors such as, diurnal variations in air temperature and seasonal variations in fertiliser application (Anon, 1990). Peak NH\(_3\) concentrations often coincide with peak noon day temperatures, resulting from temperature driven volatilisation in agricultural areas.

The localised nature of NH\(_3\) emissions and the influence of several environmental factors, has restricted regional monitoring of NH\(_3\) in the atmosphere. Similarly, measurements of the dry deposition
of NH$_3$ to ground receptors are complicated and not fully understood (Anon, 1990). The deposition of gaseous NH$_3$ takes place at or very near to the source of emission, as NH$_3$ tends not to travel long distances in the atmosphere, due to its high affinity for H$_2$O. As a result, the highest concentrations of gaseous NH$_3$ are found near to agricultural areas and are often centred around the distribution of livestock (ApSimon et al., 1987). Gaseous NH$_3$ deposition can occur over greater distances in countries that experience high emissions of NH$_3$ (Asman, 1994). However, a high concentration of NH$_3$ does not always lead to an increased deposition velocity, due to the influences of temperature, humidity and the pH of the receptor. In addition, some receptors, such as intensively N fertilised agrosystems can emit more NH$_3$ than they receive, thus complicating measurements of NH$_3$ deposition even further. The deposition of NH$_3$ to some receptors such as forest canopies and moorland is known to be quite rapid, resulting in the quick removal of NH$_3$ from the surrounding atmosphere (Sutton et al., 1992; Fangmeier et al., 1994). This most likely occurs as a result of a concentration gradient between the atmosphere and low N containing receptors such as moorland species. Also, the overall acidity of moorlands, coupled with a high water content, attract alkaline gases such as NH$_3$.

Deposition of NH$_3$ appears to be important in areas close to emission sources. However, the wet deposition of NH$_4^+$ is more important as a long-range pollutant. Since emissions of NH$_4^+$ are not thought to occur, NH$_4^+$ present in the atmosphere originates solely from the combination of H$_2$O and NH$_3$. NH$_4^+$ is initially formed as an aerosol with small particle diameters. In this form, NH$_4^+$ can travel several hundred km before eventually coalescing to form larger droplets, which are deposited in rain water. As a consequence, areas that experience high rainfall, for example Northern Europe, are often
subjected to the highest deposition of NH$_4^+$, despite the fact that they may be a long distance away from sources of NH$_3$ emissions. At high altitudes, NH$_4^+$ often remains as smaller particles for longer, forming cloud water and occult deposition. Occult deposition is a slow form of NH$_4^+$ deposition but often very concentrated, whereas, the most efficient and rapid deposition of NH$_4^+$ occurs during precipitation periods. Even so, occult deposition is important, as high altitude receptors may become constantly 'bathed' in pollution. Both cloud and rain water contain the aqueous NH$_4^+$ ion, or more commonly NH$_4^+$ salts. The most abundant salts formed by NH$_4^+$ are:

(i). NH$_3$ + HNO$_3$ $\leftrightarrow$ NH$_4$NO$_3$
(ii). NH$_3$ + H$_2$SO$_4$ $\Rightarrow$ (NH$_4$)$_2$SO$_4$
(iii). NH$_3$ + HCl $\leftrightarrow$ NH$_4$Cl
(iv). NH$_3$ + HCO$_3$ $\leftrightarrow$ (NH$_4$)$_2$CO$_3$

The formation NH$_4$NO$_3$, NH$_4$Cl and (NH$_4$)$_2$CO$_3$ are reversible, however, these compounds are often present in wet deposition. In comparison, (NH$_4$)$_2$SO$_4$ formation tends to be a one-way reaction (Asman, 1994). This feature, coupled with the abundance of sulphate (SO$_4^{2-}$) ions in the atmosphere resulting from fossil fuel burning, has led to the phenomenon of 'co-deposition' of NH$_4^+$ predominantly with SO$_4^{2-}$. Gaseous deposition of NH$_3$ is also enhanced by a synergistic interaction with SO$_2$, especially on wetted surfaces (McLeod et al., 1990). It is important not to totally discount other ammonium salts in wet deposition. Anon (1990) measured Cl$^-$ and combined marine / non-marine SO$_4^{2-}$ content of rain water at 59 monitoring sites in the UK. Cl$^-$ was comparable in concentration to SO$_4^{2-}$ at inland sites, with significantly higher concentration of Cl$^-$ at coastal sites. As a consequence, NH$_4$Cl deposition from the atmosphere could be relevant.
in both coastal and inland areas of the UK. \( \text{NH}_4^+ \) is a major neutralising compound present in the atmosphere. Despite this, \( \text{NH}_4^+ \) is often referred to as a component of acid deposition. This is due to the association of \( \text{NH}_4^+ \) with acidic compounds such as \( \text{H}_2\text{SO}_4 \) and \( \text{HNO}_3 \). The combination of these acids with \( \text{NH}_4^+ \) would eventually lead to neutralisation. However, due to the abundance of acidic compounds in rainfall such as \( \text{SO}_4^2- \), neutral conditions are seldom reached and acidity prevails (Asman, 1994). The subsequent assimilation of \( \text{NH}_x \) by plants, or by soil microbes, also leads to the release of \( \text{H}^+ \) causing increases in acidity.

Measurements of \( \text{NH}_x \) deposition in the past, have been included with measurements of total N deposition. The other main contributor to total N deposition is \( \text{NO}_x \). Substantial increases in \( \text{NH}_4^+ \) has resulted in the measurements of \( \text{NH}_x \) and \( \text{NO}_x \) both separately and in combination as total N deposition. Estimates of the contribution that \( \text{NH}_x \) makes to total N deposition vary; Anon (1990) estimates a range between 40-80%. The range depends on the dominant N pollution source, for example, \( \text{NO}_x \) is predominant in urban areas, \( \text{NH}_x \) is the main N containing pollutant in rural and agricultural areas. A range of \( \text{NH}_x \) deposition values are presented for Europe of between 2.9 - 136 kg \( \text{NH}_x \) ha\(^{-1}\) yr\(^{-1}\) (Pearson and Stewart, 1993); certain areas of The Netherlands can experience deposition rates well in excess of these values (Fangmeier \textit{et al.}, 1994; Pérez-Soba, 1995). The concentration of \( \text{NH}_4^+ \) in the atmosphere also demonstrates high variability, with ranges from \( \mu \text{mol m}^{-3} \) to mol m\(^{-3} \) concentrations. Despite the countless variations in atmospheric levels and deposition of \( \text{NH}_x \), the general opinion is that an overall increase in \( \text{NH}_x \) is occurring in the environment. As a result of this increase, the balance between influx and efflux of N in the N cycle has been affected. This will undoubtedly affect the various components of the N cycle, of which plants are one.
It is therefore necessary to consider how changes in the presence of NH$_x$ in the environment, have broadened its role as a plant nutrient, to include that of a potentially harmful atmospheric pollutant.

1.3 The effects of increased NH$_x$ on plants

The previous section has described how increases in NH$_x$ in the environment have arisen, the numerous forms that NH$_x$ can take in the atmosphere and the different ways that NH$_x$ can eventually be deposited. As mentioned earlier in section 1.1, foliar uptake of NH$_x$, in addition to root uptake, becomes more evident as atmospheric NH$_x$ increases. Foliar uptake is not a new feature, as many epiphytic and lithophytic mosses, plus wetland species, rely partly or solely on foliar uptake of N. This characteristic of mosses and wetland plants has led some workers to suggest that they are rapidly influenced by changes in the atmospheric concentration of NH$_x$ (Baxter et al., 1992; Soares and Pearson, 1996). Evidence for increases in foliar uptake for larger terrestrial species, in comparison to canopy throughfall and root uptake, has only recently begun to appear (Pearson and Stewart, 1993). The same authors have also compiled an extensive review on this subject. There are 3 major effects that can be attributed in part to increases in NH$_x$:— firstly that of eutrophication or fertilisation of ecosystems which may ultimately have a detrimental effect through changes in species composition; secondly, direct toxicity to plants arising from the presence of excess NH$_x$ and thirdly, acidification of ecosystems either directly from acid deposition containing NH$_x$ or indirectly from the assimilation of NH$_x$ by plants or soil microbes. The mechanisms by which these effects take place are undoubtedly different, however, the eventual loss of plants is the same.
1.3.1 Forest and heathland decline; the involvement of NH$_x$

Foliar damage and dieback of evergreen species such as pine and spruce, plus some deciduous broadleaf species such as oak and beech has been occurring in Northern Europe for some time. However, increased deposition of H$^+$ to the soil, was considered to be the main cause. Among the many soil effects caused by increased acidity were: - the solubilisation of heavy metals in the soil profile resulting in the generation of toxic concentrations; leaching of cations from upper soil horizons leading to decreased availability of nutrients from the soil. Some of these effects could equally apply directly to the foliage for example: - reductions in tissue cation content, foliar abrasion and needle loss were also evident (van Breemen and van Dijk, 1988). Nihlgård (1985) was one of the first workers to suggest that NH$_x$ may play a crucial role in explaining the occurrence of forest dieback in areas of Northern Europe and North America. Nihlgård postulated that the N content of acid deposition, in particular NH$_x$, was causing N saturation of forest ecosystems. Since forest ecosystems are often characterised by low N contents, severe nutrient imbalances between N and other compounds such as cations and carbohydrates would occur, leading to decreased growth and eventual death of trees. Resistance to other stresses, for example, frost hardiness is also impaired by increases in foliar N content (Dueck et al., 1990). Also, increases in N content of leaves invites more attacks from insects, fungi and bacteria (van der Eerden et al., 1991). Reports of damage by NH$_x$ to forest ecosystems are now widespread. Likewise, many more theories, centring around the effects of other atmospheric pollutants for example O$_3$, SO$_2$ and NO$_x$, have been postulated to explain forest decline; these are reviewed by Anon (1988b) and Wellburn (1994). In most cases, it is difficult to distinguish between the effects of individual pollutants. Therefore, the combined effects of all atmospheric pollutants are most
likely responsible for forest decline, the proportion of damage caused by individual pollutants will vary depending on geographical location to emission sources. One example is the close proximity of forests fringes in The Netherlands to agricultural land, where high concentrations of NH$_x$ in the atmosphere, have resulted in direct toxic effects (van der Eerden, 1992). In some instances, the extra N can be beneficial; Kenk & Fischer (1988) found a stimulation of growth in spruce and pine stands that had been subjected to levels of NH$_x$, up to 1000 kg ha$^{-1}$ for a period of 30 years. However, the sites studied were intensively managed and damage was evident on non-managed plots.

NH$_x$ in conjunction with other N-containing pollutants, is considered to be responsible for the eutrophication of ecosystems, which has led to transitions from one species or type to another. An example of this is the conversion of heathland to grassland in The Netherlands (van der Eerden et al., 1991). Heathlands are characterised by limited nutrient availability and climatic conditions that lead to the development of slow growing climax species such as *Calluna vulgaris*. Increases in N deposition to these areas, results in an increase in faster growing species, that take advantage of the greater N availability. These species of which certain grasses are an example:- *Deschampsia flexuosa, Festuca ovina* and *Molinia caerulea*, are able to out compete the slower growing species for nutrients, leading to vegetation transitions. The transition of heathland to grassland results from both eutrophication and acidification caused by NH$_x$. Heathland communities tend to be naturally acidic and are unable to buffer effectively against increases in acidity, whereas the greater vitality of the developing pioneer species offers better physiological buffering against acidity. Wetland and moorland areas of the UK, have shown a decline in their characteristic flora, for example *Calluna vulgaris*, *Sphagnum* and other moss species, as a result of NH$_x$ and acidity (Lee
& Studholme 1992). The dominant moss species are replaced by seed bearing plants that can take better advantage of the extra N and cope with increases in acidity.

1.3.2 Critical loads and levels of $\text{NH}_x$

Evidence of specific habitat damage is now accumulating, with forest vegetation, heathlands and wetlands demonstrating the most damage as a result of increases in $\text{NH}_x$ and other pollutants. Although these habitats revealed the most significant responses to atmospheric pollution, in particular acidic deposition of $\text{NH}_x$, $\text{NO}_3^-$ and $\text{SO}_4^{2-}$, other vegetation types are also subject to damage. These include both acidic and chalk grasslands, meadowlands and agricultural areas, that are subject to high levels of $\text{NH}_3$ and other pollutants such as $\text{O}_3$ and $\text{NO}_x$.

A need is identified to quantify the role of atmospheric pollutants in causing ecosystem damage. Following collaboration between scientific working groups and European governments, the concepts of 'critical levels' and 'critical loads' first appeared in the late 1980's. A critical load is defined as:

'\text{the maximum deposition of a given compound which will not cause long-term harmful effects on ecosystem structure and function, according to present knowledge}'.

A combination of geological data, climatic data and biological data leads to the production of critical loads maps that identify areas that are sensitive or tolerant to specific pollutant types. The critical loads concept is still in its infancy in terms of experimental data, although relatively high resolution maps exist for critical loads of acidity for soils, freshwaters and combined soil-vegetation systems in the UK and Europe (Anon, 1995a). The specific role of $\text{NH}_x$ in the establishment of critical loads for vegetation has yet to be considered in detail. Critical load maps of acidity include a N and S complement; in the case of N, a
distinction is made between acidifying and fertilising N. However, individual N compounds are not always identified (Posch et al., 1995) and very little effort has been made to establish critical loads for individual N compounds such as NH$_4^+$, and NO$_3^-$ (Anon, 1995b). The complexity of the N cycle, coupled with the multitude of individual plant responses, have added to the difficulty in establishing critical loads of N for specific plant species, as opposed to generalised vegetation types. Latour and Staritsky (1995) suggest a database of approximately 10,000 to 20,000 relevées for each country, plus detailed information on soil types and deposition rates, in order to relate critical loads directly to plant species. At present, data of this magnitude is not available. The importance of N as a plant nutrient has also led some workers to suggest that there is no threshold below which excess N deposition could influence ecological processes (Lee and Caporn, 1993). Critical levels are defined as:

'\text{the concentration of pollutants in the atmosphere above which adverse effects on receptors, such as plants, ecosystems or materials, may occur according to present knowledge}'.

Critical levels are not totally exclusive from critical loads, as both concepts lead to the production of 'pollutant exceedence' maps, that identify susceptible habitats (Anon, 1995a). However, critical levels are used more significantly, to distinguish between direct foliar exposure and indirect pollutant exposure by deposition to the soil. Critical levels for vegetation were most recently evaluated by Anon (1993b); critical levels of SO$_2$, NO$_2$, O$_3$, acid rain and NH$_3$ were cited. The experimental data available at that time was most consistent for SO$_2$, NO$_2$ and O$_3$. As a result, critical level exceedence maps of SO$_2$, NO$_2$ and O$_3$ are now produced for a range of vegetation types for several European countries, including the UK (Anon, 1995a). For NH$_3$ and acid rain, the data available on direct foliar effects was not as
abundant. Van der Eerden et al., (1993) calculated critical levels of \( \text{NH}_3 \) for 4 exposure times: (i) 1 hour = 3300 \( \mu g \) m\(^{-3} \); (ii) 1 day = 270 \( \mu g \) m\(^{-3} \); (iii) 1 week = 23 \( \mu g \) m\(^{-3} \); (iv) 1 year = 8 \( \mu g \) m\(^{-3} \). These levels were generalised from limited data assessing the effects of \( \text{NH}_3 \) on heathland, mosses and horticultural species. For acid rain (including occult deposition), no formal critical level is quoted. Cape (1993) estimates a range of between pH 3 - pH 3.5 for visible injury, depending on the sensitivity of the receptor and the ion content of rain and cloud water. At this stage, little indication was given of the influence \( \text{NH}_4^+ \) may have in determining critical levels for acid rain; the vast majority of work had concentrated on \( \text{SO}_4^{2-} \) content of rain and cloud water. The critical levels and loads approach has helped to further clarify susceptible and non-susceptible habitats and postulated several pollutant thresholds, above which damage may be evident. These thresholds are often calculated as a result of an accumulation of experimental data, where no standard protocols for experimental procedure exist. Complex mathematical and computational models often provide the alternative to long-term monitoring and the gathering of biological and ecological data. Thus, critical loads and critical levels are used as a guideline only, especially for pollutants like \( \text{NH}_x \) that have yet to be studied in detail. The emphasis on \( \text{NH}_x \) in critical loads and levels research, is likely to increase, as abatement protocols are finally produced for pollutants such as \( \text{SO}_2 \) and \( \text{NO}_2 \) that have been studied for a relatively longer period.

1.4 Aims of this thesis

Growth perturbations and physical damage to plants, which have also led to changes in species composition for some ecosystems, have provided disturbing evidence of the effects of atmospheric pollutants
on plants. However, the realisation of these changes has often only occurred following prolonged periods of study. Levels of atmospheric pollutants, in particular NH\textsubscript{x}, are continuing to increase with no immediate signs of reductions. As a result, emphasis is beginning to be placed on methods of rapid assessment of biological and ecological change, in advance of visible damage and species transitions from one type to another. The need for more rapid methods of assessing plant responses to atmospheric pollutants, has been identified for some time (Mathy, 1988b). Before the onset of visible damage, a multitude of physiological and metabolic changes will undoubtedly occur within plants. Physiological responses of plants to atmospheric pollutants, including NH\textsubscript{x}, have received little attention in the literature, in comparison to a large bulk of assessment of visible responses (Fangmeier \textit{et al.}, 1994). What physiological observations have been carried out, will be considered in more detail in the introductory sections of each of the experimental chapters, with particular reference to atmospheric NH\textsubscript{x} in each case. By assessing these types of response, it may be possible to evaluate potential damage from excess NH\textsubscript{x} sooner, than long-term growth and ecosystem studies. Also, it may be possible to relate physiological responses directly to critical loads and levels research, where responses are studied over a wide range of NH\textsubscript{x} concentrations and vegetation types.

This thesis sets out to identify short-term enzymatic and metabolite responses that occur in the presence of excess atmospheric NH\textsubscript{x}. The response of GS in the presence of excess NH\textsubscript{x} will be studied, along with the responses of other enzymes, nutrients and metabolites, that may reflect primary or secondary responses to NH\textsubscript{x} and acidity. In addition to enzymatic and biochemical responses, investigations into the photosynthetic response of plants to excess NH\textsubscript{x} and acidity will also be considered. This will include measurements of photosynthetic rate and
stomatal conductance. Attempts will be made to distinguish between the effects of NH$_4^+$, NH$_3$ and NH$_4^+$ plus acidity at a range of concentrations, for a wide variety of plants. The effects of direct foliar uptake will be considered, in light of the increasing importance of this method of N uptake in polluted environments. The above variety of physiological responses, will be evaluated for their potential use as indicators of short-term effects of NH$_x$ pollution. A study of the short-term physiological responses of mosses to excess NH$_4^+$ and NO$_3^-$ is undertaken, to contrast the responses of the two N compounds and also, to compare the responses of mosses and higher plants to excess NH$_x$ and acidity. N incorporation in mosses from different rural and urban pollutant exposures will be assessed, as a means of monitoring N pollution.

A survey of the physiological characteristics of plants that reflect aspects of N metabolism and possible mechanisms of acid buffering will be conducted, in an attempt to define the susceptibility of plants to atmospheric NH$_x$. Enzyme activities and metabolite concentrations in the leaves of plants, in the absence of atmospheric NH$_x$ pollution, will be assessed using multivariate techniques. Thus, intrinsic N metabolism and acid buffering in plants will be used to explain why certain species show pollution damage more rapidly than others.
CHAPTER 2

Materials and Methods

2.1 Chemicals

Laboratory chemicals used were of AnalAr@ grade and obtained from the Sigma Chemical Company Ltd (Poole, UK). Stable isotope-labelled ammonium sulphate ($^{15}$NH$_4$)$_2$SO$_4$, ammonium chloride ($^{15}$NH$_4$Cl) and potassium nitrate (K$^{15}$NO$_3$) were obtained from the Aldrich Chemical Company (Milwaukee, USA), at 98% atom enrichment. Gas cylinders of N$_2$, NH$_3$ and O$_2$ were obtained from BOC Ltd (Guildford, UK). Chemicals for automated nitrogen and carbon analysis and mass spectrophotometry (ANCA-MS) were obtained from Europa Scientific (Crewe, UK). Grade 5 purity oxygen used for combusting samples for ANCA-MS was obtained from Air Products Plc (Chineham, UK).

2.2 Experimental and collection field sites

Samples of leaf material for plants used for physiological screening (Chapter 3) were collected from sites in Hertfordshire and Bedfordshire, UK. Material was placed in sealed plastic bags and transported to the laboratory in a cooler bag.

Samples of the mosses Rytidiadelphus loreus (Hedw.) Warnst. (R. loreus) and Racomitrium lanuginosum (Hedw.) Brid. (R. lanuginosum) were treated in-situ and collected from acidic grassland (R. loreus) and rocky detritus (R. lanuginosum), at Whitewell, Aviemore, Scotland, UK, Alt. 330 m. R. loreus was collected from a mixed acid grassland, the species most commonly present were :-

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*Festuca ovina* (L.) and *Anthoxanthum odoratum* (L.). *R. lanuginosum* was collected from almost pure stands. Samples of the moss *Philonotis fontana* (Hedw.) Brid. (*P. fontana*) were collected from the side of a wet flush, at An t-Aonach on the north facing side of Cairn Gorm, Scotland, UK, Alt. 560 m. *P. fontana* was also collected from almost single species cushions. Lithophytic mosses were collected from urban sites in London and rural sites in Hertfordshire and Devon (Table 6.1). The species collected were *Homalothecium sericeum* (Hedw.) Br. Eur. (*H. sericeum*), *Tortula muralis* Hedw. (*T. muralis*) and *Grimmia pulvinata* (Hedw.) Sm., Eng. Bot (*G. pulvinata*). Moss samples were removed from wall surfaces, at a height of 1.0 metre above ground level and within 5 metres of the road side. At the point of collection, a rough estimate of the flow of motor vehicles was made, to assign each collection point to a heavy, medium, light or very light traffic exposure (Table 6.2). The above bryophyte species were used for studies in Chapter 6.

### 2.3 Plants grown under greenhouse conditions

Two year old seedlings of *Alnus glutinosa* L. Gaertner (*A. glutinosa*), *Prunus padus* L. (*P. padus*), *Quercus robur* L. (*Q. robur*) and *Pinus sylvestris* L. (*P. sylvestris*) were obtained from The Royal Holloway and Bedford New College Botanical gardens, Egham, UK. Two year old seedlings of *Picea sitchensis* (Bong.) Carriere (*P. sitchensis*) and *Populus deltoides* Marshall (*P. deltoides*) were obtained from The Forestry Commission, Edinburgh, Scotland, UK. Seedlings (approx. 0.5 - 1.0 m height) were re-potted into 25 cm diameter pots containing a ratio of 1:1:1 finely sieved topsoil, peat and medium grain horticultural sand. The plants were watered with dH₂O twice weekly and maintained in a greenhouse at 20°C : 15°C (Day : Night) under
natural daylight or under supplemented natural lighting for a 16 hour day length during winter (total approx. 88 - 120 μmol m⁻² s⁻¹ for winter; 180 - 220 μmol m⁻² s⁻¹ for summer).

Samples of *Calluna vulgaris* (L.), Hull (*C. vulgaris*) were collected from High Ash Head Moor, NE of Lofthouse, North Yorkshire Moors, UK. Intact plant and root systems were transplanted into 18 cm diameter pots containing ericaceous soil (Fisons, UK). *C. vulgaris* was grown in a greenhouse, under the same conditions as above.

Seeds of *Phaseolus vulgaris* cv tendergreen (L.) (*P. vulgaris*) were obtained from seed suppliers. Seeds were germinated in Petri dishes prior to potting out. The plants were then grown in a loam based compost (John Innes No. 2) in 18 cm diameter pots with saucers. The plants were watered twice weekly with ⅓ strength Long Ashton solution (Hewitt, 1966) and once weekly with dH₂O. For some investigations, *P. vulgaris* was grown under different NO₃⁻ regimes. The NO₃⁻ content of the ⅓ strength Long Ashton solution was altered to contain 0.6 mol m⁻³ (low NO₃⁻), 6 mol m⁻³ (moderate NO₃⁻) and 12 mol m⁻³ (high NO₃⁻). Plants were maintained in a greenhouse at 25°C : 15°C (Day : Night) under summer day length light or for a 16 hour day length, under supplemented natural lighting during shorter daylight periods (total approx. 135 - 150 μmol m⁻² s⁻¹ for winter; 250 - 275 μmol m⁻² s⁻¹ for summer). Once the plants had reached full maturity (approx. 1 month after germination) treatment regimes were applied. Nomenclature used for higher plant species follows that of Stace (1991) and for mosses, Smith (1980).
2.4 Enzyme assays

Enzyme assays were carried out on fresh current year leaf material. Activities were determined within 1 hour of collection from field sites, or immediately after a timed laboratory or greenhouse experiment. Enzyme extraction from plant material was carried out at 4°C. Incubation conditions for the enzyme assays consisted of a water bath maintained at 25°C. In establishing enzyme activities for new species, initial tests were carried out with different volumes of extract and for different lengths of time to check for linearity and proportionality of enzyme activity.

2.4.1 Nitrate reductase (E.C.1.6.6.1) (NR)

Nitrate reductase activity in leaves was determined using the *in-vivo* method adapted from Havill *et al.* (1974) by Stewart and Orebamjo (1979). 0.1 g of leaf material taken from the central area of the leaf (excluding midrib) were used for each determination, except for moss species, where 0.05 g of apical tissue (upper 2 cm of shoot) were used. Tissue was placed in glass vials and 5 ml of fresh NR assay medium containing 500 μmol KH₂PO₄, 1.5% propanol and 750 μmol KNO₃ (pH 7.5) was added (Smirnoff *et al.* 1984). Glass vials containing samples were placed in a desiccator and the tissue was vacuum infiltrated for 1-2 min, during which time the vacuum was released several times. The desiccator, containing samples, was then incubated in a covered water bath for 1 hour. 1 ml of sample was removed and 1 ml of 1% (w/v) sulphanillic acid plus 1 ml of 0.02% N-naphthyl ethylene diamine dihydrochloride (NEDD) were added. Colour was left to develop for 20 min, after which the optical density (OD) of the solution was determined at 540 nm using a Beckman DU-7 UV / Visible spectrophotometer.
2.4.2 Glutamine synthetase (E.C.6.3.1.2) (GS)

Extracts of 0.2 g of leaf material were used to determine GS activity. The extraction was carried out using a modified method of Stewart et al. (1988). The extraction buffer contained 50 mol m$^{-3}$ Tris-Cl pH 7.6, 2 mol m$^{-3}$ EDTA.Na$_2$, 2 mol m$^{-3}$ MgSO$_4$, 20.0 mol m$^{-3}$ 2-mercaptoethanol, 5 mol m$^{-3}$ dithiothreitol and 5 mol m$^{-3}$ reduced glutathione. The use of the latter 3 sulphhydryl compounds help to maintain the integrity of the extracted enzyme (Dawson et al. 1986). Non - Idet p40 (detergent) was used to extract optimum enzyme activity (Vezina et al. 1988) at a concentration of 1.5% for Q. robur, P. sitchensis and P. sylvestris, 1.0% for all other plant species (Pearson and Ji, 1994). The extraction was carried out in a chilled mortar and pestle (4°C) with 6.0 ml of extraction buffer, acid washed sand and polypyrrolidine. Extracts were then centrifuged at 10,000 g for 20 minutes at 4°C. The semi-biosynthetic method of Rhodes et al. (1976) was used to determine GS activity. 0.1 ml of the supernatant was incubated in a final volume of 1.0 ml containing 4 μmol ATP, 15 μmol MgSO$_4$, 4 μmol hydroxylamine, 30 μmol glutamic acid and 40 μmol Tris-Cl buffer (pH 7.5), at 25°C for 1 hour. 1 ml of ferric chloride reagent (FeCl$_3$ 26 g L$^{-1}$; trichloroacetic acid 40 g L$^{-1}$; conc HCl 80 ml L$^{-1}$) was added to the above to stop the reaction. The protein precipitate formed on addition of the ferric chloride solution was removed by centrifugation at 10,000 g for 5 min. The OD of the supernatant was read at 500 nm against a blank without ATP for each sample.

2.4.3 NADP$^+$ malic enzyme (E.C.1.1.1.40) (ME)

Extracts of 0.2 g of leaf material were used to determine ME. The extraction method and buffer used were identical to those used for GS. Activity of ME was determined using the method of Davies et al.
(1974). The assay was started with the addition of 0.1 ml of extract to a final volume of 2.0 ml containing 160 μmol MOPS buffer (pH 7), 50 μmol MnCl₂·4H₂O, 10 μmol L-Malic acid and 0.5 μmol NADP. The change in OD at 340 nm was measured over 300 seconds to check for linearity of the response. The stability of the reaction was determined by leaving out substrate and cofactors in turn and ensuring zero change in OD in their absence.

2.5 Protein determination

The GS / ME extract (25 μl) was added to 2.5 ml of protein reagent (Bio-rad, Hemel Hempstead, UK) (Bradford, 1976). OD of the resultant solution was determined at 595 nm. Small volumes of extract were used to prevent interference in colour development by detergent and sulphydryl compounds (Bradford, 1976).

2.6 Organic acid determination

Extraction of organic acids was carried out on current year leaf material. 0.5 g of fresh leaf tissue was ground in a chilled mortar and pestle (4°C), with acid washed sand and 0.5 ml of 1 M perchloric acid. Extracts were then centrifuged at 10,000 g for 20 min at 4°C. The above extraction was carried out within 1 hour of collection from field sites, or immediately after a timed laboratory or greenhouse experiment. The supernatant was then neutralised using 5 M K₂CO₃ and methyl orange as an indicator.

2.6.1 L-(-)-Malate

Malate was determined using the version of Mollering (1985a). 0.1 ml of supernatant was added to a volume of 1.89 ml containing 153
μmol glycine (pH 10), 100 μmol L-glutamate (final pH 10), 4.0 μmol β-NAD and 61 μg glutamic-oxalacetic-transaminase (E.C.2.6.1.1) (GOT). The OD (340 nm) of the solution was determined. 30 μg malic dehydrogenase (E.C.1.1.1.37) (MDH) was added to the solution to start the reaction, creating a final volume of 2.0 ml. The rise in OD was followed at 340 nm until a plateau was reached and δ OD was calculated. δ OD was used to determine the concentration of malate in the extract.

2.6.2 Citrate
Citrate was determined using the version of Mollering (1985b). 0.1 ml supernatant was added to a volume of 1.89 ml containing: - 352 μmol Tris-Cl buffer (final pH 7.6), 3.0 μg MDH, 74 μg lactic dehydrogenase (E.C.1.1.1.27) (LDH) and 1 μmol β-NADH. The OD (340 nm) of the solution was determined. 2.0 mg of citrate lyase (E.C. 4.1.3.6) (CL) was added to the solution to start the reaction, creating a final volume of 2.0 ml. The decline in OD was followed at 340 nm until a plateau was reached and δ OD was calculated. δ OD was used to determine the concentration of citrate in the extract.

2.6.3 2-Oxoglutarate
2-Oxoglutarate was determined using the version of Burlina (1985). 0.1 ml supernatant was added to a volume of 1.89 ml containing: - 370 μmol Tris-Cl buffer (final pH 7.6) and 0.4 μmol β-NADH. The OD (340 nm) of the solution was determined. 136.0 μg of L-glutamic dehydrogenase (E.C.1.4.1.3) (GDH) was added to the solution to start the reaction, creating a final volume of 2.0 ml. The decline in OD was followed at 340 nm until a plateau was reached and δ OD was calculated. δ OD was used to determine the concentration of 2-oxoglutarate in the extract.
2.6.4 Pyruvate

Pyruvate was determined using the version of Lamprecht and Heinz (1984). 0.1 ml supernatant was added to a volume of 1.89 ml containing: 370 µmol Tris-Cl buffer (final pH 7.6) and 0.4 µmol β-NADH. The OD (340 nm) of the solution was determined. 124.0 µg of LDH was added to the solution to start the reaction, creating a final volume of 2.0 ml. The decline in OD was followed at 340 nm until a plateau was reached and Δ OD was calculated. Δ OD was used to determine the concentration of pyruvate in the extract.

2.7 Leaf buffering capacity index

Leaf buffering capacity index (BCI) was determined using a modified version of Bender et al. (1990). Current year leaf material (0.5g) was ground with liquid nitrogen in a mortar and pestle. The ground material was added to 15 ml of boiled ddH₂O and whirlly mixed for 23 min. The solution was left to stand for 20 min after which time the pH of the solution was recorded using a Radiometer PHM82 standard pH meter with a Hanna HI 12306 gel filled electrode. This was used as a measurement of initial leaf pH. 0.02 M HCL was then added to the plant extract in 10 -100 µl aliquots until the initial pH dropped by 1 pH unit; the volume of acid used to achieve this was recorded. The index was calculated as:

\[
\text{BCI} = \frac{\mu_{eq} H^+}{(\Delta \mu_{eq} H^+)(W)}
\]
where $\mu_{eq}$ is the amount of acidity required to alter the leaf homogenate by 1 pH unit, divided by the calculated change of $H^+$ $(\Delta \mu_{eq}, H^+)$ concentration, per gram fresh weight ($W_f$).

2.8 Cation determination

Extraction of the cations calcium ($Ca^{2+}$), magnesium ($Mg^{2+}$), and potassium ($K^+$) took place by acid digest, following the routine of Allen (1974). 0.1 g of current year leaf material was added to 4.4 ml of acid digest mix (350 ml $H_2O_2$ (30% w/v), 420 ml 18 M $H_2SO_4$, 0.42 g selenium and 14.0 g $LiSO_4$) in acid washed, thick walled boiling tubes. Tubes were then heated to 360°C in 60°C steps for 10 min each. Once full temperature was achieved, the samples were left at 360°C for 2 hours until all plant material had been digested and the liquid became clear. Samples were left to cool to room temperature and dd$H_2O$ was added to achieve a total volume of 50 ml. Concentrations of $Ca^{2+}$, $Mg^{2+}$ and $K^+$ were determined from the 50 ml samples, using a Pye Unicam SP9 atomic absorption spectrophotometer.

2.9 Total nitrogen and phosphate determination

Total N and phosphate ($PO_4$) were measured from the 50 ml samples prepared as above. Total N was determined from 1.0 ml of neutralised sample by the ammonia method of McCullough (1967). $PO_4$ was determined from 1.0 ml of sample by the molybdate-antimony method of Golterman (1978).
2.10 \(^{15}\)N stable isotope and total nitrogen analysis using ANCA-MS

The technique of ANCA-MS used for stable isotope \(^{15}\)N and total N analysis, is described by Barrie & Lemley (1989). Samples of plant material exposed to labelled nitrogen sources were firstly washed 3 times in ddH2O to remove surface adsorbed \(^{15}\)N, then dried in a microwave oven on low power for 10 min. Dried material was ground to talcum powder consistency (< 250 \(\mu\)m particle diameter) using a MM2 ball mill (Retsch GmbH and Co., W.Germany) and sealed in 6 mm X 4 mm methanol washed tin capsules (2 - 10 \(\mu\)g sample per capsule). Samples were then analysed for \(^{15}\)N and total N using ANCA-MS, (Europa Scientific Ltd, Crewe, UK). Plant samples analysed for \(\delta^{15}\)N natural abundance were prepared as above and analysed using ANCA-MS.

2.11 Photosynthetic measurements

2.11.1 \(O_2\) evolution

Measurements of PS using \(O_2\) evolution were adapted from the method of Walker (1987). Leaf portions excluding the midrib, whole leaflets or needles were detached from plants and immediately placed inside an air tight chamber (7 cm\(^3\) volume), forming part of a gas phase leaf disc oxygen electrode (Hansatech, King’s Lynn, UK). The chamber allows for a 10 cm\(^2\) leaf to be placed inside. Leaves greater than 10 cm\(^2\) in area were cut with a 10 cm\(^2\) circular cutter to provide an accurately sized leaf portion. In the case of needle shaped leaves, or leaflets that were smaller than 10 cm\(^2\) in total area; these were placed along side each other in the leaf chamber to avoid overlapping. After measurement of PS, the leaves were placed under a clear acetate sheet
and their combined area was scanned using a hand held scanner connected to a PC. This gave an exact area measurement, which could then be incorporated into the calculation of PS. A mixture of 5% CO$_2$ balanced with air was injected into the chamber via a gas port. This concentration of CO$_2$ is required to allow continuous PS measurements, without the rapid depletion of CO$_2$ (Walker, 1987). A high concentration of CO$_2$ also allows uptake of CO$_2$ directly via the cuticle, as stomata may close in excised leaves (Walker, 1987). The entire chamber and electrode assembly were maintained at a temperature of 20°C with water circulating inside an integral water jacket. A tungsten-halogen light source was connected directly to the top of the chamber and electrode assembly, providing a light intensity of 1800 μmol m$^{-2}$ s$^{-1}$ at the leaf surface. O$_2$ evolution from the leaf surface was recorded polarographically using a platinum cathode and silver anode embedded in epoxy resin (Walker, 1987). The poles of the electrode were linked using an electrolyte consisting of 1 part 0.4 M H$_3$BO$_4$ + 0.4 M KCl (final pH 9); 1 part saturated KCl; 2 parts 1.0 M NaHCO$_3$ (pH 9). The electrode assembly was separated from the leaf chamber using an O$_2$ permeable polythene membrane. The electrode was connected via an analogue interface to an IBM computer allowing ‘real-time’ measurements of O$_2$ evolution to be viewed on screen and recorded. The period of time between leaf detachment, placement in the chamber, the introduction of 5% CO$_2$ and the establishment of steady state O$_2$ evolution was approximately 2 minutes. O$_2$ evolution was then recorded for a further 2 min to give control rates of PS. Prior to introduction of plant material, the chamber and electrode assembly were calibrated using zero grade N$_2$ gas, to create optimal conditions for the measurement of O$_2$ evolution.
2.11.2 CO₂ fixation and water exchange

In-situ leaf (6.25 cm²) was clamped inside an air tight portable leaf chamber (LCA 4, ADC Ltd, Hoddesdon, UK). Leaves were clamped routinely in the same position to standardise the leaf portion used. This consisted of a central portion of the leaf, excluding the midrib where possible. For species with a leaf area smaller than the leaf chamber, several non detached leaflets or needles were clamped inside the chamber, then scanned as described above after PS was measured to give a measurement of their combined leaf area. Measurements of PS using CO₂ fixation were recorded using a portable LCA-4 Infra Red Gas Analyser (IRGA) (ADC Ltd, Hoddesdon, UK) connected to the portable leaf chamber. A reference gas of measured ambient CO₂ balanced with air was allowed to pass over the leaf surface at 300 ml min⁻¹ for 2 min for equilibration. The IRGA contrasted the reference ambient CO₂ concentration to the CO₂ concentration within the chamber, to give a measurement of the rate of CO₂ fixation. In addition, the IRGA was also used to determine stomatal conductance and transpiration rates, by comparisons of the water vapour content the reference gas and the leaf chamber.
CHAPTER 3

Physiological responses of plants to atmospheric NH$_x$

3.1 Introduction

3.1.1 Visible versus invisible responses of plants to NH$_x$

The exposure of plants to atmospheric pollutants, such as NH$_x$, can result in an abundance of responses. Saxe (1991) conveniently separated these into two distinct categories: visible responses, where changes caused by the presence of a pollutant, can be visually distinguished from a non-exposed individual; invisible responses, where changes occur at a biochemical and cellular level.

Previous work on visible responses has included Firstly, growth responses, for example changes in root / shoot ratios, crop yield, quality and dry matter accumulation; secondly, foliar damage such as leaf fall, leaf chlorosis and necrosis; and thirdly, changes in species survival and composition at the level of the ecosystem, for example forest dieback and heathland to grassland transitions. The above studies account for the bulk of research into the effects of NH$_x$ on plants and have been extensively reviewed by Mathy (1988a); Anon (1988a); Anon (1988b); Zöttl (1990); Pearson and Stewart (1993) and Fangmeier et al. (1994). Studies of visible effects can contribute towards greater understanding of biological responses and biochemical change. They also provide one approach to the biological assessment of critical loads and levels for ecosystems (Anon 1993a; Anon 1993b).

From initial exposure to atmospheric NH$_x$, to eventual changes in leaf integrity, growth dynamics and species composition, a period of
time exists where a multitude of invisible physiological and biochemical changes occur (Figure 3.1). Although combined studies of both visible and invisible perturbations provide a useful and complete approach to studying the impact of atmospheric pollutants on plants (Wild and Schmitt, 1995), recent research has begun to concentrate specifically on invisible changes. This is partly due to a general reduction in some pollutant levels for example, \( \text{SO}_2 \) in developed countries, which can cause visible foliar damage (Treshow and Anderson, 1989). Where levels of pollutants greatly exceed background levels, such as \( \text{NH}_x \) emissions from livestock in the Netherlands (Voorburg and Monteny, 1990), visible injury does not always occur (van der Eerden and Pérez-Soba, 1992; van der Eerden et al., 1992). More importantly, visible injury cannot always distinguish the type of pollutant, i.e. gaseous or wet deposited, or whether it is a single pollutant or pollutant mixture that is causing the damage. Likewise, it is difficult to distinguish between direct effects from foliar uptake and indirect effects from soil uptake. This situation may be particularly applicable to \( \text{NH}_x \) pollution.

Given the above points, it is clear that a more sensitive and representative method of determining the effects of \( \text{NH}_x \) on plants will, in some instances, be required. The use of physiological and biochemical responses has the potential to achieve this, by revealing more subtle reactions to atmospheric pollution, which can occur long before visible evidence is present. Wellburn (1994) describes the use of physiological and biochemical responses as being infinitely superior to the use of visible injury, especially where visible injury may not be solely associated with air pollution. Evaluation of physiological and biochemical responses may lead to the rapid assessment of the potential damage plants may experience in the presence of excess \( \text{NH}_x \), as well as being relevant to more detailed assessment of underlying causes.
Figure 3.1 Stages in foliar plant responses to atmospheric pollutants.
(Adapted from Anon, 1988b)
3.1.2 Pre-requisites for physiological responses to excess \( \text{NH}_x \)
In order for a physiological response to be used as an indicator of \( \text{NH}_x \) pollution, there are several factors that need to be considered. \( \text{NH}_x \) pollution will undoubtedly cause a complex chain of reactions, many of which may reflect other stress factors. Physiological responses therefore need to be specific to the pollutant being investigated and easily and quickly measurable, producing unambiguous results over a wide range of pollutant concentrations and plant species. Physiological processes in plants are however often non-uniformly assigned to metabolic processes and reactions (Wild and Schmitt, 1995). This potential ambiguity can be overcome by considering a mixture of physiological parameters at the same time, ideally those with a known or hypothesised involvement with the particular pollutant under study.

3.1.3 Previous work on physiological responses to \( \text{NH}_x \)
Using physiological responses as indicators of damage to plants in the presence of atmospheric pollutants is by no means a new concept. Rabe and Kreeb (1979), Darrall and Jäger (1984), Darrall (1989) and Saxe (1991) have conducted extensive reviews of physiological responses to a range of atmospheric pollutants such as \( \text{SO}_2 \), \( \text{NO}_2 \) and \( \text{O}_3 \). As a result, a variety of enzymatic and metabolic responses have been isolated and characterised that are specific to these pollutants. However, little mention was made in these instances to \( \text{NH}_x \) pollution. Previous work on \( \text{NH}_x \) pollution has concentrated on \( \text{NH}_x \) effects in association with total N deposition and acid rain (Wellburn, 1994). Research has however been carried out on tissue N content, nutrient and amino acid changes, in response to \( \text{NH}_x \) pollution. Fangmeier et al. (1994) found a general increase in the above parameters in plants treated with \( \text{NH}_x \). However, the responses were variable and did not
always reflect the concentration of applied NH₄⁺, or the duration of the experiment. Clough (1993) and Richter et al., (1995) also commented on the large degree of seasonal variability in N and amino acid contents of plants and the non uniformity of individual amino acid changes, in relation to atmospheric pollution. These factors make it potentially difficult to quantify changes in amino acids and in some cases total N, as a result of excess NH₄⁺ deposition, especially where similar changes also take place in the presence of NOₓ pollution.

It is only recently that enzymatic and metabolite responses specific to NH₄⁺ have been considered in more detail. One such enzymatic response is the induction of GS activity in the presence of excess NH₄⁺ (Pérez-Soba et al., 1994). GS is the major enzyme of NH₄⁺ assimilation in higher plants and its enzymatic response will be considered in more detail in this chapter.

### 3.1.4 Identifying physiological responses to excess NH₄⁺

The role NH₄⁺ plays in the normal N nutrition of a plant offers the possibility of identifying physiological responses that may show a significant change in the presence of excess atmospheric NH₄⁺. This investigation will consider aspects of N metabolism in plants that are directly and indirectly associated with the assimilation of NH₄⁺. These include enzyme activities of GS and NR, the main enzymes of N assimilation in plants. Changes in the concentration of organic acids will also be considered, as these are indirectly involved in the metabolic pathways of NR and GS, thus can be related to the uptake of NH₄⁺ and subsequent effects. Methods of pH regulation in response to H⁺ inputs from NH₄⁺ assimilation, which may involve enzyme activities of ME and organic acids, will also be considered.
3.2 Experimental design

3.2.1 Applications of NH$_4^+$

Eight plant species were screened for their short-term responses to misting with NH$_4^+$, both in the field and under greenhouse conditions. The species were: *Brachythecium rutabulum* (Hedw.) Br. Eur., *B. rutabulum*; *Glechoma hederacea* L., *G. hederacea*; *Lamium album* L., *L. album*; *Prunus padus* L., *P. padus*; *Pinus sylvestris* L., *P. sylvestris*; *Quercus robur* L., *Q. robur*; *Erica tetralix* L., *E. tetralix* and *Populus deltoides* Marshall., *P. deltoides*. Mistings in the field were applied to *B. rutabulum*, *G. hederacea*, *E. tetralix* and *L. album* and took place at field sites described in Chapter 2. A 1 L solution containing 3 mol m$^{-3}$ NH$_4$Cl at pH 5 was applied evenly over a 1 m$^2$ area, which contained at least 3 individuals of each species. Solutions were applied with a continuous flow hand-held mister. The treatment for each species took place at midday. Plant material was collected 24 hours later from treated and nearby control plants and returned to the lab immediately for analysis.

Tree species misted in a greenhouse (*P. padus*, *P. sylvestris*, *Q. robur* and *P. deltoides*) were grown as described in Chapter 2. Individuals of each species (n = 3) were placed inside a 1 m$^3$ misting cabinet. A 1 L solution of 3 mol m$^{-3}$ NH$_4$Cl was applied, using a rotating spray head attached to the roof of the cabinet. In addition, solutions containing 3, 6 and 12 mol m$^{-3}$ NH$_4$Cl at pH 7, 5 and 3 were applied to plants of *P. sylvestris* and *P. deltoides* for separate experiments. The pH values were obtained using 1 M HCl and 1 M NaOH. The misting episodes lasted approximately 1 hour. Control plants (n = 3, for each species) were grown under identical greenhouse conditions as treated plants. The soil was covered with a plastic bag sealed around the plant stem for the duration of the misting episode.
As a result, only foliar uptake of misted or fumigated NH$_x$ could occur. Physiological investigations took place 24 hours after treatment with NH$_4^+$. 

3.2.2 Fumigations with NH$_3$

Fumigations took place in a sealed 1 m$^3$ perspex cabinet under greenhouse conditions. NH$_3$ was generated using a solution containing 3000 μg of NH$_4^+$, to which an excess of strong alkali was added. The solution was immediately placed inside the fumigation cabinet and agitated to promote the release of gaseous NH$_3$. A rotating fan was used to create air flow within the cabinet. The cabinet was unsealed after a 1 hour exposure and plants were sampled at varying time intervals afterwards. The cabinet was vented and washed to remove any remaining NH$_3$ that had not been taken up. Once the fumigation episode was complete, plants were removed from the cabinet and placed in the main compartment of the greenhouse along with the control individuals. Colorimetric analysis of the NH$_4^+$ solution at the end of the fumigation was carried out using the method of McCullough (1967), to measure any NH$_4^+$ not released from the solution. The final concentration of NH$_3$ in μg m$^{-3}$ released into the cabinet could therefore be calculated.

3.2.3 The relevance of the concentrations of NH$_x$ applied

Mistings with NH$_4$Cl were carried out using 1 L of solution over a 1 m$^2$ area at 3, 6 and 12 mol m$^3$ concentrations. These concentrations are approximately equivalent to 0.4, 0.8, and 1.6 kg N ha$^{-1}$. Recent reports of N deposition in the UK (Sutton and Fowler, 1993; Sutton et al., 1993) have measured N deposition between 13.4 kg ha$^{-1}$ y$^{-1}$ for an upland moor site and 60.8 kg ha$^{-1}$ y$^{-1}$ for a lowland forest site. In these instances, NH$_x$ deposition accounted for between 59-80% of the total N
deposition. These figures are representative of 1 year's worth of deposition and therefore are not immediately comparable with the dose used in this investigation. The concentrations used here would, therefore, be representative of acute single treatments, as the atmospheric concentration would not be expected to be static over the duration of one year. Pollution episodes tend to be episodic and dependant on climate and emission source conditions. It is interesting to note that in a recent review of NH\textsubscript{4} concentrations in the Netherlands, reports of between 100 and 200 kg N ha\textsuperscript{-1} y\textsuperscript{-1} are common (Pérez-Soba, 1995). Therefore, acute single high doses are potentially representative of deposition rates in certain areas of Europe and are similar to levels used by previous workers (Flaig and Mohr, 1992; Pearson and Stewart, 1993; Fangmeier \textit{et al.}, 1994).

The pH of the misted solutions were either pH 7, 5, or 3. Cape (1993) estimates a threshold for foliar injury of between pH 3 - pH 3.5. A pH 5 incident would therefore indicate a moderate incident with pH 3 more severe. Although pH 7 is generally unrepresentative of rainfall, this pH was used to help distinguish between the effects of NH\textsubscript{4} and acidity \textit{per se}.

The concentration of gaseous NH\textsubscript{3} applied was 3000 µg m\textsuperscript{-3} for a 1 hour duration. This compares favourably to a critical level of 3300 µg m\textsuperscript{-3} NH\textsubscript{3} for a 1 hour duration set at the UNECE workshop on critical levels in 1992 (van der Eerden \textit{et al.}, 1993). It is unlikely that plants in the wild will experience exposure levels similar to the above on many occasions. However, high concentrations are often required in short-term laboratory experiments to elicit a measurable response, which can then be extrapolated to longer term field investigations.
3.2.4 Experiments using *P. vulgaris*

Both misting and fumigation experiments were carried out using *P. vulgaris* grown under controlled greenhouse conditions as described in Chapter 2.3. Experiments with NH$_x$ were conducted using separate control and treatment plants, grown from the same seed batch under identical conditions. In addition, some experiments used a ‘temporary covered leaf’. This consisted of individual leaflets forming one third of a trifoliate leaf, being covered in a clear plastic bag which was sealed around the stem. This allowed the isolation of leaves from treatment, whilst exposing the remainder of the same plant. The bags were removed immediately after timed exposure to NH$_4^+$, typically 1 hour. This method provided control tissue of identical genetic material, age and physiological status. For repeat mistings, fumigations and time course studies, small amounts of leaf material (approximately 0.8g) were periodically detached from control and treatment plants for enzymatic and metabolite extraction. In all cases, leaf material of a similar age was used. The soil was covered for the duration of all NH$_x$ treatment episodes, to prevent uptake via the roots. All repeat misting and fumigations using *P. vulgaris* were conducted with a 3 day interval between each treatment episode.

3.3 Results

3.3.1 Preliminary screening of 8 plants for physiological responses to NH$_4^+$

Changes in the enzyme activities of GS, ME and NR can be seen, 24 hours after a single misting with 3 mol m$^{-3}$ NH$_4$Cl at pH 5 (Figures 3.2a-c). Measurements of GS activities were taken at the same time on different days but as far as possible, under the same environmental conditions. These conditions helped to eliminate any fluctuations in GS
activity based on changing light levels and the production of NH$_4^+$ via photorespiration (Edwards and Coruzzi, 1989). This methodology significantly reduced intra-specific variation of readings, however, a large degree of variation in control GS activities was seen between species possibly reflecting differences in N nutrition and photosynthesis. GS activity can be seen to increase significantly for all the plant species, both in the field and under greenhouse conditions. Increases in GS activity are greater in proportion to control GS activities, where control activity is low to begin with. For example, $P$. deltoides and $Q$. robur demonstrate 9.37% and 11.60% increases in activity respectively and have control GS activities above 100 $\mu$mol h$^{-1}$ g$^{-1}$ fwt. Previous measurements of control GS activities for these two species, have been found to be high in comparison to other plant species (Pearson and Stewart, 1993). $P$. padus and $G$. hederacea however, both of which have GS activities of approx. 33-42 $\mu$mol h$^{-1}$ g$^{-1}$ fwt, show increases in activity of 42.66% and 53.08% respectively. This relationship between control levels of GS and subsequent inducibility of GS activity in the presence of excess NH$_4^+$ is independent of whether the plant is a woody or herbaceous species.

Activities of ME show a significant increase in all species except $L$. album and $E$. tetralix. Increases vary from 20% to 70% and show a high degree of variation between species, as do control activities. The relationship between control activities and subsequent increased activities with NH$_4^+$ treatment is not as concise as with changes in GS, except possibly in the case of $B$. rutabulum. A high control ME activity to begin with may limit the inducibility of this enzyme in the presence of NH$_4^+$.

Activities of NR measured for the 8 species show a wide range of control values, probably reflecting variations in N metabolism. Where NH$_4^+$ is applied, a decline in NR activity is seen in species with
Figure 3.2 (a-c) Changes in GS, NR and ME activities for 8 plant species, 24 hours after misting with 3 mol m$^{-3}$ NH$_4$Cl at pH 5. Plant species are: 1, Brachythecium rutabulum; 2, Glechoma hederacea; 3, Lamium album; 4, Prunus padus; 5, Pinus sylvestris; 6, Quercus robur; 7, Erica tetralix; 8, Populus deltoides. Clear columns refer to values for separate control plants in all cases. Bars = SD of data (n = 3).
Figure 3.3 (a-d) Concentrations of organic acids for 8 plant species, 24 hours after misting with 3 mol m\(^{-3}\) NH\(_4\)Cl at pH 5. Plant species are: 1, Brachythecium rutabulum; 2, Glechoma hederacea; 3, Lamium album; 4, Frunus padus; 5, Pinus sylvestris; 6, Quercus robur; 7, Erica tetralix; 8, Populus deltoides. Clear columns refer to values for separate control plants in all cases. Bars = SD of data (n = 3).
moderate to high NR control activities. Reductions in activity vary from 13% (B. rutabulum), 32% (P. deltoides), 37% (L. album) to 43% (G. hederacea). Species which have low control NR activities, show little or no decline in NR activity in the presence of NH₄⁺ (P. padus, P. sylvestris, Q robur and E. tetralix).

Data for changes in organic acids are shown in Figures 3.3a-d. A high degree of variation is seen in organic acid contents for malate, citrate, 2-oxoglutarate and pyruvate, for all species, with malate and citrate generally present at greater concentration. A reduction in malate and citrate contents after treatment with NH₄⁺ can be seen for virtually all species, with citrate levels showing the most consistent changes. No significantly consistent changes can be seen in pyruvate and 2-oxoglutarate levels, with some species showing no detectable amounts of these two organic acids (E. tetralix and B. rutabulum). The remaining species show both increases and decreases in pyruvate and 2-oxoglutarate levels with NH₄⁺ treatment.

3.3.2 Time course study of physiological responses for P. vulgaris
Changes in GS, ME, NR activities and organic acid contents in leaves of P. vulgaris, were recorded over a 72 hour period, following misting with 3 mol m⁻³ NH₄Cl at pH 3 (Figures 3.4a-c). No significant differences were found between control activities of each enzyme and organic acid contents, at each sampling interval, over the 3 day period. Control measurements taken at each sampling interval, for the duration of the experiment are, therefore, not included. Values determined immediately before misting with NH₄⁺ are presented as control values for this and all investigations using temporarily covered leaves. Changes in enzyme activities of GS, ME and NR can be seen as early as 3 hours after misting. For GS (Figure 3.4a), the highest
Figure 3.4 (a-c) Changes in GS, ME and NR activities for *P. vulgaris* over a 72 hour period, following a single misting with 3 mol m⁻³ NH₄Cl at pH 3. Clear columns represent control values measured immediately before misting with NH₄⁺ (See 3.3.2). Bars = SD of the data (n = 3).
activity occurred between 18 and 24 hours after misting (13% increase), with a slight indication of activity beginning to decline towards control levels after 72 hours. There was a relatively rapid increase in ME activities (Figure 3.4b) up to 6 hours following treatment. ME activities then increased more gradually and remained elevated for the duration of the experiment (27-30% increase). NR activity (Figure 3.4c) rapidly declined after 3 hours, remaining low for a further 24 hours (28% reduction from control). NR activity gradually returned towards control levels by the end of the experiment (92% of control activity).

Organic acid levels (Figure 3.5) were measured every 24 hours for 3 days following misting with NH$_4^+$. No significant changes were found in levels of 2-oxoglutarate and pyruvate. Levels of these organic acids were low to begin with. Malate and citrate however, were present at high levels and exhibited significant reductions in leaf content 24, 48 and 72 hours after treatment with NH$_4^+$. Levels of these two organic acids showed some evidence of recovery towards previous levels after 72 hours.

3.3.3 The effect of NH$_4^+$ concentration and pH on enzyme activities

Figures 3.6-3.8 show the influence of pH and NH$_4^+$ concentration on GS, NR and ME activities for Pinus sylvestris and Populus deltoides. These two species demonstrated widely contrasting control enzymatic activities when grown under identical conditions in the greenhouse, most likely reflecting their different physiological and ecological properties. P. sylvestris had much lower control levels of GS, NR and ME than P. deltoides. GS activity in P. sylvestris and P. deltoides (Figures 3.6a-b) increased with NH$_4^+$ application, with highest activities found at 12 mol m$^{-3}$ treatments. However, increases were greater in
Figure 3.5 Concentrations of organic acids in P. vulgaris over a 72 hour period, following a single misting with 3 mol m\(^{-3}\) NH\(_4\)Cl at pH 3. Control values were measured immediately before misting with NH\(_4^+\) (See 3.3.2). Bars = SD of the data (n = 3).
Figure 3.6 (a-b) Changes in GS activity for *Pinus sylvestris* and *Populus deltoides*, 24 hours after a single treatment with NH$_4$Cl at 3, 6 or 12 mol m$^{-3}$ and pH 7, 5 or 3. Clear columns are pH 7 treatments, dotted columns are pH 5 treatments and solid columns are pH 3 treatments. Control plants were misted with distilled H$_2$O adjusted to the desired pH with 1 M HCl or 1 M NaOH. All values are for separate control and treatment plants; Bars = SD of data (n = 3).
Figure 3.7 (a-b) Changes in ME activity for *Pinus sylvestris* and *Populus deltoides*, 24 hours after a single treatment with NH$_4$Cl at 3, 6 or 12 mol m$^{-3}$ and pH 7, 5 or 3. Clear columns are pH 7 treatments, dotted columns are pH 5 treatments and solid columns are pH 3 treatments. Control plants were misted with distilled H$_2$O adjusted to the desired pH with 1 M HCl or 1 M NaOH. All values are for separate control and treatment plants; Bars = SD of data (n = 3).
Figure 3.8 (a-b) Changes in NR activity for *Pinus silvestris* and *Populus deltoides*, 24 hours after a single treatment with NH$_4$Cl at 3, 6 or 12 mol m$^{-3}$ and pH 7, 5 or 3. Clear columns are pH 7 treatments, dotted columns are pH 5 treatments and solid columns are pH 3 treatments. Control plants were misted with distilled H$_2$O adjusted to the desired pH with 1 M HCl or 1 M NaOH. All values are for separate control and treatment plants; Bars = SD of data (n = 3).
proportion to control levels for *P. sylvestris* than for *P. deltoides*. Also, a gradual increase in GS activity was seen with increasing NH$_4^+$ concentration for *P. sylvestris*, whereas for *P. deltoides*, increases in GS activity were similar for 3, 6 and 12 mol m$^{-3}$ concentrations of NH$_4^+$. Increasing the acidity of the misted solution, generally increased the activity of GS at all concentrations of NH$_4^+$, for both plant species, although, acidic treatments with no NH$_4^+$ had no effect on GS activity. Changes in ME (Figures 3.7a-b) demonstrated a similar pattern of responses as GS after treatment with NH$_4^+$. ME activity increased both with increased NH$_4^+$ concentration and pH. Increases were also gradual with *P. sylvestris* but of a similar level at each concentration for *P. deltoides*. NR activity declined with NH$_4^+$ application for both *P. sylvestris* and *P. deltoides* (Figures 3.8a-b). No significant difference was found in the degree of inhibition at different NH$_4^+$ concentrations. Likewise no differences were found with NH$_4^+$ applications at different pH except for *P. deltoides* at pH 3, where greater inhibition of NR occurred. A small but significant effect of pH treatment alone was evident for both species at pH 3 only.

### 3.3.4 Changes in nitrate reductase activities with soil NO$_3^-$ fertilisation and NH$_4^+$ application to leaves of *P. vulgaris*

This investigation was carried out using leaves that were temporarily covered as control leaves on treated plants. Preliminary experiments found no significant differences in background activities of NR, between covered and uncovered leaves on the same plant. The method proved to be a useful approach for single treatment episodes with *P. vulgaris* and helped to further eliminate physiological variation between plants. This method was not adopted for repeat treatments as earlier investigations found that continuous covering or repeated recovering of leaves, led to physical and physiological damage. Also,
Figure 3.9 The response of NR in *P. vulgaris*, 24 hours after misting with 3 mol m\(^{-3}\) NH\(_4\)Cl at pH 3. Dotted columns denote plants that were watered twice weekly throughout their growth cycle with \(\frac{1}{2}\) strength Long Ashton solution containing 0.6 mol m\(^{-3}\) NO\(_3\); hatched columns = 6 mol m\(^{-3}\) NO\(_3\); solid columns = 12 mol m\(^{-3}\) NO\(_3\). Controls are represented by 0 mol m\(^{-3}\) NH\(_4^+\) on the horizontal axis, using temporarily covered leaves (See 3.2.4). Bars = SD of data (n = 3).
transport of N between adjacent leaves may become a feature with longer experiments.

Plants of *P. vulgaris* watered twice weekly with \( \frac{1}{2} \) strength Long Ashton solution containing different concentrations of \( \text{NO}_3^- \) (Chapter 2.3), demonstrated different background NR activities. NR activities were higher in individuals watered with greater concentrations of \( \text{NO}_3^- \) (Figure 3.9). A decline in NR activity was measured 24 hours after \( \text{NH}_4^+ \) application, at all concentrations of soil \( \text{NO}_3^- \) fertilisation. The amount of reduction was however, proportionally greater where NR activity was higher to begin with.

### 3.3.5 Physiological responses of *P. vulgaris* after repeat mistings with \( \text{NH}_4^+ \) or fumigations with \( \text{NH}_3 \)

Increases in GS and ME activities (Figure 3.10 a & d) occurred after the first misting, with activities steadily increasing with each subsequent treatment. Increases in GS activity became less significant as the treatments continued, suggesting an upper limit of enzyme activity at approx. 100 \( \mu \text{mol h}^{-1} \text{g}^{-1} \text{fwt} \) for this species. A steady increase in leaf protein content is also seen with a 9% increase in protein content after the final episode (Figure 3.10b). ME activities show an almost linear increase over time, with activities almost doubled after the final misting episode. NR (Figure 3.10c) and organic acids (Figures 3.11a-d) show reductions in activities and concentrations as the experiment proceeded. NR shows a decline of 50% activity after 6 days, with no further significant decline following the last two misting episodes. Of the 4 organic acids measured, citrate and malate show the most consistent changes, with citrate demonstrating an almost linear decline with each misting episode.

Analysis of the remaining \( \text{NH}_4^+ \) solution used to generate \( \text{NH}_3 \) by the addition of strong alkali, as described in Section 3.2.2, revealed a
Figure 3.10 (a-d) Changes in enzyme activities and protein content for *P. vulgaris*, in response to 4 repeat mistings with 3 mol m\(^{-3}\) NH\(_4\)Cl at pH 3. Measurements were taken 24 hours after each misting, with a 3 day interval between each episode. Control values are clear circles, treatment values are solid circles. All values are for separate control and treatment plants; Bars = SD of data, \((n = 3)\).
Figure 3.11 (a-d) Changes in organic acids for *P. vulgaris*, in response to 4 repeat mistings with 3 mol m⁻³ NH₄Cl at pH 3. Measurements were taken 24 hours after each misting, with a 3 day interval between each episode. Control values are clear circles, treatment values are solid circles. All values are for separate control and treatment plants; Bars = SD of data, (n = 3).
Figure 3.12 (a-c) Changes in enzyme activities for *P. vulgaris*, in response to 4 repeat fumigations with 3000 μg m⁻³ NH₃. Each fumigation episode lasted 1 hour. Measurements were taken 24 hours following exposure to NH₃, a 3 day interval occurred between each fumigation episode. Control values are clear circles, treatment values are solid circles. All values are for separate control and treatment plants; Bars = SD of data, (n = 3).
Figure 3.13 (a-b) Changes in organic acids concentrations for *P. vulgaris*, in response to 4 repeat fumigations with 3000 µg m⁻³ NH₃. Each fumigation episode lasted 1 hour. Measurements were taken 24 hours following exposure to NH₃, a 3 day interval occurred between each fumigation episode. Control values are clear circles, treatment values are solid circles. All values are for separate control and treatment plants; Bars = SD of data, (n = 3).
virtually zero NH$_4^+$ content after 1 hour. Tests also revealed that the majority of NH$_3$ was released from the solution within 5 min after the addition of strong alkali. The system provided an efficient method of producing NH$_3$ to a known concentration, in a fumigation cabinet of 1 m$^3$ area.

Plants of *P. vulgaris* were exposed to repeat fumigations with 3000 $\mu$g m$^{-3}$ NH$_3$ for 1 hour to compare the effects of gaseous and wet applied NH$_x$. NR activity and leaf contents of malate and citrate showed similar responses to previous experiments conducted with NH$_4^+$, with declines in NR activity and the concentrations of malate and citrate (Figures 3.12c and 3.13a-b). ME and GS activities increased with NH$_3$ fumigation, achieving greater levels of increase with NH$_3$ treatment in comparison to NH$_4^+$ (Figures 3.12a-b). For example, the increases in GS and ME activities above control levels, after 4 fumigations with NH$_3$ were 120% and 350% respectively, compared to 20% (GS) and 100% (ME) after 4 mistings with NH$_4^+$.  

### 3.4 Discussion

#### 3.4.1 Glutamine synthetase activity

The potential toxicity of NH$_x$ suggests that NH$_x$ taken up from the soil or the atmosphere, needs to be readily assimilated into organic form at, or close, to the point of entry (Pearson and Stewart, 1993). Similarly, NH$_3$ produced by photorespiration in the leaves needs to be assimilated readily. As a consequence, both the leaves and roots of higher plants contain GS, to aid the rapid assimilation of both primary and secondary sources of NH$_x$. The series of experiments presented here has highlighted the responses of foliar GS to the presence of excess NH$_x$, revealing rapid increases in GS activity for a wide range of NH$_x$ concentrations. Increases in protein content were also seen for plants.
misted with NH$_4^+$ (Figure 3.10b), reflecting NH$_x$ assimilation into amino acids and eventually proteins. These results compare favourably to the work of Pérez-Soba et al., (1994), who demonstrated increases in GS activities and protein content in needles of *P. Sylvestris*, after exposure to NH$_x$.

The degree of increased GS activity appears however, to be governed by a number of factors. One such factor is the proportionality of increase in relation to control activities (Figure 3.2a). Control plants with high GS activities show a smaller range of increased GS activity with NH$_x$ treatment; perhaps indicating that they are already near maximal activity. Even so, high background levels of GS activity may offer a more immediate protection against harmful concentrations of NH$_x$. Plants with lower control GS levels for example *E. tetralix* and *B. rutabulum* demonstrate greater increases in GS activity in response to NH$_x$ (Figure 3.2a). Although, these increases are in some cases in excess of 100%, the activity remains relatively low and may not be sufficient to cope with an extended period of high exposure to NH$_x$. Pérez-Soba and Visser (1994) also indicated similar differences in plant vitality in relation to the capacity for assimilating excess NH$_x$ via GS.

Comparisons between GS activities in *P. sylvestris* and *P. deltoides* (Figure 3.6a-b) further highlight dissimilarities between species. Increased concentrations of NH$_4^+$ up to 12 mol m$^{-3}$ lead to greater increases in GS activity for *P. sylvestris* but only slight increases in *P. deltoides*. However, a maximum GS activity of 38 $\mu$mol h$^{-1}$ g$^{-1}$ fwt for *P. sylvestris* with a pH 7 misting, is only one third that of the maximum value for *P. deltoides*. Thus high levels of NH$_4^+$ may only elicit large increases in GS activity in some species and not others, based on the level of GS activity before the application of excess NH$_x$. The application of H$^+$ at pH 3 with NH$_4^+$ misting appears to further
increase GS activities, most noticeably in *P. sylvestris*. Thus, an increase in H\(^+\) ions would appear to have some effect on the behaviour of GS. H\(^+\) ions present in the misting solution, together with those produced from the assimilation of NH\(_x\) (Raven, 1988), may cause alterations in membrane permeability (Nieboer *et al.*, 1984), allowing increased uptake of NH\(_x\) and potentially higher GS activities. This synergistic relationship is not entirely clear and increases in GS activity may only be a temporary occurrence. With prolonged exposure at high levels of NH\(_x\) and acidity, detrimental effects on the function of GS may occur.

The form in which NH\(_x\) is applied ie. NH\(_4^+\) or NH\(_3\), can also lead to differences in GS responses. These differences are best highlighted by the responses of *P. vulgaris*. For example, initial control activities of GS in *P. vulgaris* fumigated with NH\(_3\) (Figure 3.12a) were 40% lower than those of plants misted with NH\(_4^+\) (Figure 3.10a). However, after 4 repeat treatments with either NH\(_3\) or NH\(_4^+\), the levels of GS activity reached were within 5 \(\mu\)mol h\(^{-1}\) g\(^{-1}\) fwt of each other. The amount of NH\(_3\) applied was approximately 5% that of the total amount of NH\(_4^+\) in a 3 mol m\(^{-3}\) misting with NH\(_4\)Cl. Rapid responses to NH\(_3\) may, therefore, be a result of increased efficiency of uptake of NH\(_3\) through the stomata, in gaseous form. With an NH\(_4^+\) misting, a high proportion of the solution will not impact on the leaf surface. As a result, differences in NH\(_4^+\) uptake may also occur, leading to variable increases in GS activity. The differences in GS responses highlighted here, will be important for plants in habitats that receive different proportion of NH\(_4^+\) and NH\(_3\).

Recent work by Pérez-Soba *et al.*, (1994) has demonstrated significant increases in GS activity after exposing *Pinus sylvestris* to 60 and 240 \(\mu\)g m\(^{-3}\) NH\(_3\) for 14 weeks. Increases in GS activity of 76% measured after 6 weeks continuous exposure at 240 \(\mu\)g m\(^{-3}\) NH\(_3\) by
Pérez-Soba et al., (1994), were similar in increases in GS activity in *P. sylvestris* exposed to a single 3 mol m\(^{-3}\) NH\(_4^+\) misting (54% increase in GS activity) (Figure 3.2a). This reflects the versatility of GS in the presence of excess atmospheric NH\(_x\) and further emphasises, the similarity of response between NH\(_4^+\) and NH\(_3\). Changes in GS activity can also be measured over much shorter durations of exposure. The time course study using *P. vulgaris* (Figure 3.4a) is one further proof of this, as increases in GS activity could be detected as little as 3 hours after a misting episode. Work, also on repeated exposure of *P. vulgaris* to NH\(_4^+\) and NH\(_3\) (Figures 3.10a and 3.12a), further indicate the similarity of response of GS in the presence of atmospheric NH\(_x\). In comparison to GS, Pérez-Soba et al., (1994) also measured leaf activities of GDH in *P. sylvestris*, in response to the above treatments with NH\(_3\) and found no significant changes in activity. It would appear that GS is the main enzyme of NH\(_x\) assimilation in this species. However, Schlee et al., (1994) found higher GDH activities in *P. sylvestris* collected from polluted areas, in contrast to non polluted areas. Reports of GDH induction in the presence of excess NH\(_x\) are few; this enzyme is considered to have a minimal NH\(_x\) assimilatory role (Chapter 1). The Michaelis-Menten kinetics (K\(_m\)) of GS for NH\(_4^+\) is several orders of magnitude lower than the K\(_m\) of GDH for NH\(_4^+\), thus GDH would have difficulty competing with GS for NH\(_4^+\), even at high concentrations of NH\(_x\) (Stewart et al., 1995b). Thus, the results of Schlee et al., (1994) may need to be treated tentatively, as Schlee also suggests, until further work is done.

The correct function of GS and the NH\(_x\) assimilatory GS-GOGAT cycle is dependant on several precursor molecules being present, one of which is 2-oxoglutarate. 2-oxoglutarate is incorporated into glutamic acid and eventually into protein via the GS-GOGAT cycle. 2-oxoglutarate itself is formed from precursor molecules in the
TCA cycle which include citrate and pyruvate. The results have shown that levels of citrate (Figures 3.3b, 3.11b and 3.13b), in some cases levels of pyruvate (Figure 3.3c) and 2-oxoglutarate (Figure 3.11c) have declined after NH₄ treatment. Increases in GS activity will lead to increased demand for the above organic acids, thus their availability may be temporarily reduced under conditions of high NH₄. This response is shown most clearly with citrate, as levels of this organic acid are in general significantly higher than pyruvate and 2-oxoglutarate, thus providing more scope for change. The low levels of pyruvate and 2-oxoglutarate measured, may also reflect their rapid incorporation into the TCA cycle (pyruvate) and particularly for 2-oxoglutarate, the GS-GOGAT cycle and other transaminase reactions.

3.4.2 Nitrate reductase activity

Treatments with NH₄ demonstrated a reduction in leaf NR activity in all the plants investigated. Högbom and Högberg (1991) found reduced NR activities in Deschampsia flexuosa growing in high N deposition areas of Sweden, which suggested a higher proportion of NH₄⁺ deposition than NO₃⁻. Previous work on moss species (Woodin and Lee, 1987; Padidam et al., 1991), has also shown reductions in NR activity, in the presence of excess NH₄⁺.

Reductions in NR activity appear, like GS activity, to be influenced by previous control activities of the enzyme. Where activities of NR are high to begin with, the subsequent reduction in activity is greater than for plants with a lower control NR activity (Figure 3.2c). NR is known to be a substrate inducible enzyme and experiments with P. vulgaris (Figure 3.9) have further demonstrated this. However, induced control activities of NR as a result of soil NO₃⁻ feeding, still decline in the presence of NH₄⁺. Also, not all plants demonstrate the capacity for significant NR induction, in the presence
of excess NO$_3^-$ (Pearson and Soares, 1995). NO$_3^-$ assimilation in plants leads to the production of NH$_4^+$, which is then readily metabolised by GS so as not to reach harmful concentrations. Reductions in NR activity in the presence of excess NH$_x$, may be a protective mechanism preventing the build up of NH$_x$ in leaf tissues. Plants that have low levels of foliar NR activity, often carry out most of their NO$_3^-$ reduction in their roots (Stewart et al., 1988). These plants may also favour alternative N sources such as NH$_4^+$ or mycorrhizal N (Pearson and Stewart, 1993). Although this investigation deals specifically with foliar responses, previous workers have shown that root NR activity can also be affected by excess NH$_4^+$ deposition, with reductions in NR activity (Muller et al., 1994) and eventual stimulation of NO$_3^-$ efflux from the roots into the soil medium (Aslam et al., 1994). Intercepted precipitation can often contain both NH$_4^+$ and NO$_3^-$ ions, which could cause induction or inhibition of NR, depending on the proportion of each ion present. Brumme et al., (1992) has shown that where both types of N source are available to the foliage, NH$_4^+$ is often the favoured N source. Thus, inhibition of foliar NR may be more evident in precipitation containing similar amounts of both NH$_4^+$ and NO$_3^-$.

Likewise, for plants that are unable to induce foliar NR activity, inhibition of NR activity would be most likely event in the presence of both excess NH$_4^+$ and NO$_3^-$. This situation may not be the same for predominantly soil intercepted NH$_4^+$ and NO$_3^-$; Morecroft et al., (1994) found an increase in foliar NR activity, 48 hours after watering chalk and acidic grassland species with the equivalent of 14 g N m$^{-2}$ yr$^{-1}$ in the form of NH$_4$NO$_3$. The increases in NR activity found with NH$_4$NO$_3$ watering, may be due in this instance to increased rates of nitrification in the soil, which were evident in the chalk soil and at high N applications for the acid soil. Despite the fact that acidity in combination with NH$_4^+$, had no conclusive effect on NR activities in
this investigation, acidity may prove to be more influential to soil processes like nitrification. NH$_4^+$ is considered to be the main form of N in acid deposition, therefore, direct foliar effects are likely to reflect this. The influence of combined soil and foliar intercepted NH$_4^+$, NO$_3^-$ and acidity, on the induction or inhibition of foliar NR, would require further long-term field studies.

Whether reductions in either foliar or root NR activities are triggered directly by high concentrations of NH$_x$ or by feedback from products of NH$_x$ assimilation is unclear. The time course study (Figure 3.4c) shows a decline in NR activity within 3 hours of treatment with NH$_x$, providing only a short time for newly synthesised products of NH$_x$ assimilation to trigger inhibition of NR activity. Filner (1966), Lee et al., (1992) and recently Xiu-Zhen et al., (1995) have suggested that rapidly produced amino acids such as asparagine and glutamine, may have a negative feedback on the regulation of NR. This feedback mechanism may take place in some cases since GS activity, leading to the production of glutamine is also stimulated rapidly in the presence of excess NH$_x$. The time-course study also shows that NR activities begin to recover 72 hours after a single treatment with NH$_x$. Continued exposure to NH$_x$ over longer periods would mean that both the concentration of NH$_x$ as well as products of NH$_x$ assimilation could lead to NR inhibition. Inevitably, NR activities may decline to a minimum level with less scope for recovery after high or prolonged exposures to NH$_x$. Morecroft et al., (1994) found a loss in NR inducibility and an eventual decline in NR activity for the moss Rytidiadelphus squarrosus, after 1 year exposure to 14 g N m$^{-2}$ NH$_4$NO$_3$ or (NH$_4$)$_2$SO$_4$.

A physiological response possibly linked with declines in NR activity after NH$_x$ application is a decline in malate content, (Figures 3.3a, 3.5, 3.11a and 3.12a). BenZioni et al., (1971) demonstrated that
NO$_3^-$ uptake by the roots, typically in the form of KNO$_3$ is transported to the shoots, where NO$_3^-$ can be assimilated and K$^+$ is returned to the roots in the form of K-malate. Therefore, a reduction in NO$_3^-$ assimilation in the shoots of NH$_4$ treated plants, may lead to a reduction in malate levels. This in turn, may lead to a decreased uptake of NO$_3^-$ by the roots as less K-malate will be returned to the roots (Touraine et al., 1990). The reduction of NO$_3^-$ is also considered to be stoichiometric to the synthesis of malate (BenZioni et al., 1971). Further evidence for this is provided by the work of Touraine et al., (1990), who showed an increase in foliar malate levels with increased NR activity for tobacco leaves, after root feeding with NO$_3^-$ solutions. Similarly, NO$_3^-$ starved plants showed a decline in both malate and NR activity. It would appear therefore, that a clear link between NR and malate exists, which can go some way towards explaining the changes in malate levels seen with foliar NH$_4$ application.

3.4.3 NADP malic enzyme activity and organic acids
Malate levels may also play an important role as part of the functioning of ME, as well as the functioning of NR highlighted above. ME is considered to be one half of a cellular pH-stat working in conjunction with PEP carboxylase to control pH changes in plant cells (Davies, 1980, 1986). ME is thought to control pH changes, by catalysing the conversion of malate to pyruvate, producing CO$_2$ and forming NADPH from NADP$^+$. Thus excess H$^+$ ions are utilised in the formation of NADPH and the CO$_2$ produced helps to buffer against acidity. PEP carboxylase on the other hand, controls increases in alkalinity by forming a strong acid, malate. ME itself has a wide ranging pH optimum, tending towards acidity (Edwards and Andreo, 1992), with increasing activities as leaf pH declines from neutral conditions (Davies, 1986). These properties provided the first proof of a possible
pH regulatory role (Davies, 1986). Studies by Gerant et al., (1987) have shown increases in ME activity for *Picea sitchensis* exposed to acidic pollutants. More recently Pearson and Soares (1996), have shown substantial increases in ME activity for *P. deltoides* and *Hordeum vulgare*, misted with \( \text{NH}_4^+ \) containing solutions. Increases in ME activity shown for 8 plant species treated with \( \text{NH}_4^+ \) (Figure 3.2b) agree with the work of Pearson and Stewart, (1993) and Pearson and Soares (1996). The assimilation of \( \text{NH}_4^+ \) leads to the release of \( \text{H}^+ \) ions. The induction of ME activity can therefore, be seen as one possible mechanism of alleviation of cell acidosis. Variations in induction of ME for different plants, in the presence of potential acidifying conditions, are less defined than for GS and NR and do not reflect previous control levels. Nevertheless, ME demonstrates a rapid response to \( \text{NH}_4^+ \) with continued induction after repeated treatments. Two further indicators of the activity of ME is the reduction in malate content of plants treated with \( \text{NH}_4^+ \) and an increase in pyruvate content (Edwards and Andreo, 1992). Under virtually all experimental conditions, decreases in malate content were seen, as ME activities increased after repeated exposures to \( \text{NH}_4^+ \). Where malate concentrations begin to stabilise after repeated fumigations (Figure 3.13a), ME activity can also be seen to do the same (Figure 3.12b). Thus ME activity is dependant on the presence of malate or *vice-versa* and plants with higher levels of control malate, may benefit from extended periods of ME induction in the presence of acidifying pollutants. Pyruvate levels may be expected to increase as a result of induced ME activity. This was the case for *G. hederacea* and *P. sylvestris*, although, other species showed significant reductions in pyruvate content (Figure 3.3c). The levels of pyruvate in the plant species investigated, including *P. vulgaris*, were in general very low in comparison to other organic acids and no real conclusions can be
drawn from this work. The low concentrations of pyruvate in all the species studied, suggests that pyruvate is readily metabolised and possibly does not form storage pools, which may occur with other organic acids such as citrate and malate.

The organic acids malate, citrate, 2-oxoglutarate and pyruvate investigated here, have revealed responses that show possible links to treatments with NH₄. These include, 2-oxoglutarate, pyruvate and citrate as precursory carbon skeletons for the TCA, GS-GOGAT cycles (Section 3.4.1); malate circulation in relation to NO₃ uptake, transport and subsequent assimilation by NR (Section 3.4.2); and malate as a substrate for ME activity (Section 3.4.3), with pyruvate as a bi-product of ME activity.

The possibility also exists that changes in organic acid concentrations, may also reflect short-term regulation of cellular pH. Raven (1988) states that the assimilation of inorganic NH₄⁺ into organic form in plant tissue leads to a net increase in H⁺. Thus a decline in organic acids, which is a common feature of virtually all the NH₄ treated species, would be beneficial to maintaining pH homoeostasis. Plant species that assimilate NO₃⁻ predominantly in their leaves, generate an excess of OH⁻ ions which can be neutralised by the presence of organic acids (Raven, 1988). As a consequence, plants with high NR activities have high organic acid contents (Soares et al., 1995). Where excess NH₄ is present, these plants show the greatest reductions in organic acid content, most likely as a direct result of increased H⁺ ion content and inhibition of NR.

Changes in organic acids may therefore, reveal direct responses to acidity and indirect responses via related enzyme activities, to both excess N and acidity. As NH₄ pollution is often combined with acidity and other N containing pollutants, it is difficult to totally distinguish between direct and indirect effects on organic acids. A combination of
responses is the most likely situation. However, the influence of acidity may be the predominant factor.

3.5 Conclusions

The screening of 8 plant species and the more detailed work with *P. vulgaris*, has provided evidence of physiological responses to NH$_x$ pollution. The enzymatic and organic acid responses have provided consistent results over a range of concentrations of NH$_x$, for different plant species and for different levels and types of exposure. They also demonstrate conclusively that plant responses to NH$_4^+$ and NH$_3$ are often identical, with the exception that NH$_3$ responses are generally more rapid due to ease of uptake.

Enzymatic responses studied here, may be specific to N metabolism and pH regulation. However, they will undoubtedly be influenced by a number of factors, other than the presence of NH$_x$. These could include seasonal variations in enzyme activities (Pearson and Ji, 1994; Stadler and Gebauer, 1992), influences of other stress factors such as climatic conditions, soil fertility and other pollutants (Wild and Schmitt, 1995) and plant susceptibility to NH$_x$ pollution based on physiological properties (Soares *et al.*, 1995). The complexity of plant biochemical pathways, especially where an 'overlapping' response may occur, make interpretation of results difficult. An example of this could be changes in malate content, in response to either NR activity, ME activity, direct acidity or indeed any combination of the three.

Despite these potential difficulties, the use of more sensitive and representative physiological responses remains a viable option in the present pollution climate, where visible injury is less evident. The variety of primary and secondary responses which could be
investigated is enormous; the enzymes and metabolites here represent only a minute fraction of possible physiological responses. This short-term investigation has provided an introductory study of specific responses to NH₃. The need for longer term studies is clearly evident as the influences of seasonal and climatic variations for example, may then be taken into consideration. Likewise, longer term field studies are required to distinguish more clearly between the possible effects of soil and foliar interception of mixed N compounds with acidity.
CHAPTER 4

Susceptibility of plants to atmospheric NH$_x$

4.1 Introduction

The degree to which plants are affected by the presence of an atmospheric pollutant such as NH$_x$ is determined by a combination of factors. These include: firstly, the frequency, concentration and longevity of exposure and subsequent uptake rates; secondly, the ability to detoxify the pollutant by storage or assimilation; thirdly, acclimation and adaptation to pollutant conditions and finally, the interaction of NH$_x$ with other pollutants both in the atmosphere and the plant tissue. Plants have little control over the external concentration and longevity of exposure. The presence of a cuticular barrier offers a protective barrier to some pollutants, the effectiveness of which varies markedly between species. The cuticle can however become damaged where abrasion, insect attack and acidic pollutants dissolve the waxy covering, thus decreasing the integrity of the structure and aiding the entry of pollutants. The presence of stomatal pores provide an easy route by which pollutants, particularly in gaseous form, can enter into a plant, as normal photosynthetic processes require that stomata are open for most of the daytime. Wet deposited NH$_x$ can enter the plant through the foliage as described above and also, via deposition to the soil and subsequent uptake by roots. Although variation in mechanical resistance of the leaf cuticle is undoubtedly an important aspect of species susceptibility, it is evident from the literature that this cannot be the full explanation for species damage.

There is a growing awareness that biochemical and physiological properties may also have an important role in defining a plant's
tolerance to atmospheric pollution in general. The physiological properties of a plant, which regulate the assimilation and detoxification of NH₃ may be of paramount importance in ensuring the survival of plants in polluted environments.

Investigations in Chapter 3 have identified a variety of physiological variables as indicators of NH₃ pollution. Changes in NR, GS and ME activities plus organic acids, show consistent and reproducible changes in NH₃ treated plants. However, no definitive link has been established between physiological responses and overall plant susceptibility to excess NH₃. Some authors have suggested links for other pollutants for example: Rabe and Kreeb, (1979) studied the changes in several enzyme activities and chlorophyll content in response to SO₂ pollution. Of the 7 plant species studied, Rabe and Kreeb concluded that some species were more susceptible than others, based on their previous ‘pollution conditioning’, before exposure to SO₂. Similarly, a different level of enzymatic response was found to occur in plants studied in the previous chapter, based on the intrinsic metabolism of the plant, before exposure to NH₃. It would appear therefore, that plant susceptibility to NH₃ is likely to be decided by a combination of factors, which reflect differences in plant types, location and overall plant metabolism under natural environmental conditions.

NH₃ deposition is not exclusive to specific ecosystems, thus a wide variety of plants are subjected to excessive NH₃ levels. Even so, reports of damage from NH₃ are often concerned with specific plant types, most notably woody species, for example coniferous trees, climax broad leaf trees and ericaceous species (Soares et al., 1995). These woody species are notable for their sclerophyllous leaves and thick waxy cuticle. In comparison, herbaceous species and fast growing perennials are not as frequently reported as suffering
comparable damage from excess NH$_x$. In addition, the effects of NH$_x$ on plants are often localised and patchy, often with healthy individuals of one species present alongside damaged individuals of the same species (Nihlgård, 1985; Crawford, 1989).

As N metabolism is central to nearly all plant biochemistry, it is likely that the presence of excess NH$_x$ will affect plant metabolism as a whole. The difficult task is to identify which particular aspects of plant metabolism provide the best indicators of susceptibility. The role of NH$_x$ in N nutrition and the acidification problems associated with uptake and assimilation of NH$_x$, offer two areas which may be significant in determining plant susceptibility to NH$_x$. Wellburn (1994) stated that many plant species growing in nutrient deficient habitats, are often more prone to damage from NH$_x$, than plants growing in nutrient rich habitats. Differences in N metabolism in species found in both habitats will inevitably occur. It is these differences that may ultimately dictate the susceptibility of a plant to excess NH$_x$. This chapter describes how a survey of plant physiological characteristics may provide some indication of plant susceptibility to NH$_x$ pollution.

4.2 Experimental design

4.2.1 Rationale

A total of 32 plant species including woody perennials, herbaceous species, ferns and mosses were collected from various field sites in Hertfordshire and Bedfordshire between May and August, 1992 (Table 4.1). The location of the field sites are detailed in Chapter 2.2. The emphasis on the collection was to provide a varied and non-biased selection of plant material encompassing different plant types from different habitats. Individuals of *P. vulgaris* (n = 3) were grown under greenhouse conditions as outlined in Chapter 2.3 and included in the
<table>
<thead>
<tr>
<th>ID No.</th>
<th>Plant Species</th>
<th>Common name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>Lamium purpureum</em> L.</td>
<td>Red dead-nettle</td>
</tr>
<tr>
<td>2.</td>
<td><em>Lamium album</em> L.</td>
<td>White dead-nettle</td>
</tr>
<tr>
<td>3.*</td>
<td><em>Sambucus nigra</em> L.</td>
<td>Elder</td>
</tr>
<tr>
<td>4.*</td>
<td><em>Corylus avellana</em> L.</td>
<td>Hazel</td>
</tr>
<tr>
<td>5.**</td>
<td><em>Phaseolus vulgaris</em> L. cv tendergreen</td>
<td>Dwarf French bean</td>
</tr>
<tr>
<td>6.*</td>
<td><em>Prunus padus</em> L.</td>
<td>Bird cherry</td>
</tr>
<tr>
<td>7.*</td>
<td><em>Populus deltoides</em> Marshall</td>
<td>Poplar</td>
</tr>
<tr>
<td>8.*</td>
<td><em>Calluna vulgaris</em> (L.), Hull</td>
<td>Heather</td>
</tr>
<tr>
<td>9.*</td>
<td><em>Crataegus monogyna</em> L.</td>
<td>Hawthorn</td>
</tr>
<tr>
<td>10.*</td>
<td><em>Aesculus hippocastanum</em> L.</td>
<td>Horse chestnut</td>
</tr>
<tr>
<td>11.*</td>
<td><em>Picea sitchensis</em> (Bong.), Carriere</td>
<td>Sitka spruce</td>
</tr>
<tr>
<td>12.*</td>
<td><em>Alnus glutinosa</em> (L.), Gaertner</td>
<td>Alder</td>
</tr>
<tr>
<td>13.*</td>
<td><em>Carpinus betulus</em> L.</td>
<td>Hornbeam</td>
</tr>
<tr>
<td>14.*</td>
<td><em>Erica tetralix</em> L.</td>
<td>Cross-leaved heath</td>
</tr>
<tr>
<td>15.*</td>
<td><em>Acer pseudoplatanus</em> L.</td>
<td>Sycamore</td>
</tr>
<tr>
<td>16.*</td>
<td><em>Pinus sylvestris</em> L.</td>
<td>Scots pine</td>
</tr>
<tr>
<td>17.*</td>
<td><em>Quercus robur</em> L.</td>
<td>Pedunculate oak</td>
</tr>
<tr>
<td>19.*</td>
<td><em>Betula pendula</em> Roth</td>
<td>Silver birch</td>
</tr>
<tr>
<td>20.</td>
<td><em>Brachythecium rutabulum</em> (Hedw.) Br.,Eur.</td>
<td>Moss</td>
</tr>
<tr>
<td>21.*</td>
<td><em>Fraxinus excelsior</em> L.</td>
<td>Ash</td>
</tr>
<tr>
<td>22.*</td>
<td><em>Fagus sylvatica</em> L.</td>
<td>Beech</td>
</tr>
<tr>
<td>23.</td>
<td><em>Urtica dioica</em> L.</td>
<td>Stinging nettle</td>
</tr>
<tr>
<td>24.*</td>
<td><em>Ilex aquifolium</em> L.</td>
<td>Holly</td>
</tr>
<tr>
<td>25.</td>
<td><em>Glechoma hederacea</em> L.</td>
<td>Ground ivy</td>
</tr>
<tr>
<td>26.</td>
<td><em>Senecio jacobaea</em> L.</td>
<td>Ragwort</td>
</tr>
<tr>
<td>27.*</td>
<td><em>Taxus baccata</em> L.</td>
<td>Yew</td>
</tr>
<tr>
<td>28.</td>
<td><em>Dryopteris filix-mas</em> (L.), Schott</td>
<td>Male fern</td>
</tr>
<tr>
<td>29.</td>
<td><em>Dryopteris dilatata</em> (Hoffm.), A. Gray</td>
<td>Broad buckler fern</td>
</tr>
<tr>
<td>30.</td>
<td><em>Pteridium aquilinum</em> (L.), Kuhn</td>
<td>Bracken</td>
</tr>
<tr>
<td>31.</td>
<td><em>Hippocrepis comosa</em> L.</td>
<td>Horseshoe vetch</td>
</tr>
<tr>
<td>32.*</td>
<td><em>Salix babylonica</em> L.</td>
<td>Weeping willow</td>
</tr>
<tr>
<td>33.*</td>
<td><em>Larix decidua</em> Miller</td>
<td>European larch</td>
</tr>
</tbody>
</table>

The ID number is used in subsequent statistical analysis and figures

* Refer to woody species only

** *P. vulgaris* L. cv tendergreen was grown under greenhouse conditions as outlined in Chapter 2.3.
analysis, thus making a total of 33 species surveyed. It is important to note at this stage that the collection of samples is only representative of a certain stage in the development of plants, namely at peak leaf growth or expansion. The implications of these conditions, in particular, how observed results may or may not change with time will be considered.

4.2.2 Physiological investigations
Fifteen physiological variables with emphasis on aspects of N metabolism and known or hypothesised mechanisms of pH regulation, were recorded for each species. These were: enzyme activities of NR, GS, ME; leaf content of protein, malate, citrate, 2-oxoglutarate, pyruvate, Ca, Mg, K, nitrogen and phosphorus; leaf pH and buffering capacity index (Chapter 2.7). Plant material was collected at the same time each morning between 9-10 am and placed in sealed polythene bags in a cool box, maintained with ice packs for transportation to the laboratory. Details of analysis for the above variables are outlined in Chapter 2.

4.2.3 Statistical analysis
Multivariate analysis was performed using the Clustan program (Wishart, 1987). Measurements for each of the 33 species were carried out in triplicate for each variable. The mean value for each variable was then calculated for each species. Data for each species and each variable (averaged and non-averaged) was firstly standardised to give equal weighting and a similarity matrix, based on squared euclidean distance was calculated. Variables that did not conform to a normal distribution were either $\log_{10}$ transformed or square root transformed. Hierarchical agglomerative clustering based on the calculated similarity matrix, was performed for the whole data set using Ward’s method and average linkage (UPGMA). Ordination,
using principal component analysis (PCA) was used as a further means of validation and interpretation of the data. For PCA, data was standardised and a similarity matrix calculated. Factor scores (1-5) were then calculated to give each species a series of weighted values based on the variables measured. Calculated factor scores were linearly regressed against the averaged data for selected variables to analyse key components of the PCA. Clustering and PCA results were plotted as species dendrograms or scatter plots with super-imposed cluster circles, allowing visual analysis of the groupings formed.

A subset of the data (21 woody species only) was used to further test the multivariate approach. The two methods of hierarchical clustering were repeated for the reduced data set. Cluster diagnostics for Ward’s method only, calculated by the Clustan program, were interpreted on the basis of intra-cluster variability (F ratio) and inter-cluster variability (T value). PCA as described above was also carried out for the reduced data set. In addition, comparisons of data for individual variables against each other, were performed using bi-plots and linear regression.

4.3 Results

4.3.1 Hierarchical cluster analysis of plant species
The summarised results for the 15 recorded variables (woody and herbaceous species) are shown in Table 4.2. The standard deviation within species for a given parameter was found to be small. Consequently, a preliminary cluster analysis of the full data set (ie. not averaged) for all variables and species demonstrated the same species groupings as shown for the averaged data set. Therefore, the mean values of each variable for each species were used to further simplify calculations and presentation of data. The averaged data set can be seen
<table>
<thead>
<tr>
<th>Variable</th>
<th>Min value</th>
<th>Max value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf pH</td>
<td>2.90</td>
<td>7.00</td>
</tr>
<tr>
<td>BCI</td>
<td>0.03</td>
<td>7.90</td>
</tr>
<tr>
<td>NR Activity</td>
<td>0.04</td>
<td>8.00</td>
</tr>
<tr>
<td>ME Activity</td>
<td>0.57</td>
<td>12.70</td>
</tr>
<tr>
<td>GS Activity</td>
<td>9.30</td>
<td>233.40</td>
</tr>
<tr>
<td>Citrate</td>
<td>0.00</td>
<td>12.62</td>
</tr>
<tr>
<td>2-Oxoglutarate</td>
<td>0.00</td>
<td>2.20</td>
</tr>
<tr>
<td>Malate</td>
<td>0.55</td>
<td>54.93</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.00</td>
<td>1.54</td>
</tr>
<tr>
<td>Protein</td>
<td>6.43</td>
<td>77.80</td>
</tr>
<tr>
<td>N</td>
<td>3.00</td>
<td>46.05</td>
</tr>
<tr>
<td>PO₄</td>
<td>0.57</td>
<td>4.97</td>
</tr>
<tr>
<td>K</td>
<td>0.61</td>
<td>37.16</td>
</tr>
<tr>
<td>Mg</td>
<td>0.91</td>
<td>32.23</td>
</tr>
<tr>
<td>Ca</td>
<td>0.04</td>
<td>7.40</td>
</tr>
</tbody>
</table>

Units for each variable are: NR, ME and GS enzyme activities = μmol h⁻¹ g⁻¹ fwt leaf; leaf BCI = g⁻¹ fwt leaf; citrate, malate, pyruvate and 2-oxoglutarate = μmol g⁻¹ fwt leaf; protein = mg g⁻¹ fwt leaf; N, PO₄, Ca, Mg and K = mg⁻¹ g⁻¹ dwt leaf.
in appendix 1. Table 4.2 shows a wide numerical range for each variable measured. This highlights the broad spectrum of plant species sampled.

Hierarchical cluster analysis of the mean values for 15 variables, measured for 33 species using Ward’s and UPGMA algorithms are shown in Figures 4.1a and 4.1b respectively. The dendrograms have grouped together similar plant types for example, the genus *Lamium* (Species no’s, 1 and 2); further herbaceous species (Species no’s, 1, 2, 5, 23, 25, 26 and 31); pteridophyte species (Species no’s, 28, 29 and 30); bryophyte species (Species no’s, 18 and 20) and ericaceous species (Species no’s, 8 and 14). Comparisons of the two clustering algorithms suggested the formation of 4 main clusters, which divided on the above species groupings. Differences between Ward’s method and UPGMA were minimal, with only species 9 & 19 changing cluster groups. Both methods have previously shown good correlation with plant taxonomical data (Sneath and Sokal, 1973). More recent work by Ingrouille and Pearson (1987) and Wishart (1987) has shown good correlation with biological data using Ward’s method only. Ward’s method was, therefore, adopted as the standard clustering method for this investigation (Soares *et al*., 1995).

Hierarchical cluster analysis of the data subset for woody species only (Table 4.1), using Ward’s and UPGMA are shown in Figures 4.2a-b. The species groupings formed were in broad agreement with the groupings shown for woody species in the whole data set (Figures 4.1a-b). Comparisons of the two clustering algorithms once again suggested the formation of 4 main clusters, with minimal differences in cluster formations between the two methods. As a consequence, Ward’s method was once again adopted as the standard (See 4.3.1). Close examination revealed that clusters containing species with high NR activities (Species no’s, 3 and 7; 7.42 and 8.0 μmol h⁻¹ g⁻¹ fwt
Figure 4.1 (a-c) Hierarchical cluster analysis of 33 plant species, for 15 variables, using (a) Ward's method, (b) UPGMA and (c) Ward's method with the variables GS, ME, PO₄ and organic acids masked from the procedure. Numbers on the horizontal axis refer to species identification (Table 4.1)
Table 4.3 Cluster diagnostic values for 21 woody species derived by Ward's method

**Cluster 1: Species 3 & 7**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean</th>
<th>SD</th>
<th>F ratio</th>
<th>T value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR</td>
<td>7.714</td>
<td>1.050</td>
<td>0.002</td>
<td>2.151</td>
</tr>
<tr>
<td>Total N</td>
<td>25.725</td>
<td>5.183</td>
<td>0.448</td>
<td>1.467</td>
</tr>
<tr>
<td>Total N</td>
<td>25.725</td>
<td>5.183</td>
<td>0.448</td>
<td>0.467</td>
</tr>
<tr>
<td>pH</td>
<td>6.240</td>
<td>0.010</td>
<td>0.512</td>
<td>1.296</td>
</tr>
<tr>
<td>Cations (Mg, Ca, K)</td>
<td>51.890</td>
<td>18.653</td>
<td>1.168</td>
<td>1.335</td>
</tr>
</tbody>
</table>

**Cluster 2: Species 4,6,9,10,19,21,22,27 & 32**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean</th>
<th>SD</th>
<th>F ratio</th>
<th>T value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR</td>
<td>0.867</td>
<td>0.694</td>
<td>0.382</td>
<td>0.857</td>
</tr>
<tr>
<td>BCI</td>
<td>0.680</td>
<td>2.070</td>
<td>0.380</td>
<td>0.090</td>
</tr>
<tr>
<td>Total N</td>
<td>24.384</td>
<td>4.784</td>
<td>0.376</td>
<td>0.294</td>
</tr>
<tr>
<td>pH</td>
<td>5.170</td>
<td>0.398</td>
<td>0.230</td>
<td>0.513</td>
</tr>
<tr>
<td>Cations (Mg, Ca, K)</td>
<td>37.466</td>
<td>12.103</td>
<td>0.492</td>
<td>0.499</td>
</tr>
</tbody>
</table>

**Cluster 3: Species 12,13,15,17,24 & 33**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean</th>
<th>SD</th>
<th>F ratio</th>
<th>T value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR</td>
<td>0.500</td>
<td>0.240</td>
<td>0.227</td>
<td>-0.168</td>
</tr>
<tr>
<td>BCI</td>
<td>0.140</td>
<td>0.380</td>
<td>0.431</td>
<td>-0.852</td>
</tr>
<tr>
<td>Total N</td>
<td>23.490</td>
<td>10.788</td>
<td>1.942</td>
<td>0.179</td>
</tr>
<tr>
<td>pH</td>
<td>4.240</td>
<td>0.645</td>
<td>0.604</td>
<td>-0.607</td>
</tr>
<tr>
<td>Cations (Mg, Ca, K)</td>
<td>20.741</td>
<td>10.098</td>
<td>0.342</td>
<td>-0.470</td>
</tr>
</tbody>
</table>

**Cluster 4: Species 8,11,14 & 16**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean</th>
<th>SD</th>
<th>F ratio</th>
<th>T value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR</td>
<td>0.240</td>
<td>0.140</td>
<td>0.004</td>
<td>-0.490</td>
</tr>
<tr>
<td>BCI</td>
<td>0.250</td>
<td>0.035</td>
<td>0.350</td>
<td>-0.450</td>
</tr>
<tr>
<td>Total N</td>
<td>13.090</td>
<td>0.170</td>
<td>0.005</td>
<td>-0.490</td>
</tr>
<tr>
<td>pH</td>
<td>4.003</td>
<td>0.895</td>
<td>1.164</td>
<td>-0.893</td>
</tr>
<tr>
<td>Cations (Mg, Ca, K)</td>
<td>10.125</td>
<td>1.589</td>
<td>0.451</td>
<td>-1.085</td>
</tr>
</tbody>
</table>

NB. Cluster formations are shown in Figure 4.2a
respectively) and low NR activities (Species no’s, 8, 11, 14 and 16; 0.240, 0.293, 0.042 and 0.384 μmol h⁻¹ g⁻¹ fwt respectively were clearly separated from the other clusters present.

Cluster diagnostics for woody species are shown in Table 4.3, for the 4 main clusters formed using Ward’s method. The variables shown represent the most significant components of the cluster formations. Small F ratio values suggest variables that have comparatively low variance within each cluster (intra-cluster) and are therefore good diagnostic variables for the formation of a particular cluster. T values are used as an indication of inter-cluster variability based on the differences from the mean of the whole data set. The T values for cluster 1, suggest that the mean values for the variables shown, are higher than the total sample means, for each variable. Noticeable here, is the very small F ratio and large T value for NR, indicating a particularly good cluster diagnostic. For clusters 2 and 3, T values suggest closer mean values for the variables shown, to those of the total sample mean for each variable. For cluster 4, T values suggest lower mean values for the variables shown, than those of the total sample mean for each variable, particularly for cation content, N content and NR.

4.3.2 The integrity of Ward’s cluster formations
The integrity of the cluster dendrograms produced by Ward’s method was tested to eliminate those variables which have no significant influence on their formation. Preliminary investigations of cluster formations were carried out by masking individual variables from the cluster procedure and comparing these cluster dendrograms with the cluster formation for the whole data set. Figure 4.1c shows a cluster dendrogram with the variables GS, ME, protein content, PO₄ and organic acids masked from the statistical analysis. This cluster
Figure 4.2 (a-c) Hierarchical cluster analysis of 21 woody plant species for 15 variables, using (a) Ward's method, (b) UPGMA and (c) Ward's method with the variables GS, ME, PO$_4$ and organic acids masked from the procedure. Numbers on the horizontal axis refer to species identification (Table 3.1).
formation represents the minimum configuration of variables, whereby the original integrity of the species clusters shown with all variable present (Figure 4.1a) are conserved, with the fewest exceptions. The exceptions are species no’s 6, 9, 17, 27, 31 and 32, which migrate to different clusters after the variables are masked.

The integrity of the cluster dendrogram produced by Ward’s method for 21 species was also tested as above. Figure 4.2c shows a cluster dendrogram of 21 species, also with the variables GS, ME, protein content, PO₄ and organic acids masked from the statistical analysis. This once again represents the minimum configuration of variables conserving the original integrity of the clusters shown in Figure 4.2a, in this case with no changes in species groupings. For Ward’s method using masked / unmasked variables, no significant species separation was shown between the central two clusters (Species no’s, 4, 6, 9, 10, 12, 13, 15, 17, 19, 21, 22, 24, 27, 32 and 33) for 21 woody species. However, it was found that by masking Ca content from the cluster procedure, species no’s 15 and 21 which are high leaf NO₃⁻ assimilators, migrated towards the high NR activity cluster. A similar response was observed for low leaf NO₃⁻ assimilators, species no’s 6, 17 and 27, which migrated towards the low NR activity cluster. Due to the importance of Ca as a major plant nutrient, it was decided not to exclude Ca from the clustering procedure.

4.3.3 PCA analysis for plant species
Calculated factor scores no’s 1 and 2 produced by PCA were found to account for over 60% of the total variation of the data set. As this accounts for a substantial part of the total variation, these scores were used for the presentation of the PCA. The species groupings produced by PCA of the 15 measured variables for 33 species (Figure 4.3), gives good agreement with Ward’s method (Figure 4.1a), with only species
Figure 4.3 PCA of 33 plant species, for 15 variables, using factor scores 1 (vertical axis) and factor scores 2 (horizontal axis) in respect of the 15 measured variables. Numbers next to each point refer to species identification (Table 4.1). Cluster circles denote 4 groupings obtained by Ward's method. Average NR activities are shown for each cluster.
Figure 4.4 (a-b) Cation content (a) and BCI (b) plotted against factor 1 values calculated by PCA for 33 plant species. $R^2$ values of 0.708 (cations) and 0.673 (BCI) ($p = < 0.001$) show significant correlations between factor scores and the two variables.
Figure 4.5 PCA of 21 woody species, for 15 variables, using factor scores 1 (vertical axis) and factor scores 2 (horizontal axis) in respect of the 15 measured variables. Numbers next to each point refer to species identification (Table 4.1). Cluster circles denote 4 groupings obtained by Ward's method.
no's 12 (*Alnus glutinosa*) and 22 (*Fagus sylvatica*) changing cluster groups.

PCA was found to group species together with similar NR activities. Figure 4.3 shows the average NR activities between each cluster grouping varying accordingly, with increasing NR activity shown along the horizontal axis. It can therefore be reasonably assumed that factor 2 scores (horizontal axis) are best represented by NR activities of the individual species. Calculated values for factor 1, showed the most significant correlation's with cation content (Ca, Mg and K), and BCI (Figures 4.4a and 4.4b respectively), indicating these variables as the major determinants of the calculated weighted scores for factor 1.

PCA for 21 woody species only (Figure 4.5) showed similar results to the cluster analysis for 21 species using Ward’s method, with only specie no. 12 (*Alnus glutinosa*) changing cluster group. Similarities can also be drawn between PCA for 21 species and groupings formed with the whole data set (Figures 4.1a and 4.3). PCA for 21 species was found to group species together with similar NR activities, particularly low and high extremes of NR activity. Factor 2 scores (horizontal axis) were once again represented most significantly by NR activities with an increase in average NR for each cluster along this axis. As with previous PCA, calculated values for factor 1 demonstrated the most significant correlation's with cation content (Ca, Mg and K), and BCI (data not shown).

**4.3.4 Bi-plots of variables**

Bi-plots of variables for 33 species show that the potential capacity of plants to buffer against acidic input is strongly related to cation content (Ca, Mg and K) and also NR activity. Plant species with high BCI, also have high NR activities (Figure 4.6a) and greater cation content in
Figure 4.6 (a-b) BCI plotted against (a) NR activity and (b) cation content for 33 plant species. R^2 values of 0.847 (NR) and 0.724 (cations) (p = < 0.001) show significant correlation between BCI and the 2 variables.
Figure 4.7 (a-b) N content plotted against PO$_4$ content for (a) 33 species and (b) 21 woody species.
their leaf tissue (Figure 4.6b). The converse is true for plant species with lower BCI. Similar plots were also achieved for 21 species only (data not shown). No significant correlations were found between any of the other measured variables for 33 or 21 species. However, a possible trend was found between increasing N content and increasing PO$_4$ content for both data sets (Figures 4.7 a-b).

4.4 Discussion

4.4.1 Validation of mathematical techniques
Multivariate analysis, using hierarchical agglomerative clustering and PCA ordination are common techniques used for the analysis of large data sets (Gauch, 1982). These techniques are often applied to biological and ecological data, as they enable analysis of similar and contrasting variables for inter and intra-specific relationships (Gauch, 1982). Recently, these techniques have been used to assess potential damage to ecosystems, using physiological and biochemical parameters as data (Wild and Schmitt, 1995, Soares et al., 1995).

For this investigation, the multivariate techniques used have enabled consistent grouping of plant species of the same type and similar physiological characteristics. Ward's method and UPGMA perform slightly different similarity calculations. Ward's calculation is based on minimum distance between the centre of each cluster and the cluster members, whereas, UPGMA calculations are based on the average linkage distance of pairs of individuals from each cluster. Despite this difference in calculation, both methods show comparable results, with only minimal disagreement in species groupings. Similarities between the two clustering techniques, offer further validation of the multivariate analysis used and more confidence in the formation of the species groupings.
Ordination using PCA is one of the most widely used statistical techniques for the interpretation of ecological data (Ludwig and Reynolds, 1988). The previous cluster analysis provided the initial separation of plant species. PCA is a means of isolating the main constituents of the database that show the most influence in the cluster formations. The PCA showed consistent agreement with the cluster formations produced by Ward’s method. Also, PCA conducted for different data sets such as the whole data set (Figure 4.3) and the smaller woody data set (Figure 4.5), were comparable. These similarities enabled the isolation of specific variables, based on the calculated factor scores, that were most influential in deciding the species ordination. Both hierarchical clustering and PCA ordination did on occasion result in small inconsistencies between cluster formations. These occasions occurred most notably with the reduced data set (21 woody species) and where specific variables were masked from the statistical analysis. Although no specific minimum size of data set is outlined for multivariate analysis, larger data sets tend to give more consistent results (Wishart, 1987). There is always a degree of subjectivity when interpreting groupings formed from such methods, especially where rigorous mathematical techniques are used to make biological or ecological sense. Nevertheless, the use of PCA in conjunction with other multivariate tests like Ward’s method, can be seen as reliable means of statistically analysing and interpreting large data sets.

4.4.2 Cluster and ordination of plant species
The potential for foliar NO$_3^-$ assimilation, shown by NR activity, appears to be a major determinant of the species groupings produced by both hierarchical clustering and PCA. A clear distinction can be seen between plants with high and low levels of foliar NR activity.
Plant species that typically exhibit low levels of leaf NO$_3$- assimilation are often slow growing climax species (Stewart et al., 1990; Stadler and Gebauer, 1992) for example $P$. *sylvestris* (16), *E. tetralix* (14), *C. vulgaris* (8) and *P. sitchensis* (11). These species tend to rely on root assimilation of N and only exhibit very small constituent levels of foliar NO$_3$- assimilation (Smirnoff et al., 1984; Clough, 1993). On the whole, species such as the above are incapable of greatly inducing foliar NO$_3$- assimilation under conditions where NO$_3$- has been applied either as a soil or shoot derived fertiliser, both in the field, or under controlled laboratory experiments (Wingsle et al., 1987; Pearson and Soares, 1995). In comparison, higher levels of leaf NO$_3$- assimilation, are commonly associated with faster growing pioneer species (Clough et al., 1989; Stadler and Gebauer, 1992). These include highly nitrophilous woody species such as *S. nigra* (3), *P. deltoides* (7) and herbaceous ruderal species such as *L. album* and *L. purpureum* (1 and 2), *U. dioica* (23) *G. hederacea* (25). These plant species can readily induce NR activity in their leaves to accommodate increased availability and translocation of NO$_3$- from the soil (Smirnoff et al., 1984) and are more physiologically active, with greater enzyme activities, nutrient pools and growth rates (Lambers and Poorter, 1992) (Table 4.3). A recent study of the relationship between growth rate and nutrient availability of 34 woody species from tropical deciduous forest in Mexico demonstrated a continuous relationship between the two variables (Huante et al., 1995).

Habitats that contain an abundance of climax species are frequently reported as being prone to damage from atmospheric pollution, especially pollutants that have an overall acidifying effect on plant cell metabolism. These habitats include coniferous forests (Nihlgård, 1985), heathlands (Aerts, 1989) and also some deciduous forests that contain an abundance of tree species exhibiting low levels
of foliar NO$_3^-$ assimilation, such as Oak and Beech (Tietema and Verstraten, 1990). A common feature of these habitats is an overall low nutrient content (Van Breemen and Dijk, 1988), especially N (Sutton et al., 1992), which can limit plant growth.

Pioneer species are associated with more dynamic habitats than climax species. These can include recently disturbed or reclaimed land, land on the fringes of agricultural areas, and generally areas where nutrient availability is high. Pioneer species have the ability to grow rapidly and compete efficiently with other species for the available nutrients. They are also species that play an important part in habitat transition from areas that have suffered prolonged nitrogen deposition (van Breemen and van Dijk, 1988). In this situation, alterations of plant and habitat nutrient balances caused by excess N deposition, lead to a gradual transition from climax to more nitrophilous species. An example of this is the eutrophication of Dutch heathlands (van der Eerden et al., 1991; Bobbink et al., 1992). Further evidence might also be found in the Rhine Valley, S. Germany, where on SW facing slopes, a marked increase in the nitrophilous species $S$. nigra has occurred over the past 20 years. A decline in climax species has resulted on the same slopes. This change is thought to be due to NO$_x$ deposition from prevailing SW winds. In contrast, plant species on the eastern side are not so badly affected (Karin Ullrich, Freiburg University, personal communication).

The separation of plant species revealed by clustering and PCA shows the most distinctive separations based on the capacity for foliar NO$_3^-$ assimilation. There are however, several intermediary species groups between the two limits of NO$_3^-$ assimilation. Although the members of these clusters showed no significant distributions, this may only be as a result of the time of sampling. Inter-cluster transitions may be expected to occur at different times of the year, when
physiological and enzymatic properties fluctuate, due possibly to changes in the growth cycle and the nutrient status of the plants. Several of the species contained in the intermediary groups are capable of NR induction for example, *Betula pendula* (species no. 19) and *Ilex aquifolium* (species no. 24), (Clough, 1993). These species may then migrate to the high leaf NO₃⁻ assimilating cluster of species, when there is a better supply of NO₃⁻. Clough (1993) has demonstrated the diurnal and seasonal variability in NR activity in a wide range of plants, most notably broadleaf pioneer trees. Although NR activities can vary quite markedly throughout the season in pioneer species, they seldom decline to the levels measured in climax species (Clough, 1993; Pearson and Ji, 1994). In contrast, climax species show a much lower diurnal or seasonal variation in NR activity (Pearson and Ji, 1994) and little or no capability for foliar NR induction with increasing NO₃⁻ supply (Pearson and Soares, 1995). The groupings formed by climax species may remain relatively stable throughout the year.

Where rigorous mathematical techniques are employed to interpret biological data, there will always be some anomalous results. In these cases, it may be important to consider the ecology of individual plant species. An example is *Alnus glutinosa* (species no. 12), which changes cluster grouping between PCA ordination and hierarchical clustering. The ability of *A. glutinosa* to fix atmospheric N₂ via a symbiotic fungal association, thus not having to totally rely on NH₄⁺ or NO₃⁻ for a source of N, may well contribute towards its anomalous behaviour. Similarly, *Taxus baccata* (species no. 27) is often found growing in chalk grassland communities. The influence of Ca in these environments may influence the changes in groupings demonstrated, especially when Ca is masked from the clustering procedure. Likewise, when GS is masked from the clustering, *Quercus robur* (species no. 17) is one species that changes grouping. GS
activities in this species are particularly high, with activities in excess of 200 μmol hr⁻¹ g⁻¹ fwt (Pearson and Ji, 1994). Individual species characteristics as described above cannot explain all the anomalous results. However, they can help to interpret the data more completely.

4.4.3 NO₃⁻ assimilation and acidic buffering

The differential capacity for NO₃⁻ assimilation, as shown for climax and pioneer species, may offer one explanation as to the broad differences in susceptibility between the two species groups to atmospheric pollutants.

Amundson and Maclean (1982) suggested a link between the location of NO₃⁻ assimilation and the susceptibility of plants to NOₓ. Here it was stated, that root NO₃⁻ assimilators are more prone to damage by NOₓ pollution due to a lack of foliar NO₃⁻ assimilation. A similar conclusion was also stated by Stewart et al., (1986) for several pteridophyte species.

The mechanism by which NO₃⁻ assimilation conveys pH buffering potential against pollutants like NH₃, SO₂ or H⁺ can be considered by closer examination of the process of NO₃⁻ assimilation in plants. The assimilation of NO₃⁻ in leaves and roots of plants is a two stage process: the first involves the conversion of NO₃⁻ to nitrite (NO₂⁻) by the action of NR:

\[
\text{NR} \quad \text{NO}_3^- + \text{NADPH} + \text{H}^+ \xrightarrow{}\text{NO}_2^- + \text{NADP} + \text{H}_2\text{O}
\]

Secondly, the conversion of NO₂⁻ to NH₃ by nitrite reductase (NiR) (EC 1.7.7.1)

\[
\text{NiR} \quad \text{NO}_2^- + 3\text{NADPH} + 3\text{H}^- \xrightarrow{}\text{NH}_3 + 3\text{NADP} + \text{H}_2\text{O} + \text{OH}^-
\]
Both NR and NiR found in roots and shoots are substrate inducible enzymes, with rapid turnover of enzyme in the presence of $\text{NO}_3^-$ and $\text{NO}_2^-$ (Oaks and Hirel, 1985; Solomonson and Barber, 1990). The production of $\text{NH}_3$ is potentially toxic and is generally rapidly assimilated by GS. Hydroxyl ions ($\text{OH}^-$) generated by leaf $\text{NO}_3^-$ assimilation cannot be excreted directly from the leaf surface nor readily transported to the roots where disposal to the rhizosphere can occur (Pate, 1980). The resultant accumulation of $\text{OH}^-$ can be neutralised by the presence of organic acids in the cytoplasm (Raven, 1988). Organic acids such as malate, citrate, 2-oxoglutarate and pyruvate were in general found to be more abundant in high leaf $\text{NO}_3^-$ assimilators (Appendix 1).

The possibility also exists that $\text{OH}^-$ produced from $\text{NO}_3^-$ assimilation could help to neutralise excess acidity, by the combination of $\text{OH}^-$ and $\text{H}^+$ to form $\text{H}_2\text{O}$. The potential for acid buffering as a result of $\text{NO}_3^-$ assimilation is demonstrated by species with high levels of foliar $\text{NO}_3^-$ assimilation having a greater BCI (Figure 4.6a). Conversely, plant species with lower buffering capacity have lower levels of foliar $\text{NO}_3^-$ assimilation (Figure 4.6a). The subsequent shortage of $\text{OH}^-$ may be one contributing factor to a decreased potential for physiological buffering.

### 4.4.4 The contribution of base cations to acidic buffering

Preliminary cluster analysis of all the variables, excluding NR, revealed dendrogram formations that differed only marginally from those produced by the whole data set (data not shown). Although the capacity for $\text{NO}_3^-$ assimilation appears to have a major influence on cluster formations, the data suggests the involvement of other variables. The multivariate analysis demonstrated that when plant
species associated with low nutrient habitats are clustered together, levels of nutrients such as N, Ca and Mg are significantly lower than other clusters containing pioneer species (Table 4.3). The availability of nutrients such as Ca, Mg and K, which carry a positive charge in aqueous solution and have basic properties may offer a further means to alleviate H⁺ input from acidifying pollutants. A charge balancing relationship, creating a stabilised pH gradient across plant cell membranes using cations has been envisaged by Nieboer et al., (1984). The potential for this is clearly shown in Figure 4.6b, where increased buffering capacity is shown in plant species with a higher combined concentration of Ca, Mg and K ions. Conversely, Figure 4.6b also demonstrates that plant species with lower levels of base cations, are less able to buffer against acidic inputs. K is also commonly associated with uptake from roots and subsequent transport of NO₃⁻ to the leaves for assimilation (Ben-Zioni et al., 1971). Thus low levels of foliar NO₃⁻ assimilation in climax species may be one contributory factor to the ensuing low cation levels.

The ability of cations to provide buffering potential in plants may be limited by acid deposition into the soil medium. When NH₄⁺ or H⁺ enters the soil medium, base cations like Ca, Mg and K become detached from their exchange sites and are rapidly leached out of the soil in rain water. This limits their availability for root uptake and subsequently reduce the potential for providing physiological buffering. Leaching of cations from the soil will undoubtedly have more impact in nutrient poor soils for example, upland areas dominated by climax species. In nutrient rich soils, the cation reservoir may be sufficient to provide buffering in plants as well as the soil, for longer periods of time.
4.4.5 The role of organic acids, glutamine synthetase, malic enzyme, protein and phosphate

Foliar contents of organic acids, protein and PO$_4$, plus enzyme activities of GS and ME showed no direct correlations with the species groupings formed. The contributions that these variables may make to overall plant susceptibility to NH$_x$ is, however, not completely discounted.

The previous chapter has shown that where excess NH$_x$ is present, organic acids such as malate, citrate, 2-oxoglutarate and pyruvate, GS, ME and protein show significant and consistent changes in foliar levels and activities. These changes not only reflect direct responses to NH$_x$ but also mechanisms of pH regulation. Previous workers have also noted significant changes in GS and ME (Pearson and Stewart, 1993; Pearson and Soares, 1996), protein (Rabe and Kreeb, 1979) and PO$_4$ (Dueck and Elderson, 1992) in response to treatments with acidifying atmospheric pollutants, including NH$_x$. In this study, a slight positive correlation was also found between PO$_4$ and N (Figures 4.7a-b), possibly reflecting a higher demand for other essential nutrients in nitrophilous species, to maintain a nutrient balance (Nihlgård, 1985). The differential responses found between species in the previous chapter, in some instances, closely represent the climax and pioneer separations highlighted by the multivariate analysis. Examples of this are, *P. sylvestris* and *E. tetralix* (climax species), *L. album*, *G. hederacea* and *S. nigra* (pioneer species) (See 3.3-3.4).

Seasonal changes in physiological parameters may have some influence on the lack of correlation found in some of the variables. One example of this is the seasonal variability in enzyme activities of GS as shown by Pearson and Ji (1994). Higher GS activities were often found in early season, with activities declining towards the end of the year. Similarly, GS in leaves is thought to exist as two isoforms, the
predominant form being chloroplastic GS. Chloroplastic GS is known to be present with quite a high activity, primarily to re-assimilate photorespiratory NH$_3$. Photorespiration is greater under high temperature and light, thus diurnal as well as seasonal variation may regulate GS activities. This fluctuation in GS may also have some influence on organic acid levels that contribute to the GS-GOGAT cycle and N transformations (See 3.4.1). Although this study represents only a 'snap shot' of the growth cycle of the plants studied, it is reasonable to suggest that some of the above variables, may be significant determinants of a plant's susceptibility to excess NH$_3$. This suggestion is possibly best represented under different experimental conditions, where changes in enzyme activities and organic acids can be assessed over longer periods of time for instance, a full growing season. Multivariate analysis can be a dynamic process allowing new information to be added to existing databases at any time. However, a degree of caution needs to be maintained, so that the database is not saturated, which will inevitably make biological and ecological conclusions more difficult to make. Even so, several approaches may still exist which with future work, could augment the data already collected. Data collected from experiments where plant species have been exposed to atmospheric pollutants, could be incorporated into the clustering procedure. This could be represented by laboratory experiments or by collecting similar species from contrasting pollution environments for example, the Netherlands, which experiences particularly high concentrations of NH$_3$ pollution in comparison to less polluted areas in the UK. Individual plant characteristics could be manipulated to see how species grouping may alter under different conditions. Examples are :- soil NO$_3^-$ feeding to induce NR activities in some of the intermediate species; altering the soil nutrient components in order to change the nutrient status of
greenhouse grown plants; using enzyme and metabolite blocking agents such as methionine sulphoxamine, (an inhibitor of GS activity) to change N assimilation characteristics. Experiments such as these, in addition to the species screening carried out in this study, may provide more information on the role of individual physiological variables, as well as a greater knowledge of the susceptibility of plants to NH\textsubscript{x} pollution.

4.5 Conclusions

Multivariate analysis of physiological characteristics has provided a means of firstly, categorising plants into different ecological types and secondly, along with a consideration of N metabolism, a means of assessing plant susceptibility to NH\textsubscript{x} pollution. Where the cluster and PCA analysis has identified discrete divisions of species, the bi-plots and to some extent the PCA, suggest continuous relationships between different variables. Several physiological factors have been isolated that may contribute to plant susceptibility to NH\textsubscript{x} inputs. These notably include the capacity for foliar NO\textsubscript{3}\textsuperscript{-} assimilation and foliar levels of base cations. Both these variables were found to contribute significantly to a plants ability to buffer against acidifying inputs. Buffering capacities may remain relatively stable especially in climax species, as they often lack the ability for foliar induction of NR activity, despite changes in NO\textsubscript{3}\textsuperscript{-} availability. Climax species that can induce NR activity in the presence of extra NO\textsubscript{3}\textsuperscript{-}, still maintain relatively low NR activities (Pearson and Soares, 1995). In contrast, pioneer species demonstrate a more responsive NR capabilities, often maintaining a higher NO\textsubscript{3}\textsuperscript{-} assimilation capacity than climax species. As a result, pioneer species may benefit from a higher buffering capacity. The ability to buffer against pH changes may therefore, be an intrinsic
feature of a plant's physiological characteristics (Soares et al., 1995; Pearson and Soares, 1995). Other variables that were not significant in the formation of species clusters, possibly due to seasonal variations, may still contribute towards overall susceptibility and buffering capacity, over longer periods. The differential ability of plants to buffer against acidity is of paramount importance when assessing the impact an acidifying pollutant or pollutants may have on an ecosystem.

There are a multitude of variables that can be assessed to determine the susceptibility of plants to atmospheric pollutants. The ones studied here represent only a small minority, with specific interest in NH₃ pollution and acid deposition. Atmospheric pollution often contains a cocktail of pollutants and each pollutant may cause a different physiological effect. Where a basic understanding of the pollutants overall effects are known, physiological screening could potentially be used to assess plant susceptibility to atmospheric pollution.
CHAPTER 5

Photosynthetic and dose responses of plants to atmospheric NH$_x$

5.1 Introduction

Photosynthesis (PS) is a major process in the primary metabolism of plants. As a result, PS is one of the most widely researched topics in the area of physiological responses to environmental changes including atmospheric pollution.

Previous work on the effects of air pollution on PS has centred around SO$_2$, O$_3$ and acid rain, where a sizeable literature has developed. This literature has recently been extensively reviewed by Saxe (1991) and Wellburn (1994). Pollutants such as the above can cause a variety of effects on PS, for example: (i). direct effects via oxidative damage to the PS apparatus (chloroplasts, thylakoid membranes, chlorophyll content) leading to disruption of electron transport, denaturing of protein complexes involved in PS and changes in fluorescence (Muthuchelian et al., 1994; Dietz et al., 1988; Wild et al., 1988); (ii). direct effects on gaseous exchange for example, disruption of stomatal function and subsequently alterations of transpiration rates and CO$_2$ uptake (Anon, 1988b; Christodoulakis, 1993; Eamus, 1993); (iii). indirect and possibly feedback changes in PS via secondary physiological processes, such as transport mechanisms for PS substrates or products and in the context of this thesis, perturbation of N metabolism (Anon, 1988b). The above responses vary dramatically according to the type of plant, the level and longevity of exposure to a pollutant and environmental factors,
such as climatic conditions and water availability (Strand, 1993). Saxe (1991) attempts to summarise the overall effect of SO$_2$, O$_3$ and acid rain on PS as a net decline in PS, resulting from direct toxicity, oxidation and in some cases acidification, caused by the individual pollutants or pollutant mixtures in combination with H$^+$ ions. Saxe (1991) also noted that exceptions to the rule can occur, with stimulation of PS by increases in stomatal aperture often occurring with low concentration, short duration treatments. Alterations of physiological status, whether a stimulation or inhibition of PS are potentially harmful, as prolonged exposure to pollution would inevitably change the integrity of plant physiological processes (Anon, 1993b).

Concerns over increasing levels of N compounds in the environment, in particular NO$_x$ and NH$_3$ has focused attention on their possible effects on PS. The uptake of NO$_x$ (most commonly as gaseous NO$_2$) and its subsequent solubilisation in water, leads to the formation of NO$_2^-$ and NO$_3^-$ ions. NO$_2^-$ is highly toxic and is rapidly taken up by chloroplasts via a specific carrier on the chloroplast envelope (Krämer et al., 1988). Once inside the chloroplast electrons are donated from ferredoxin and in conjunction with the enzyme nitrite reductase (NiR), NO$_2^-$ is reduced to NH$_3$. NO$_3^-$ is not thought to be toxic and can be converted into NH$_3$ in the cytoplasm and chloroplast by NR followed by NiR, or transported to the vacuole for storage. The above mechanisms offer a potential for detoxification and are possibly one reason why short-term experiments, conducted with high concentrations of NO$_x$ (20,000 ppb), have shown no effects on PS (Heber et al., 1994). Long-term experiments with NO$_x$, have also revealed little effect on PS, for example; van Hove et al., (1992) continuously exposed shoots of Pseudotsuga menziesii to 96 µg NO$_2$ m$^{-3}$ for 8 months with no apparent reduction in PS. Wellburn (1990,
1994), has concluded that most herbaceous species and many non-herbaceous species have the ability to detoxify NO\textsubscript{x} rapidly, with species that have very low levels of foliar NO\textsubscript{3}\textsuperscript{-} assimilation being most prone to damage. Evidence such as the above, has also led some workers to conclude that the indirect toxic action of NO\textsubscript{x} in the formation of O\textsubscript{3} is potentially more damaging than direct toxicity from NO\textsubscript{x} itself (Heber et al., 1994).

Studies of the effects of NH\textsubscript{x} on PS have been much fewer than those of NO\textsubscript{x}, despite the role of NH\textsubscript{x} as a major plant nutrient. The lack of studies is due partly to the more recent awareness of NH\textsubscript{x} as a pollutant and also, the difficulty of treating plants with such a volatile gas. Whilst the overall emphasis for SO\textsubscript{2}, O\textsubscript{3} and acidity tends to favour inhibition of PS, with NO\textsubscript{x} showing little effect except with long duration, high concentration treatments, stimulation of PS with NH\textsubscript{x} is more frequently reported than inhibition. Fangmeier et al., (1994) summarise the responses of *Populus euramericana* and *Pinus sylvestris* to NH\textsubscript{3} fumigations as a stimulation of PS, for applications of between 0.05 - 0.24 mg m\textsuperscript{-3} over 6 - 12 weeks. Similarly, van Hove et al., (1992) found an increase in PS for *Pseudotsuga menziesii* after 8 months continuous exposure with NH\textsubscript{3} at 66 μg m\textsuperscript{-3}. In most cases, inhibition of PS only occurs after long-term exposures to NH\textsubscript{3} at high concentrations. There have been a limited number of investigations into the effects of NH\textsubscript{4}\textsuperscript{+} on PS. Van Elsacker et al., (1988) found a reduction in net PS, stomatal conductance and water use efficiency in *Populus* cv. Beaupré, following exposure to acidic mists containing NH\textsubscript{4}\textsuperscript{+} at pH 4.3 and pH 3.5. In contrast, Eamus and Fowler (1990) found substantial increases in the rate of PS, chlorophyll production and stomatal conductance in *Picea rubens*, following exposure to acid mists containing NH\textsubscript{4}\textsuperscript{+} at pH 2.5. Conflicting results such as these, appear to be common place in the literature, where acidity, NH\textsubscript{4}\textsuperscript{+} and
PS are concerned. Despite the inclusion of \( \text{NH}_4^+ \) and other compounds in acid mists, few workers consider their possible influence on PS (Ashmore \textit{et al.}, 1989) and tend to concentrate on pH only (Cape, 1993). Studies considering the effect of N compounds, in particular \( \text{NH}_4^+ \), short-term fumigations with \( \text{NH}_3 \) and comparisons between the effects of \( \text{NH}_3 \), \( \text{NH}_4^+ \) and acidity have yet to be considered in detail.

In view of the above statements, this investigation sets out to consider the short-term responses of plants to \( \text{NH}_4^+ \) in more detail. Comparisons between the effects of \( \text{NH}_3 \), \( \text{NH}_4^+ \) and acidity are also made. Short-term changes in PS were also assessed for their use as physiological indicators of \( \text{NH}_x \) pollution, complimenting the enzymatic responses in chapter 3. Emphasis is placed on the rapid assessment of PS changes, without the need for time consuming tissue extraction and biochemical analysis of photosynthetic pigments or products. The use of a wide range of \( \text{NH}_x \) concentration and in some instances, stable isotope labelled \( ^{15}\text{NH}_4^+ \), enabled inter and intra-species dose response comparisons to be made. The differential susceptibility of plants to \( \text{NH}_x \) pollution, as outlined in chapter 4, is also considered using PS responses to \( \text{NH}_x \).

5.2 Experimental design

5.2.1 Rationale

Four plant species were initially screened for PS responses to \( \text{NH}_3 \). The species were \( \text{P. sylvestris} \), \( \text{C. vulgaris} \), \( \text{P. deltoides} \) and \( \text{A. glutinosa} \). \( \text{P. sylvestris} \) and \( \text{C. vulgaris} \) were selected as species that are commonly accepted as being susceptible to atmospheric pollution; likewise \( \text{P. deltoides} \) and \( \text{A. glutinosa} \) were selected as being less susceptible (Soares \textit{et al.}, 1995; Chapter 4). In addition, \( \text{P. vulgaris} \) was used for investigations comparing the effects of \( \text{NH}_3 \) and \( \text{NH}_4^+ \).
All plants were grown under greenhouse conditions as described in chapter 2.3.

5.2.2 Treatment with NH₃, NH₄⁺ and acidity

'Real time' measurements of PS in the presence of NH₃ were conducted using the O₂ electrode (See 2.11.1). After steady state PS was reached and control readings had been taken, 1 cm³ of air containing NH₃ at different concentrations was injected into the leaf chamber. The electrode was allowed to re-equilibrate for approx. 2 min and then, O₂ evolution was recorded in the presence of NH₃ for a further 2 min. This enabled an exact comparison between the presence and / or absence of NH₃ for the same leaf material, over a standard time period. The final concentration of NH₃ in the leaf chamber ranged from 0 - 20 μg cm³. The concentrations of NH₃ used for 'real time' measurement of PS are several orders of magnitude higher than a proposed critical level of NH₃ at 3300 μg m⁻³ for a 1 hour exposure (van der Eerden et al., 1993). The high concentrations of NH₃ were used to facilitate cuticular uptake of NH₃ in the event of stomatal closure by excised leaves (Walker, 1987; See 2.11.1). At this stage in the investigation, high concentrations of NH₃ were also used to gain a measurable response over a very short exposure time, with more realistic concentration used for later experiments. A response dose 50 (RD₅₀), which equals 50% of the initial maximum change in PS in the presence of NH₃, was used for inter-species comparison.

Repeat NH₃ fumigations at 3000 μg m⁻³, single mistings with NH₄Cl in the range 0 - 12 mol m⁻³ and repeat mistings with 1 mol m⁻³ ^¹⁵NH₄Cl (98% atom) were also carried out on 1 month old seedlings of *P. vulgaris*. Treatments were carried out in a 1 m⁻³ sealed perspex cabinet, using the method described in chapter 3.2.1 and 3.2.2 for greenhouse treatments. Identical control regimes to those described in
chapter 3.2.4 for temporarily covered leaves were used for all single treatment episodes. For repeat mistings and fumigations, separate control and treatment plants were used. In all cases, leaf material of a similar age and position was used. Both $O_2$ electrode and IRGA were used to measure changes in PS, with IRGA used for non-destructive measurements of PS in long duration experiments, with several treatment applications. Stable isotope $^{15}N$ determination for plant material was carried out as described in chapter 2.10. A 10 cm$^2$ area of leaf was detached after each misting episode and used to determine $^{15}N$ content.

Comparisons of the effects of $NH_4^+$ and acidity were carried out using a 'floating leaf' technique. 10 cm$^2$ leaf discs of *P. vulgaris* were cut from whole leaves and immediately placed in Petri dishes containing solutions of $dH_2O$ at pH 6 (control), $dH_2O$ at pH 3 and 1 mol m$^{-3}$ $NH_4^+$ at pH 3. The control value of pH 6 was used for *P. vulgaris*, as this compares closely to previous measurements of internal leaf pH conducted by Soares *et al.*, (1995) (Appendix 1). The Petri dishes were gently agitated on a flat-bed rotating shaker for 1 hour under greenhouse conditions, after which time PS activity of the leaf discs were determined using the $O_2$ electrode.

5.3 Results

5.3.1 The effects of $NH_3$ on photosynthesis using an $O_2$ electrode
Injection of $NH_3$ into the leaf chamber without the presence of leaf material, revealed no change in the electrode signal. The high concentrations of $NH_3$ were therefore, found not to affect the normal working of the electrode. Likewise, when leaf material was allowed to photosynthesise without the addition of $NH_3$, PS rate did not change
Figure 5.1 (a-b) Changes in photosynthetic rate (A) of 10 cm² leaf discs of *Alnus glutinosa*, in response to increasing concentration of NH₃. Measurements were taken using an O₂ electrode. Figure 5.1 (a) shows the actual values for A; control values for leaf discs are clear columns, treatment values measured for the same leaf discs are solid columns. Bars = SD of data, n = 3 individual plants (1 leaf disc from each). Figure 5.1 (b) shows the % change in A between control and treatment.
Figure 5.2 (a-b) Changes in photosynthetic rate (A) of 10 cm² leaf discs of *Populus deltoids*, in response to increasing concentration of NH₃. Measurements were taken using an O₂ electrode. Figure 5.2 (a) shows the actual values for A; control values for leaf discs are clear columns, treatment values measured for the same leaf discs are solid columns. Bars = SD of data, n = 3 individual plants (1 leaf disc from each). Figure 5.2 (b) shows the % change in A between control and treatment.
Figure 5.3 (a-b) Changes in photosynthetic rate (A) of 10 cm$^2$ leaf area of *Calluna vulgaris*, in response to increasing concentration of NH$_3$. Measurements were taken using an O$_2$ electrode. Figure 5.3 (a) shows the actual values for A; control values for leaf discs are clear columns, treatment values measured for the same leaf discs are solid columns. Bars = SD of data, n = 3 individual plants. Figure 5.3 (b) shows the % change in A between control and treatment.
Figure 5.4 (a-b) Changes in photosynthetic rate (A) of 10 cm² leaf area of *Pinus sylvestris*, in response to increasing concentration of NH₃. Measurements were taken using an O₂ electrode. Figure 5.4 (a) shows the actual values for A; control values for leaf discs are clear columns, treatment values measured for the same leaf discs are solid columns. Bars = SD of data, n = 3 individual plants. Figure 5.4 (b) shows the % change in A between control and treatment.
until the CO$_2$ concentration in the chamber became limiting. The longevity of steady state PS, was dependant on the initial rate of PS for each species, with lower rates of PS enabling longer duration within the chamber. Typically, PS could be maintained for between 10-15 min after the introduction of NH$_3$.

Exposure of $A$. glutinosa, $P$. deltoides, $C$. vulgaris and $P$. sylvestris to NH$_3$ causes an initial stimulation of PS (Figure 5.1-5.4 respectively). However, as the concentration of NH$_3$ increases, PS gradually declined below control levels. This is shown clearly for $A$. glutinosa (Figure 5.1) and $P$. sylvestris (Figure 5.4). Inter-species comparisons of RD$_{50}$ to NH$_3$, reveals a clear distinction between less susceptible and susceptible species. $A$. glutinosa and $P$. deltoides achieved higher increases in PS, than $C$. vulgaris and $P$. sylvestris. $A$. glutinosa and $P$. deltoides have RD$_{50}$ values of 30% stimulation (6 µg cm$^{-3}$) and 14% stimulation (8 µg cm$^{-3}$) respectively. RD$_{50}$ values for $C$. vulgaris and $P$. sylvestris are 4.25% stimulation (2 µg cm$^{-3}$) and 10% stimulation (4 µg cm$^{-3}$) respectively, indicating that peak stimulation of PS occurred at a lower concentration of NH$_3$ for these two species. A decline in stimulation responses occurred at lower concentrations of NH$_3$ in $C$. vulgaris and $P$. sylvestris than $A$. glutinosa and $P$. deltoides.

5.3.2 IRGA measurements of photosynthesis with NH$_3$ and NH$_4^+$

The PS responses of $P$. vulgaris to repeat fumigations with 3000 µg m$^{-3}$ NH$_3$ were determined using an IRGA (Figure 5.5a-b). PS was stimulated by 15%, 24 hours after the first fumigation. Stimulation of PS was also evident after the final fumigation (19% above control values). Stomatal conductance measured by IRGA gradually increased with each NH$_3$ treatment, with a final stomatal conductance of 25% above control values (Figure 5.5c).
Figure 5.5 (a-c) Changes in photosynthetic rate ($A$) in leaves of *P. vulgaris* measured by IRGA (5.5a), in response to 3 repeat fumigations with 3000 $\mu$g m$^{-3}$ NH$_3$. Each fumigation episode lasted 1 hour. Measurements were taken 24 hours after fumigation, with a 3 day interval between each episode. Control values are clear columns, treatment values are solid columns. Figure 5.5 (b) shows the % change between control and treatment values. Figure 5.5 (c) shows changes in stomatal conductance ($g_s$) for the same plants, with control values as clear circles and treatment values as solid circles. All values are for separate control and treatment plants; Bars = SD of data, n = 3.
Figure 5.6 (a-c) Changes in photosynthetic rate (A) of *P. vulgaris*, 24 hours after single mistings with 0-12 mol m⁻³ NH₄Cl at pH 3. Figure 5.6 (a) shows A measured by O₂ electrode for 10 cm² leaf discs. Figure 5.6 (b) shows A measured by IRGA for the same leaf as used for O₂ measurements, prior to leaf disc detachment. Control values are clear columns, treatment values are solid columns. Control regimes were conducted using temporary covered leaves; Bars = SD of data, n = 3. Figure 5.6 (c) shows the % change in A between control and treatment for both O₂ electrode and IRGA measurements.
Figure 5.7 (a-b) Changes in transpiration (E) (5.7a) and stomatal conductance ($g_s$) (5.7b) in *P. vulgaris*, 24 hours after single mistings with 0-12 mol m$^{-3}$ NH$_4$Cl at pH 3. E and $g_s$ were measured by IRGA. Control values are clear circles, treatment values are solid circles. Control regimes were conducted using temporary covered leaves; Bars = SD of data, n = 3.
The effect of NH$_4^+$ on PS in *P. vulgaris* was investigated 24 hours after single mistings with 0-12 mol m$^{-3}$ NH$_4^+$ at pH 3. This investigation was carried out using temporary covered leaves as control leaves on treated plants. Preliminary experiments to test the effects of the plastic bags, found no significant differences in PS rate, transpiration or stomatal conductance as a result of covering leaves for the duration of the misting episode. Measurements of PS using both O$_2$ electrode and IRGA revealed an initial stimulation in PS (Figure 5.6a-c). Stimulation of PS reached a peak value of 28% (O$_2$ electrode measurements) and 24% (IRGA measurements) for NH$_4^+$ treatments at 3-6 mol m$^{-3}$. Further treatment with NH$_4^+$ at 12 mol m$^{-3}$ resulted in no further increase in PS rate. A small but consistent difference was detected between O$_2$ electrode and IRGA measurements at all concentrations of NH$_4^+$. Even though IRGA measurements were taken from the same leaf as O$_2$ electrode measurements, before leaf detachment and placement in the electrode chamber, O$_2$ electrode measurements revealed a higher rate of PS in all cases. These higher rates of PS shown with O$_2$ electrode measurements are most likely due to the supra-optimal conditions provided within the electrode chamber.

Changes in stomatal conductance and transpiration rate were recorded using IRGA for *P. vulgaris*, 24 hours after misting with 0-12 mol m$^{-3}$ NH$_4^+$ at pH 3 (Figure 5.7a-b). No significant change was seen in the transpiration rate, although treatment values remained above those of control values at all concentrations of NH$_4^+$. Significant differences in stomatal conductance were revealed at the 3, 6 and 12 mol m$^{-3}$ NH$_4^+$ concentrations.

In order to distinguish between the effects of NH$_4^+$ and acidity, a ‘floating leaf’ experiment was conducted as described in 5.2.3. Incubating leaf discs in dH$_2$O at pH 3 and 1 mol m$^{-3}$ NH$_4^+$ at pH 3 for 1 hour, resulted in stimulation of PS, in comparison to leaf discs
Figure 5.8 Changes photosynthetic rate (A) in 10 cm² leaf discs of *Phaseolus vulgaris*, incubated in dH₂O at pH 6 (clear columns), dH₂O at pH 3 (grey columns) and 1 mol m⁻³ NH₄Cl at pH 3 (solid columns) for 1 hour at 20 °C. Leaf discs were placed in petri dishes containing each solution and continuously agitated using a rotary shaker. PS was determined using an O₂ electrode. Bars show SD of data; (n = 3 discs) for each treatment, from 3 individual plants.
Figure 5.9 (a-b) Changes in photosynthetic rate (A) measured by IRGA for *Phaseolus vulgaris*, 24 hours after successive mistings with 1 mol m$^{-3}$ $^{15}$NH$_4$Cl at pH 3. A 3 day interval occurred between each misting. Control values are clear columns, treatment values are solid columns. All values are for separate control and treatment plants; Bars = SD of data, n = 6. Figure 5.7 (b) shows the % change in A between control and treatment.
Figure 5.10 Changes in stomatal conductance ($g_s$) measured by IRGA for *Phaseolus vulgaris*, 24 hours after repeat mistings with 1 mol m$^{-3}$ $^{15}$NH$_4$Cl at pH 3. A 3 day interval occurred between each misting. Control values are clear circles, treatment values are solid circles. All values are for separate control and treatment plants; Bars = SD of data, n = 6.
incubated in dH₂O at pH 6 only (Figure 5.8). The stimulation of PS was greater for leaf discs incubated in NH₄⁺ at pH 3 (37% ± 7.5), than those incubated at pH 3 only (19% ± 8).

The PS responses of *P. vulgaris* to repeat mistings with 1 mol m⁻³ ^1⁵NH₄Cl at pH 3 were determined using an IRGA (Figure 5.9a-b). A 9% increase in PS was found 24 hours after the first misting. Increases in PS continued over 7 consecutive misting episodes, with peak stimulation occurring after the third misting episode (18% PS activity above control levels). A gradual decline in the enhancement of PS occurred as treatments were continued, resulting in a final PS activity 10% above control levels. Stomatal conductance also showed significant increases after 4 misting episodes (Figure 5.10).

5.3.3 ^1⁵N incorporation in *P. vulgaris* after repeat mistings with ^1⁵NH₄⁺

Repeat mistings with 1 mol m⁻³ ^1⁵NH₄Cl were carried out using 6 separate control and treatment plants, grown under identical conditions outlined in chapter 2. The total N content of control plants increased from 5.97% to 6.51% over the duration of the experiment (21 days). This was equivalent to an increase in N of 5.44 mg N g⁻¹ dwt. The total N content of misted plants increased from 6.57% to 7.75% over 21 days. This was equivalent to an increase in N of 11.78 mg N g⁻¹ dwt, (6.34mg N g⁻¹ dwt above control plants). The ^1⁵N content in leaf samples after the final misting was 3.85 mg ^1⁵N g⁻¹ dwt, indicating further uptake of N, equivalent to 2.49 mg N g⁻¹ dwt. Changes in ^1⁵N content for treated plants after each misting episode are shown in Table 5.1. A rapid uptake of ^1⁵N occurs over the first 3 misting episodes, with uptake stabilising from episode 4 onwards. Peak stimulation of PS occurs at episode 3-4. A gradual decline in the stimulation of PS occurred over the remaining episodes.
Table 5.1 $^{15}$N incorporation in leaves of *P. vulgaris*, 24 hours after repeat treatments with $^{15}$NH$_4$Cl

<table>
<thead>
<tr>
<th>Misting Episode</th>
<th>Regime</th>
<th>$\mu g$ $^{15}$N per 10 cm$^2$ leaf</th>
<th>% Change in A</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control Treatment</td>
<td>11.05</td>
<td>9</td>
</tr>
<tr>
<td>2</td>
<td>C</td>
<td>11.48</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>93.40</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>C</td>
<td>12.92</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>135.25</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>C</td>
<td>12.25</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>170.62</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>C</td>
<td>12.24</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>162.70</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>C</td>
<td>11.86</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>182.15</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>C</td>
<td>12.40</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>192.41</td>
<td></td>
</tr>
</tbody>
</table>

$A =$ net photosynthesis $\mu$mol O$_2$ m$^2$ s$^{-1}$
5.4 Discussion

5.4.1 Photosynthetic measurements and plant susceptibility to NH$_x$

The O$_2$ electrode provided a highly controlled environment within which to make direct comparisons between the responses of different species at varying concentrations of NH$_3$. As previously discussed in chapters 3 & 4 and presented in Soares et al., (1995), plant species that are regarded as being less susceptible to atmospheric pollutants, often have greater physiological vitality, with higher enzymatic rates and metabolite concentrations. These features contrast with more susceptible species that are less physiologically active. By taking two putative examples of each, the O$_2$ electrode also revealed differences in PS responses between A. glutinosa and P. deltoides (less susceptible) and P. sylvestris and C. vulgaris (more susceptible), (Figure 5.1-5.4). Although the two less susceptible species did not show significantly higher rates of control PS than C. vulgaris and P. sylvestris, they demonstrated higher increases in PS when exposed to NH$_3$. RD$_{50}$ values for A. glutinosa and P. deltoides were also higher than those for C. vulgaris and P. sylvestris. Consequently, the concentration of NH$_3$ required to illicit an inhibition of PS in less susceptible plants was found to be higher. It would appear therefore, that less susceptible plants are able to endure higher concentrations of NH$_3$ before inhibition of PS occurs, possibly as a result of their greater ability to assimilate and detoxify excess NH$_x$ (Soares et al., 1995). Extrapolation of these results into the field may be more problematic, as highly controlled conditions do not exist and PS could be influenced by environmental variables, such as light intensity, water availability and temperature. Even so, this method of PS measurement provided a relatively quick means of assessing the different responses of plants to
NH₃. By combining PS responses presented here, with enzymatic and metabolite responses presented in chapter 3, further clarification of the physiological status and possibly the susceptibility of a plant to atmospheric NH₃ can be made.

Gaseous exchange measurements using an O₂ electrode are a well established means of determining maximal rates of PS. Previous work has also employed this method for determining the effects of atmospheric pollutants on plants (Strand, 1993). In most instances, measurements of PS are taken on pre-treated material, at some time interval after exposing the plant to atmospheric pollutants. The O₂ electrode has in this investigation demonstrated the ability to measure PS responses to NH₃ exposure in ‘real time’ (Figure 5.1-5.4). A similar response may also be possible using an IRGA, by altering the composition of the gas flow through the leaf chamber. However, the concentrations of NH₃ required to achieve this response, typically in the μg cm⁻³ range, are greatly above those normally experienced in a polluted environment, despite a very short exposure time. It is unlikely that pollution levels required to elicit a ‘real time’ response to NH₃ occur very often, possibly only at or very near to a major pollution point source.

5.4.2 Stimulation of photosynthesis by NH₃ and acidity
The stimulation of PS following the application of NH₃ and acidity, appears to be a consistent response in the experiments reported here. Similarities can also be made with results obtained by previous workers: van der Eerden and Pérez-Soba (1992) demonstrated a 24% increase in PS for *P. sylvestris* continuously exposed to 240 μg m⁻³ NH₃ for 3 months, measured by IRGA. It is important therefore to establish which aspects of increased NH₃ uptake and acidity may be causing the increases in PS.
The use of $^{15}\text{NH}_4^+$ provided a means of associating N uptake with changes in PS (Table 5.1). The percentage increases found after applying labelled N may be greater in the field as the soil could act as a further sink for N allowing root uptake. Likewise, in these misting experiments, much of the misting solution is lost to the cabinet walls or floor. Some evidence of root uptake of non-labelled N exists, as the increase in N content in misted plants is greater than the measured increase in $^{15}$N. This could be due to the promoted onset of nodulation of *P. vulgaris*, in the presence of excess N, causing increased uptake of non-labelled N from the soil medium. The uptake of $^{15}$N may not have been sufficient to fuel demands for N, in light of increased PS rate. The plants may therefore, have taken up extra N from the soil to combat the demand for N. The results indicate that the availability of N is an important factor in determining the rate of PS. A close relationship between PS rate and N content of leaves is widely recognised (Evans, 1989) and may go some way to explaining the responses shown here. The availability and production of RuBP carboxylase-oxygenase (Rubisco), the main enzyme of CO$_2$ fixation in plants, is considered to be the most fundamental factor determining the rate of PS in plants (Fitter and Hay, 1987). Evans (1989) described a direct relationship between the production of Rubisco and leaf N content, with Rubisco consisting of up to 70% of the total leaf N content. The availability of N is also a major pre-requisite for the formation of other photosynthetic components for example, chlorophyll, thylakoid membrane proteins and other thylakoid constituents (Evans, 1989). Andreeva *et al.*, (1992) also found a 50% reduction in PS caused by a decrease in chlorophyll and Rubisco content of *Brassica juncea* grown under low soil N conditions. The possibility exists that the uptake of excess NH$_x$, may cause an increase in the production of Rubisco and / or chlorophyll, which in turn could
promote an increase in PS rate. The above relationship has indeed been established by van Hove et al., (1989,1992) and Fangmeier et al., (1994).

The synthesis of Rubisco and chlorophyll are dynamic processes in plants which generally occur rapidly under favourable conditions. Increased production of these compounds as a result of atmospheric sources of NH₃, will be regulated by the assimilation of NH₃ by GS and the subsequent production of amino acids and proteins. Chapter 3 provided evidence for the induction of GS within hours after exposure to excess NH₃. Thus increases in PS rate measured 24 hours after exposure to NH₃ and over longer periods could be due in part to increases in Rubisco and chlorophyll production. The increases in PS measured with the O₂ electrode occur within a few minutes after the introduction of NH₃. GS activities under control conditions vary between species but are often found to be relatively high in comparison to other enzyme activities (Soares et al., 1995). The 4 species investigated using the O₂ electrode were found to have a range of GS activities of between 40-120 μmols hr⁻¹ g⁻¹ fwt (Appendix 1), which is equivalent to 4-12 μmols hr⁻¹ fwt leaf disc (0.066-0.2 μmols min⁻¹ fwt leaf disc). At the lowest concentration of NH₃ used (1 μg cm⁻³), the electrode chamber contains approximately 0.406 μmols NH₃. It is therefore, possible for the assimilation of NH₃ to have an effect on PS within a few minutes of introducing NH₃ into the O₂ electrode. However, it is unlikely that complex molecules such as Rubisco, chlorophyll and thylakoid proteins could be synthesised as quickly. The changes in PS measured by O₂ electrode, suggest that other features of NH₃ uptake and assimilation may also influence changes in PS.

The rapid uptake of NH₃ by leaves, especially under high light intensities (van Hove et al., 1988) as used in the O₂ electrode, followed
by rapid assimilation by GS, allows for the release of H⁺ ions and potential increases in acidity. Likewise, acidity is often present in conjunction with the wet deposition of NH₄⁺. Both NH₄⁺ and H⁺ ions are known to cause uncoupling of photosynthetic electron transport, which can lead to a rapid increase in PS and O₂ evolution (Nicholls and Ferguson, 1992). The floating leaf experiment also demonstrated an increase in PS above control levels, when leaf discs were incubated in dH₂O at pH 3. Eamus (1993) found significant increases in PS in *Picea rubens* after 5 months of treatment with acid mists as low as pH 2.5. Increases in PS are also evident where NH₄⁺ is combined with acidity (Figure 5.8). Likewise, Eamus (1993) demonstrated higher rates of PS in *Picea rubens* subjected to NH₄NO₃ at pH 2.5 as opposed to NH₄NO₃ at pH 5.6. However, several workers have found significant reductions in PS as a result of treatments with acidity. Muthuchelian *et al.*, (1994) exposed *Phaseolus mungo* to acid mists of pH 5, 4 and 2 for 5 days and found significant reductions in PS of 60% (pH 2) and 20% (pH 4). Van Elsacker *et al.*, (1988) also found a decrease in PS in *Populus sp* after 3 weekly mistings at pH 4.3 and pH 3.5 for a whole growing season. Wellburn (1994) suggests that the direct effects of acidity on plants are little or no changes in PS, unless unrealistic levels of acidity are used (< pH 2). Likewise, Saxe (1991) reported increases in PS of several plant species treated with acid of between pH 1.7-pH 5.6, for short and long-term studies. However, the same author also mentions a variety of responses resulting in inhibition of PS by acidity. The conflicting literature makes it difficult to attribute increases in PS directly to acidity, or acidity in combination with NH₄⁺. As a consequence, there is a lack of published discussion towards possible mechanisms by which acidity may promote or inhibit an increase in PS. Changes in stomatal conductance in the presence of NHₓ and
acidity, as shown in this investigation, may however, provide some evidence of one such mechanism.

*P. vulgaris* treated with NH₃ displayed an increase in stomatal conductance. This occurred with both repeat fumigations and mistings and single high concentration mistings with NH₄⁺. Other workers have also noted an increase in stomatal conductance with NH₃ treatment (van der Eerden, 1992; Fangmeier *et al.*, 1994). An increase in stomatal conductance would inevitably allow a greater uptake of CO₂ to promote a higher rate of PS. Whether these increases in stomatal aperture occur as a direct result of NH₃ assimilation and / or the generation of acidity is still unclear. Stomatal apertures are controlled by adjacent guard cells, which increase in cell turgor in order to increase the size of the aperture. The turgor of guard cells is regulated by several factors; these include, K⁺, Cl⁻, and organic acids (Ilan *et al.*, 1994). Influxes of one or several of these compounds causes an increase in cell turgor and subsequent stomatal aperture. The same author states that the driving force behind influxes and effluxes of the above components, is a H⁺ ion pump, situated in the plasma membrane of the guard cell. Studies using pH-sensitive fluorescent dyes have shown rapid increases in acidity of the apoplastic solution of the guard cell walls when stomata open (Ilan *et al.*, 1994). Stomatal opening results from the extrusion of H⁺ ions followed by the uptake of K⁺, Cl⁻ and malate, causing water influx and an increase in cell turgor. Increases in H⁺ ion content of the apoplast from NH₃ assimilation or from H⁺ ions in acid deposition, would require the uptake of a balancing ion into the guard cell, which would promote stomatal opening. Fangmeier *et al.*, (1994) has reviewed several articles which show an increase in the N : K ratio with gaseous NH₃ treatments, thus K⁺ in the guard cell may be important in providing a balancing ion to H⁺ in the apoplast. Guard cells are not thought to contain chloroplasts,
therefore, GS activity will be low or negligible in such cells, as there is little demand for rapid assimilation of photorespired NH₃. Adjacent cells forming part of the stomatal complex may, however, contain chloroplasts and GS, thus providing a nearby source of acidity, following NH₃ assimilation and the extrusion of H⁺ ions into the apoplast. Regulation of stomatal aperture can be influenced by many factors; Van Hove et al., (1989) have suggested that increases in stomatal conductance result from increased CO₂ fixation, as the demand for C skeletons to compliment NH₃ assimilation increases. Thus stomatal conductance may be controlled by the sub-stomatal CO₂ concentration more directly than the presence of excess NH₃ or changes in acidity. The above mechanisms which may promote stomatal opening in the presence of excess NH₃ are by no means clearly defined, nor independent of other physiological process that cause stomatal closure. The growth hormone abscisic acid (ABA) is known to have a strong influence on stomatal regulation, causing rapid closure under drought conditions. The mobilisation of ABA from roots or from mesophyll cells in close proximity to guard cells, to ABA’s primary site of action on the outer surface of guard cells, is influenced by pH gradients created between the cytosol and apoplast. A decline in pH, which can also occur under drought conditions causes stomatal closing. Chapter 3 has also shown how levels of malate and other organic acids decline in the presence of excess NH₃. The involvement of malate and other organic acids in stomatal opening, may be inconsistent in the presence of excess NH₃. It is likely that a combination of biochemical changes arising from the presence of excess NH₃ may influence stomatal apertures. Whatever the mechanism behind these changes, decreased resistance to gaseous exchange as a result of increased stomatal aperture, provides the conditions for an increase in PS. The possibility of higher transpiration rates also exists as a result of
increased stomatal conductance. Although an increase in transpiration rate was not demonstrated significantly in this investigation, other workers have shown increases with long-term studies (Fangmeier et al., 1994). The resultant increase in water loss may prove to be damaging, where water availability is limited.

5.5 Conclusions

This chapter has provided further evidence for the effects of NH$_x$ on PS rate in plants. It has been shown that both stimulation and inhibition of PS can occur. The work presented here predominantly reveals a stimulation of PS, where plants are exposed to high and low concentrations of NH$_x$ for short-term durations only. Changes in physiology of a plant will undoubtedly alter as the pollution episodes become more persistent or increase in concentration. Even though the experiments presented here are essentially short-term investigations, repeat mistings with realistic concentrations of $^{15}$NH$_4^+$ at a 1 mol m$^{-3}$ concentration, demonstrated the potential for N saturation to lead to a decline in PS. It is important to remember once again that both stimulation and inhibition of PS can be damaging.

Several links are established in the literature between PS and N content; the synthesis of products such as Rubisco, chlorophyll and thylakoid proteins, dependant on the supply of N to a plant, represents just one. The synthesis of these products is an important stage in PS, however, there are countless other PS processes that can also be influenced by the presence of excess N. Acidity, either in combination with NH$_x$ or alone has an influence on the rate of PS. The changes in stomatal aperture discussed here represent one apparent mechanism of action; the interaction of acidity, stomata and other factors such as water availability, nutrient uptake and ABA make a full explanation
difficult. The complexity of possible interactions between NH\textsubscript{x}, acidity and PS is probably one reason why there are few details in the literature on the mechanisms of action of these compounds in relation to PS.

The ability of a plant to photosynthesise efficiently is subject to many environmental influences, which will ultimately make it difficult to assess the effects of atmospheric pollutants on PS in the field. It can be potentially difficult to distinguish between the influence of atmospheric pollutants, water availability, attacks by pests, nutrient deficiencies and seasonal variations. The O\textsubscript{2} electrode and to some extent the leaf chamber of an IRGA provide stable environments to study changes in PS. These techniques need to be applied to long-term studies in the field, where more variation will inevitably take place but hopefully lead to a fuller understanding of events.

The difficulty in explaining the effects of NH\textsubscript{x} and acidity on PS, does not detract from the established short-term stimulation and long-term inhibition, that have been demonstrated here and by other workers. The use of PS changes for biological assessment of the effects of NH\textsubscript{x} pollution, may be limited until further understanding of the effects are achieved.
CHAPTER 6

Physiological responses of mosses to atmospheric NH$_4^+$ and NO$_3^-$

6.1 Introduction

6.1.1 The atmosphere as a source of nutrition and pollution
The absence of a defined root system for nutrient uptake in terrestrial bryophytes, conveys a potential dependence on the atmosphere for the acquisition of major nutrients (Steinnes et al., 1994). The lack of a cuticular barrier in bryophytes will undoubtedly facilitate the rapid uptake of atmospheric compounds. The reliance of mosses on direct atmospheric sources of N can be beneficial under some circumstances, one such instance being mosses growing in nutrient poor habitats. These habitats which include upland, heathland and moorland communities, are typically low in productivity with limited nutrient cycling. Plant species in these areas predominantly consist of slow-growing climax species, which are adapted to the low nutrient availability. The deposition of N in these remote habitats is predominantly in the form of wet deposited NH$_4^+$ and NO$_3^-$, with a much smaller deposition of gaseous NH$_3$ and NO$_x$ than habitats closer to urban areas (Anon, 1990). Historically, the availability of N in these habitats has been at a level that limits growth (Aerts et al., 1992). However, levels of both NH$_4^+$ and NO$_3^-$, most often in association with acidity, are steadily increasing in these and other environments (Anon, 1990). The lack of a cuticle, coupled with a reliance on the atmosphere for nutrient acquisition, suggests that mosses may be sensitive to changes in levels of atmospheric compounds.
6.1.2 Previous work on mosses and atmospheric nitrogen pollution

The sensitivity of mosses to atmospheric pollutants (Wellburn, 1994) has been utilised by workers in Scandinavian countries, where several species are used as bio-monitors of trace element deposition. Steinnes et al., (1994) and Berg et al., (1995), have constructed detailed maps of trace element deposition in Norway, using *Hylocomium splendens* growing in natural communities. The sensitivity of mosses to atmospheric pollutants has also been exploited in historical studies of N deposition (Baddeley et al., 1994 and Pitcairn et al., 1995). Using field investigations and material from herbarium collections, increases in N content of moss tissue have been positively correlated with increases in N deposition from the atmosphere. An example of this is *R. lanuginosum* which demonstrates regional differences, with higher N content in polluted areas (Baddeley et al., 1994 and Pitcairn et al., 1995). Where studies have been carried out in the same area for long periods, for example *R. lanuginosum* collected between 1879 and 1989 from a site in N. Yorkshire, an increase in N content from 4.6 mg g\(^{-1}\) dwt to 12.3 mg g\(^{-1}\) dwt was found (Baddeley et al., 1994). This corresponds to an increase in wet deposited N from 9.25-25 kg ha\(^{-1}\) y\(^{-1}\) in the approximate area over a 90 year period (Pitcairn et al., 1995).

Studies such as these, have outlined the ability of mosses to accumulate atmospheric compounds to what might be considered concentrations greatly in excess of natural background levels. Although in many cases, our knowledge of so called ‘clean’ background levels of nutrients is scarce. Studies, predominantly using *Sphagnum* species, have demonstrated growth limitations and adverse changes in physiology with excess N deposition, (Rudolph and Voigt, 1986; Press et al., 1986 and Lee and Studholme, 1992). N accumulation in moss
tissue is considered to contribute towards the decline of some moss species including *R. lanuginosum*, as noted by Lee *et al.*, (1988) and Baddeley *et al.*, (1994). Transitions from non-nitrophilous to nitrophilous moss species have also been demonstrated in areas of high N deposition in Northern Europe (Hallingbäck, 1992; Greven, 1992).

6.1.3 Physiological effects of NH$_4^+$, NO$_3^-$ and acidity on mosses

Previous work on this subject has been very limited (Farmer *et al.*, 1992). Studies which have considered physiological effects have concentrated mainly on NO$_x$, predominantly as gaseous NO$_2$. Morgan *et al.*, (1992) carried out fumigation experiments with NO$_2$ on *Ctenidium molluscum, Homalothecium sericeum, Pleurozium schreberi* and *Hylocomium splendens*. These experiments showed that after 21 days of continuous exposure to NO$_2$, initial induction of NR activity was lost when extra NO$_3^-$ was applied. A similar response was also found for NR in *Rytidiadelphus squarrosus* after exposure to NH$_4^+$ (Morecroft *et al.*, 1994). Other workers have looked at physiological effects of NH$_4^+$ on *Sphagnum* species. Baxter *et al.*, (1992) exposed *S. cuspidatum* to mmol m$^{-3}$ concentrations of NH$_4^+$ for 30 days and found reductions in chlorophyll content and changes in amino acid contents. These responses were found to reflect previous exposure of *S. cuspidatum* to pollution; the greatest physiological changes occurred for species from unpolluted sites.

The effects of excess acidity on mosses has been considered in greater detail, although these studies have focused mainly on growth and moss cover. Hutchinson *et al.*, (1986) and Hutchinson and Scott (1988) conducted field mistings on *Pleurozium schreberi* at pH values of between pH 2.5 - pH 5.6. A significant loss in moss cover was
found over short and long-term treatments with all acidic treatments. In this instance, the main ions present in the misting solution were \( \text{H}^+ \), \( \text{SO}_4^{2-} \) and \( \text{NO}_3^- \). Later experiments reviewed by Farmer et al., (1992) have considered a few physiological parameters, such as a reduction in chlorophyll content and fluctuations in cation contents. However, these studies once again have focused mainly on \( \text{H}^+ \), \( \text{SO}_4^{2-} \) and \( \text{NO}_3^- \) induced acidity, with no reference to the possible influence of \( \text{NH}_4^+ \) as part of excess N deposition.

### 6.1.4 Aims of this investigation

The relative sensitivity of mosses to atmospheric pollutants (Morgan et al., 1992), suggests that physiological responses to \( \text{NH}_4^+ \) and \( \text{NO}_3^- \) can be detected with realistic concentrations of pollutants, far sooner than for higher plants (Hutchinson et al., 1986). Soares et al., (1995) have shown how certain physiological properties connected with N metabolism and pH regulation in tissues, for example, nitrate reductase activities, cation and organic acid contents, can determine the overall susceptibility of higher plants and mosses to atmospheric pollutants. Pearson and Stewart (1993) and Pearson and Soares (1995) also put forward the idea that, in higher plants at least, intrinsic nitrogen metabolism may play a role in species susceptibility to acidifying atmospheric pollutants.

In view of the above points, the aims of this study were firstly, to study the uptake of different N sources in bryophytes after short-term applications of \( \text{NH}_4^+ \) and \( \text{NO}_3^- \). The interaction of these individual inorganic N sources with acidity in applied mists was also considered. In addition, the use of stable isotope \( ^{15}\text{N} \) for some of the investigation enabled closer examination and quantification of \( ^{15}\text{NH}_4^+ \) and \( ^{15}\text{NO}_3^- \) uptake and assimilation at different concentrations of pollutant. Mosses
from contrasting habitats, for example rocky surfaces, grassland and semi-aquatic environment were also selected for comparisons of uptake of N and subsequent metabolic responses. Secondly, by examining changes in direct (enzymatic) and secondary effects (organic acids) of N metabolism, to attempt to identify consistent physiological indicators of short-term \( \text{NH}_4^+ \) and \( \text{NO}_3^- \) pollution. Thirdly, a comparison between the responses of mosses and responses known to occur in higher plants is considered. Fourthly, the ability of mosses to counteract the effects of atmospheric \( \text{NH}_4^+ \) and \( \text{NO}_3^- \) are discussed. Finally, a preliminary study is included to assess levels of total N and \( \delta^{15} \text{N} \) in lithophytic mosses, growing on walls in rural and urban areas. These mosses have an almost total reliance on atmospherically derived N; the hypothesis is to test whether total N and \( \delta^{15} \text{N} \) can be used to distinguish between atmospheric N sources and different levels of pollution.

6.2 Experimental design

6.2.1 Location of field sites and moss species studied
Experimental sites were established in the Cairngorm area of Scotland, as detailed in Chapter 2. The moss species studied were :- (i) \( R. \ \text{lorei} \), studied in an upland acidic grassland community, (ii) \( R. \ \text{lanuginosum} \), located amongst boulder detritus. Both of these mosses were present in abundant patches, particularly \( R. \ \text{lanuginosum} \), which was found in virtual monoculture, thus providing a sufficiently large area of material, without interference from other plant species. The third species studied was \( P. \ \text{fontana} \), was located at the side of a wet acid-flush. \( P. \ \text{fontana} \) was once again present in large, almost pure patches, and provided a comparison between the two terrestrial mosses and a semi-aquatic species.
In addition to the above, a preliminary survey was conducted of total N and $^{15}$N contents of lithophytic mosses, collected from urban sites in London and rural sites in Hertfordshire and Devon (Table 6.2). The species collected were *H. sericeum*, *T. muralis* and *G. pulvinata*. At the point of collection, a rough estimate of the flow of motor vehicles was made, to assign each collection point to a heavy, medium, light or very light traffic exposure (Table 6.2).

### 6.2.2 Treatment regimes

Plots of 25 cm x 25 cm for each species were marked out for treatments in the summer of 1993. Treatments consisted of a single misting episode of ± 500 cm$^3$ solutions of distilled water (Control); 3 mol m$^{-3}$ NH$_4$Cl pH 5 and pH 3; 3 mol m$^{-3}$ KNO$_3$ pH 5 and pH 3, applied evenly over the plot, with a continuous flow hand-held mister. The pH of the solution was adjusted using 0.1 M HCl. The misting episodes lasted approximately 30 minutes and took place from 12 noon onwards. Alternative treatments were applied to *R. lanuginosum* in the summer season of 1994. Plots of 25 cm x 25 cm, separate from the above, were misted with 500 cm$^3$ solutions of 1, 3 and 6 mol m$^{-3}$ isotope labelled $^{15}$NH$_4$Cl (98% atom) and K$^{15}$NO$_3$ (98% atom) at pH 5 and pH 3. Tissue was collected at varying time intervals after NH$_4^+$ or NO$_3^-$ application and returned to the laboratory for immediate analysis. The upper 2 cm of apical tissue (upper shoot), incorporating both the stem and leaves were used for all physiological studies. In addition, lower stem consisting of tomentum-like material, was collected for isotope analysis.

Mosses collected from London, Hertfordshire and Devon were not subjected to any treatment regimes. These samples were assessed
for total N and $^{15}$N natural abundance only, using the method described in Chapter 2.10.

6.3 Results

6.3.1 Total nitrogen and $^{15}$N content and uptake
All three moss species sampled in 1993, demonstrated increases in total N, whether the N was applied as NH$_4^+$ or NO$_3^-$ (Figure 6.1). Increases in total N after 48 hours were in the order of 1 mg g$^{-1}$ dwt or greater for both NH$_4^+$ and NO$_3^-$ treatments. This represented an increase of 20% or more above control N levels for the 3 species. R. lanuginosum demonstrated a slight preference for NH$_4^+$ incorporation over that of NO$_3^-$ whereas R. loreus and P. fontana showed no such distinction (Figure 6.1).

The total N in control plants of R. lanuginosum in the 1994 season measured by ANCA-MS, was 4.58 mg g$^{-1}$ dwt ± 0.16 (n = 10) and 10.16 mg g$^{-1}$ dwt ± 0.23 (n = 10) for upper and basal sections respectively. Comparison can be made with the total N in the upper section for the previous season's measurements for the same species shown in Figure 6.1 (4.2 mg N g$^{-1}$ dwt, 1993 season). In addition, natural abundance $\delta^{15}$N values, for control samples of upper and lower sections of R. lanuginosum were $-1.092 \pm 0.0013$ and $1.911 \pm 0.0046$ respectively (n=10).

The preference for NH$_4^+$ over NO$_3^-$ exhibited by R. lanuginosum, was further demonstrated with $^{15}$N labelled treatments at 1 and 3 mol m$^{-3}$ concentrations. However, less distinction between uptake of the two $^{15}$N sources was shown at the highest rate of application, 6 mol m$^{-3}$ (Figure 6.2 a-b) (Table 6.1). $^{15}$N uptake at 1 and 3 mol m$^{-3}$ concentrations appears to be more efficient, with up to 50% of applied
Figure 6.1 (I-III) Changes in leaf N content for 3 moss species, 48 hours after a single misting with: (1) distilled water; (2) 3 mol m\(^{-3}\) NH\(_4\)Cl pH 5; (3) 3 mol m\(^{-3}\) NH\(_4\)Cl pH 3; (4) 3 mol m\(^{-3}\) KNO\(_3\) pH 5; (5) 3 mol m\(^{-3}\) KNO\(_3\) pH 3. Bars show SD of the data (n = 3). Treatments with different letters (a-c) show LSD (** p = < 0.01). Horizontal axis labels 1-5 refer to treatments as above.
Figure 6.2 (a-b) $^{15}$N incorporation into upper stem and leaves and lower region of \textit{R. lanuginosum}, 24 hours after a single misting with $^{15}$NH$_4^+$ and $^{15}$NO$_3^-$. Labels on the horizontal axis refer to either a pH 5 or pH 3 treatment and the concentration of $^{15}$NH$_4^+$ and $^{15}$NO$_3^-$. Clear columns refer to background natural abundance $^{15}$N in moss tissues prior to treatment; hatched columns are $^{15}$NH$_4^+$ treatments and solid columns are $^{15}$NO$_3^-$ treatments. All treatment columns show $^{15}$N incorporation with background natural abundance $^{15}$N deducted. Bars show SD of the data (n = 3 for treatments, n = 10 for controls).
Table 6.1
Uptake of labelled N in 25 cm X 25 cm plots of *Racomitrium lanuginosum*

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>$^{15}$N applied to plot (mg)</th>
<th>Excess $^{15}$N harvested from plot (mg)</th>
<th>% uptake of applied $^{15}$N</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mol m$^{-3}$ $^{15}$NH$_4$Cl pH5</td>
<td>7.50</td>
<td>3.95</td>
<td>52.77</td>
</tr>
<tr>
<td>1 mol m$^{-3}$ $^{15}$NH$_4$Cl pH3</td>
<td>7.50</td>
<td>3.93</td>
<td>52.45</td>
</tr>
<tr>
<td>1 mol m$^{-3}$ K$^{15}$NO$_3$ pH5</td>
<td>7.49</td>
<td>3.06</td>
<td>40.94</td>
</tr>
<tr>
<td>1 mol m$^{-3}$ K$^{15}$NO$_3$ pH3</td>
<td>7.49</td>
<td>2.80</td>
<td>37.28</td>
</tr>
<tr>
<td>3 mol m$^{-3}$ $^{15}$NH$_4$Cl pH5</td>
<td>22.50</td>
<td>7.21</td>
<td>32.06</td>
</tr>
<tr>
<td>3 mol m$^{-3}$ $^{15}$NH$_4$Cl pH3</td>
<td>22.50</td>
<td>6.68</td>
<td>29.68</td>
</tr>
<tr>
<td>3 mol m$^{-3}$ K$^{15}$NO$_3$ pH5</td>
<td>22.47</td>
<td>6.00</td>
<td>26.67</td>
</tr>
<tr>
<td>3 mol m$^{-3}$ K$^{15}$NO$_3$ pH3</td>
<td>22.47</td>
<td>5.31</td>
<td>23.61</td>
</tr>
<tr>
<td>6 mol m$^{-3}$ $^{15}$NH$_4$Cl pH5</td>
<td>45.00</td>
<td>9.68</td>
<td>21.52</td>
</tr>
<tr>
<td>6 mol m$^{-3}$ $^{15}$NH$_4$Cl pH3</td>
<td>45.00</td>
<td>10.62</td>
<td>23.62</td>
</tr>
<tr>
<td>6 mol m$^{-3}$ K$^{15}$NO$_3$ pH5</td>
<td>44.94</td>
<td>9.35</td>
<td>20.81</td>
</tr>
<tr>
<td>6 mol m$^{-3}$ K$^{15}$NO$_3$ pH3</td>
<td>44.94</td>
<td>8.94</td>
<td>19.90</td>
</tr>
</tbody>
</table>

$^{1}$ Values for $^{15}$N natural abundance are deducted from this column.
label being recovered in the harvested plot (Table 6.1). When $^{15}$NO$_3^-$ or $^{15}$NH$_4^+$ were applied at 6 mol m$^{-3}$ concentration, there was a reduced efficiency in uptake of the two N sources, resulting in 20% or less recovery of the total $^{15}$N applied (Table 6.1). Additionally, $^{15}$N incorporation into the upper stem and leaf of *R. lanuginosum* was greater than uptake in the lower stem for all treatments, by a factor of 4 to 5 (Figure 6.2 a-b).

Mosses collected from urban and rural sites demonstrated contrasting total N and $^{15}$N contents (Table 6.2). In general, the total N content of mosses were significantly higher in urban areas than in rural areas; N in *H. sericeum* was almost 3 times higher, N in *T. muralis* and *G. pulvinata* were 2 times higher in London as opposed to Devon. In addition, samples of *H. sericeum* collected from sites near heavy traffic in London, had higher total N contents than samples collected from sites with lower traffic exposure. The same distinctions in total N content were not as evident for the other 2 species for different urban traffic exposures. Less distinction in total N content was also found between samples of *T. muralis* and *G. pulvinata* collected in London and Herts. Natural abundance measurements of $\delta^{15}$N were more positive (+ve) in urban areas, except for *G. pulvinata*, which gave a negative (-ve) $\delta^{15}$N reading at all collection sites. However, $\delta^{15}$N values became increasingly more -ve for all moss species, as traffic exposure decreased, either within London or between urban and rural sites. Variations in $\delta^{15}$N values were to some extent independent of total N contents for all 3 mosses, most notably for -ve $\delta^{15}$N values.

**6.3.2 Changes in nitrate reductase activity**

Induction of NR activity occurred for all three moss species misted with NO$_3^-$ (Figures 6.3-6.5). Both *R. loreus* (Figure 6.3) and *P. fontana*
Table 6.2 Total N and δ¹⁵N contents of mosses exposed to different traffic conditions

<table>
<thead>
<tr>
<th>Moss species</th>
<th>Location</th>
<th>Traffic Exposure</th>
<th>mg N g⁻¹ dwt</th>
<th>SD</th>
<th>δ¹⁵N/‰</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homalothecium</td>
<td>Chennies St, London</td>
<td>heavy</td>
<td>28.66</td>
<td>0.53</td>
<td>3.53</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>Regents St, London</td>
<td>heavy</td>
<td>21.80</td>
<td>0.32</td>
<td>6.06</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
<td>North End Rd, London</td>
<td>medium</td>
<td>19.17</td>
<td>0.41</td>
<td>-2.93</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>Golders Hill Park, London</td>
<td>light</td>
<td>13.44</td>
<td>0.36</td>
<td>-2.58</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>Dartmeet Bridge, Devon</td>
<td>very light</td>
<td>10.32</td>
<td>0.22</td>
<td>-6.63</td>
<td>0.68</td>
</tr>
<tr>
<td>Tortula muralis</td>
<td>Gower St, London</td>
<td>heavy</td>
<td>13.13</td>
<td>0.26</td>
<td>7.35</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>Gower Place, London</td>
<td>medium</td>
<td>18.68</td>
<td>0.37</td>
<td>4.63</td>
<td>0.43</td>
</tr>
<tr>
<td></td>
<td>University St, London</td>
<td>medium</td>
<td>17.73</td>
<td>0.40</td>
<td>2.51</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>Marshalsea Rd, London</td>
<td>light</td>
<td>17.99</td>
<td>0.64</td>
<td>-1.44</td>
<td>0.68</td>
</tr>
<tr>
<td></td>
<td>Albert Embankment, London</td>
<td>light</td>
<td>15.52</td>
<td>0.54</td>
<td>-1.78</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td>Aldbury, Herts</td>
<td>very light</td>
<td>24.34</td>
<td>1.19</td>
<td>-12.30</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>Daymer Bay, Devon</td>
<td>very light</td>
<td>7.51</td>
<td>0.52</td>
<td>-2.31</td>
<td>0.34</td>
</tr>
<tr>
<td>Grimmia pulvinata</td>
<td>Murray Terrace, London</td>
<td>heavy</td>
<td>22.47</td>
<td>0.40</td>
<td>-1.15</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>Grange Street, London</td>
<td>medium</td>
<td>14.84</td>
<td>0.31</td>
<td>-2.51</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>Hampstead Grove, London</td>
<td>light</td>
<td>20.05</td>
<td>0.40</td>
<td>-3.83</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>Regents Park, London</td>
<td>very light</td>
<td>19.99</td>
<td>0.25</td>
<td>-5.31</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
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<td>14.65</td>
<td>0.16</td>
<td>-7.68</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td>Wadebridge, Devon</td>
<td>very light</td>
<td>11.52</td>
<td>0.30</td>
<td>-5.62</td>
<td>1.39</td>
</tr>
</tbody>
</table>

Traffic exposure:
- heavy = > 20 cars passing per min
- medium = 10-20 cars min⁻¹
- light = 5-10 cars min⁻¹
- very light = < 5 cars min⁻¹

Values for total N and δ¹⁵N are the average of 3 independent moss samples
Figure 6.3 (I-VI) Physiological changes in *R. loreus* 24 hours after a single misting with: (1) distilled water; (2) 3 mol m⁻³ NH₄Cl pH 5; (3) 3 mol m⁻³ NH₄Cl pH 3; (4) 3 mol m⁻³ KNO₃ pH 5; (5) 3 mol m⁻³ KNO₃ pH 3. Bars show SD of the data (n = 3). Treatments with different letters (a-d) show LSD (* p = < 0.05; ** p = < 0.01; *** p = < 0.001). Horizontal axis labels 1-5 refer to treatments as above.
Figure 6.4 (I-VI) Physiological changes in *R. lanuginosum* 24 hours after a single misting with: (1) distilled water; (2) 3 mol m⁻³ NH₄Cl pH 5; (3) 3 mol m⁻³ NH₄Cl pH 3; (4) 3 mol m⁻³ KNO₃ pH 5; (5) 3 mol m⁻³ KNO₃ pH 3. Bars show SD of the data (n = 3). Treatments with different letters (a-d) show LSD (*** p = < 0.001). Horizontal axis labels 1-5 refer to treatments as above.
Figure 6.5 (I-VI) Physiological changes in *P. fontana* 24 hours after a single misting with (1) distilled water; (2) 3 mol m\(^{-3}\) NH\(_4\)Cl pH 5; (3) 3 mol m\(^{-3}\) NH\(_4\)Cl pH 3; (4) 3 mol m\(^{-3}\) KNO\(_3\) pH 5; (5) 3 mol m\(^{-3}\) KNO\(_3\) pH 3. Bars show SD of the data (n = 3). Treatments with different letters (a-e) show LSD (** p = < 0.01; *** p = < 0.001). Horizontal axis labels 1-5 refer to treatments as above.
(Figure 6.5) had low (possibly constitutive) NR activities in control plots (0.1 - 0.3 μmol h⁻¹ g⁻¹ dwt); this compares with a control activity of about 3.5 μmol h⁻¹ g⁻¹ dwt in *R. lanuginosum* (Figure 6.4). Induced NR activities in *P. fontana* remained low with NO₃⁻ application possibly reflecting its semi-aquatic habitat, where some of the N could be washed away from, or be more readily diluted within the plot. The time course study for NR induction in *R. lanuginosum* treated with 3 mol m⁻³ NO₃⁻ suggests that peak induction may occur between 3 and 24 hours after NO₃⁻ application (Figure 6.6). The high background rates of NR activity in this species make precise determination of peak induction difficult. Induced NR activities began to decline towards control levels, 72 hours after treatment.

Misting with NH₄⁺ led to inhibition of NR activity in all three species (Figures 6.3-6.5). Application of 3 mol m⁻³ NH₄⁺ at pH 3 generally gave greater reductions in NR activity than at pH 5. In addition, *R. lanuginosum*, which had the highest control activities, also showed the greater decline in NR with NH₄⁺ treatment (Figure 6.4).

Figure 6.7 shows the effect of NO₃⁻ and NH₄⁺ treatments, at three different concentrations and two pH levels, on *R. lanuginosum*. Control NR values measured are similar to those shown in Figure 6.4 for the previous year. Increasing the treatment concentration of NO₃⁻ caused an expected induction of NR, with induced levels at 6 mol m⁻³ NO₃⁻, twice that of the controls. In contrast, NH₄⁺ inhibits NR activity, particularly at the highest concentration. The effect of the pH treatments is too variable for detailed interpretation, however there is an indication that the pH 3 treatment may be inhibiting NR activity more so than at pH 5 (Figure 6.7).
Figure 6.6 Time course study of NR activity in *R. lanuginosum* over 72 hours, following a single misting with 3 mol m\(^{-3}\) KNO\(_3\) at pH 5. Clear columns are control treatments; solid bars are NO\(_3^-\) treatments. Bars show SD of the data (n = 3). Asterisks show LSD between control and treatments, (*p = < 0.05; ** p = < 0.01; *** p = < 0.001).
Figure 6.7 Changes in Leaf NR activity of *R. lanuginosum* 24 hours after a single misting with NH$_4^+$ and NO$_3^-$. Labels on the horizontal axis refer to either a pH 5 or pH 3 treatment and the concentration of NH$_4^+$ and NO$_3^-$. Clear columns refer to controls; hatched columns are NH$_4^+$ treatments and solid columns are NO$_3^-$ treatments. Bars show SD of the data (n = 3).
6.3.3 Changes in base cations and organic acids

Significantly consistent changes in base cations were evident in the two terrestrial species following the addition of N. Ca levels in *R. loreus* (Figure 3) and *R. lanuginosum* (Figure 4) increased with NO$_3^-$ treatment and decreased with NH$_4^+$ treatment in *R. loreus* only (Figure 3). K levels in *R. lanuginosum* increased with NO$_3^-$ but decreased with NH$_4^+$ treatment (Figure 4), with *R. loreus* demonstrating increases in K with both NH$_4^+$ and NO$_3^-$ treatments (Figure 3). No consistent differences were found in Mg concentration for all three species and no significantly consistent differences were found between pH 3 and pH 5 treatments for all three species, with any of the measured cations. The concentrations of Ca, Mg and K in *P. fontana* (Figure 5) showed some variations that cannot wholly be accounted for by the different N treatments. Changes in cation content for example, a decline in Mg at 3 mol m$^{-3}$ NO$_3$ at pH3 only (Figure 5) and declines in K with all treatments except 3 mol m$^{-3}$ NH$_4$ at pH3 (Figure 5), showed no correlation with changes found in the other two species.

The organic acids malate and citrate can be seen to alter with the different N treatments. Treatments with NH$_4^+$ lead to consistent declines in malate and citrate content, for all the moss species (Figures 3-5). The more marked quantitative changes occur in malate concentration, probably because it is present at a greater concentration than citrate, in all three species. *R. lanuginosum* (Figure 4) and *P. fontana* (Figure 5) also demonstrated an enhanced reduction in malate and citrate, when NH$_4^+$ is applied at pH3 compared to pH5, suggesting a pH sensitive reaction. Treatment with NO$_3^-$ showed significant increases in malate for all species except *P. fontana*, were no consistent changes were found (Figure 3, 4 & 5). No changes were found in citrate content with NO$_3^-$ treatments for all three species.
6.4 Discussion

6.4.1 Total nitrogen and $^{15}$N content and uptake

This study has further emphasised the possible reliance of mosses on atmospheric N and their ability to reflect the N conditions in the habitats in which they are present. The total N content found in control samples of the upper section of *R. lanuginosum* closely resembled levels measured by Thompson and Baddeley (1991) for the altitude of collection and suggested a relatively unpolluted site. Likewise, the low $^{15}$N values measured for *R. lanuginosum*, are typical for plants that gain much of their N from atmospheric sources and are generally growing in environments depleted of N (Stewart *et al.*, 1995a). It would appear therefore, that the sampling sites used in the Cairngorms, are at present relatively unpolluted. This evidence is supported by recent estimates of total N deposition in the Cairngorm area, at a low to moderate level of 9 kg N Ha$^{-1}$ yr$^{-1}$ (Pitcairn *et al.*, 1995). The 3 mosses collected from Devon had lower total N contents than the same species collected in London. N pollution levels in London, predominantly in the form of gaseous NO$_x$, are much higher than those in Devon (Anon, 1990), which may be reflected in moss tissue. Previous workers have also related total N content of mosses to deposition levels of N, most notably with regional comparisons of total N content (Baddeley *et al.*, 1994; Pitcairn *et al.*, 1995). Differences in total N content between London and Herts were not so notable, which may reflect the transport of gaseous NO$_x$ to areas in close proximity to urban sources of pollution. Also, differences between total N from species exposed to different rates of traffic flow in London, were not distinct; the most consistent contrasts were found between the two
extremes of heavy and very light traffic for all three mosses. Therefore, using total N values to give an indication of atmospheric N levels or N deposition, may not be applicable were mosses are sampled from nearby areas. However, more consistency was found with $\delta^{15}N$ values, which became more -ve as exposure to traffic decreased. Changes in $\delta^{15}N$ values were to some extent, independent of the total N content of moss tissue (Table 6.2). This suggests that $\delta^{15}N$ values are influenced more by the N source, than the amount of N present. Natural abundance values for $^{15}N$ can vary quite markedly in biological material (Handley and Raven, 1992). However, the large ranges of $\delta^{15}N$ measured for 3 mosses in this study, might not be as expected for intra-species comparisons, unless the N source is influencing the isotopic ratio of $^{15}N$ to $^{14}N$. Assuming lithophytic mosses in urban areas gain most of their N from NO$_x$, the results suggest that NO$_x$ in urban areas may have a predominantly +ve $\delta^{15}N$ signature. Heaton (1986) suggested a range of $\delta^{15}N$ values for pollution sources of NO$_x$ at -1 to +5 %oo, which offers a possible explanation for the +ve $\delta^{15}N$ values found in urban mosses. Other workers have also suggested predominantly +ve $\delta^{15}N$ values for industrial sources of gaseous and particulate NO$_x$ (Handley 1995, SCRI, personal communication). In contrast, Heaton (1990) found the $\delta^{15}N$ content of NO$_x$ directly from vehicle exhausts to be -ve, with increasing negativity as engine speed decreased. Traffic congestion in London is widespread, thus slowing down vehicles and suggesting that much of the NO$_x$ produced in London may have a -ve $\delta^{15}N$. The +ve $\delta^{15}N$ values found in mosses sampled from London are, therefore, in complete contrast to values that may be expected based on Heaton’s (1990) work. There are several possible explanations to the differences found. The gas samples measured by Heaton were collected directly from the car exhaust pipe.
on a test-bed and not from roadside conditions. As a consequence, the concentrations of NO\textsubscript{x} measured were very high, typically 130-1550 ppm (248820 µg m\textsuperscript{-3} to 2966700 µg m\textsuperscript{-3} NO\textsubscript{2}). This compares to annual mean concentrations of NO\textsubscript{2} in the range 30-80 µg m\textsuperscript{-3} NO\textsubscript{2} for London in 1984-1985 (Anon, 1993c). Although very little data is available for isotopic values of NO\textsubscript{x} at realistic urban concentrations, it is possible that isotopic ratios may change, following the release of NO\textsubscript{x} into the atmosphere. The recent incorporation of catalytic converters to many motor vehicles (which were not considered by Heaton, 1990) may also change the \textsuperscript{15}N / \textsuperscript{14}N ratio of exhaust gasses. Biological discrimination in favour of \textsuperscript{15}N may occur as a part of N assimilation in mosses. However, there is little evidence of discrimination occurring in higher plants in favour of \textsuperscript{15}N following NO\textsubscript{3}\textsuperscript{-} and NH\textsubscript{4}\textsuperscript{+} assimilation (Linda Handley 1995, SCRI, personal communication), thus discrimination may also not be evident in mosses. In the case of moss samples collected from rural areas, where N deposition is predominantly wet deposited, the \textminus ve \textdelta\textsuperscript{15}N values correspond to previous measurements of \textdelta\textsuperscript{15}N of NO\textsubscript{3}\textsuperscript{-} and NH\textsubscript{4}\textsuperscript{+} in rainwater of -1.5 \%\textsubscript{o} to +0.9 \%\textsubscript{o} for NO\textsubscript{3}\textsuperscript{-} and -8.6 \%\textsubscript{o} to -7.0 \%\textsubscript{o} for NH\textsubscript{4}\textsuperscript{+} (Linda Handley, SCRI, personal communication). Areas of Devon are not totally N pollution free; a large proportion of UK gaseous NH\textsubscript{3} emissions occurs as a result of agricultural practices in Devon (1000-2000 tonnes per 20 km\textsuperscript{2} grid square, Anon, 1990). Thus, with our current understanding of atmospheric processes and \textdelta\textsuperscript{15}N values of NO\textsubscript{x} and NH\textsubscript{x}, mosses from rural areas may indicate N pollution sources more directly, whereas more research would be needed to clarify NO\textsubscript{x} signatures if \textdelta\textsuperscript{15}N values in mosses collected from urban areas were to be related to N pollution sources.
Whatever the pollution source, the mosses sampled in this study have shown the ability to rapidly incorporate excess N into their tissues. The degree to which mosses incorporate N into their tissues can indicate past and present exposure levels to N sources. Mosses growing in low N areas will take rapid advantage of exogenous N sources, whereas mosses already growing in areas with high available N, will incorporate less N in proportion to availability. This relationship has been demonstrated by transplant studies on *Sphagnum* species by Lee *et al.* (1988). Pitcairn *et al.* (1995) have also demonstrated how several moss species show increases in total N of up to 62% over long periods of time (30 years) in polluted areas. The rate of incorporation of atmospheric N can also be significant over much shorter periods of time (20% increase in total N over 48 hours; Figure 6.1).

Distinctions between uptake of different N sources were evident, especially for *R. lanuginosum*, which favoured NH$_4^+$. The tendency towards greater NH$_4^+$ uptake as opposed to NO$_3^-$, is possibly facilitated by a high cation exchange capacity, which is common in mosses (Bates, 1992). Utilising NH$_4^+$ as an N source as opposed to NO$_3^-$, is commonly regarded as being more energy efficient, achieving greater specific growth rates (Raven *et al.* 1992a). Where NH$_4^+$ and NO$_3^-$ are both available in equal conditions and amounts, NH$_4^+$ may therefore be the favoured N source. Higher plant shoots when treated with atmospheric NH$_x$ or NO$_x$ also tend to favour NH$_x$ uptake when applied at the same concentration (Pearson and Stewart, 1993). The preference for NH$_4^+$ uptake in favour of NO$_3^-$ may not occur in all situations. Baddeley *et al.*, (1994) found slightly lower total N contents in *R. lanuginosum* misted with 14 kg ha$^{-1}$ yr$^{-1}$ NH$_4^+$-N, in comparison to treatments of 10 kg ha$^{-1}$ yr$^{-1}$ NO$_3^-$-N, after 3 years of treatments, in addition to
12 kg ha\(^{-1}\) N yr\(^{-1}\) bulk background deposition. Although the treatments were at slightly different levels, the results suggest that long-term exposure to NH\(_4^+\) may lead to inhibition of uptake, possibly resulting from acidification caused by the assimilation of NH\(_4^+\). The same authors found significantly higher N contents in *R. lanuginosum* treated with both NH\(_4^+\) and NO\(_3^-\) for the same amount of time. The need to buffer against acidity may be lower with both NH\(_4^+\) and NO\(_3^-\) uptake, allowing greater N incorporation.

The upper stem and leaf of *R. lanuginosum* were more efficient at retaining atmospherically applied \(^{15}\)N than the basal region. This is in agreement with similar investigations on *R. lanuginosum* where \(^{15}\)N accumulation was greatest in the upper 1 cm of shoot, 1 week after aerial treatment with 1 mol m\(^{-3}\) NH\(_4^+\) (Jönsdöttir *et al.*, 1995). This may be accounted for by the greater cation exchange capacity and surface area of leaves on the upper stem; leaves lower down the stem were reduced in frequency. However, the basal region of *R. lanuginosum* had over twice as much total N content, in comparison to the upper leaves and shoot. The slight differences in the \(\delta^{15}\)N natural abundance values between upper and basal regions, may be due to the higher total N content in the lower section, or could be the result of differences in N isotope mobilisation for storage. The higher total N content in the basal region may also account for the reduced uptake of \(^{15}\)N as described above. The proportion of lower stem material to upper in *R. lanuginosum* was between 5:1 to 15:1, which is typical for upland areas, where strong winds prevail (Tallis, 1959). Therefore, the more abundant basal region may capture a higher proportion of deposited atmospheric N. Likewise, rapid saturation of the upper shoot material, may lead to excess run-off of N-containing solutions directly into the lower sections of the moss. Rapid N saturation of the upper
shoot, may help to explain why uptake of 6 mol m\(^{-3}\) \(^{15}\)NH\(_{4}^{+}\) and \(^{15}\)NO\(_{3}^{-}\) was less efficient, than at 1 and 3 mol m\(^{-3}\) concentrations (Table 6.1). Therefore, the basal region of \(R. lanuginosum\) may represent a significant storage pool for N, with the possibility of translocation of N occurring some time later, when new upper shoot growth occurs. The redistribution of N sources from lower to upper regions of \(R. lanuginosum\), most likely by capillary action, has also been considered by Jónsdóttir \textit{et al.}, (1995), where soil applied NH\(_{4}^{+}\) and NO\(_{3}^{-}\) was found to increase the upper shoot N content, 1 week after application.

\subsection*{6.4.2 Nitrate reductase activity}

Treatment with NH\(_{4}^{+}\) for all the species, leads to inhibition of NR activity. This has also been demonstrated for \textit{Sphagnum} species (Woodin and Lee, 1987), cell suspensions of \textit{Funaria hygrometrica} (Padidam \textit{et al.} 1991) and \textit{Rytidiadelphus squarrosus} (Morecroft \textit{et al.}, 1994). The inhibition of NR in leaves of higher plants when treated with atmospheric NH\(_{x}\) has also been reported by Pearson and Stewart, 1993; Pearson and Soares, 1996; and presented in Chapter 3. Thus, the inhibition of NR activity by excess NH\(_{x}\) would appear to be consistent for both mosses and higher plants. As a consequence, much of the explanation behind NR reduction presented in Chapter 3 for higher plants, may be relevant here. Nitrate reduction itself leads to the production of NH\(_{4}^{+}\), which is readily assimilated into organic form so as not to reach harmful levels. Declines in NR activity may therefore be a protective mechanism to prevent an excessive build up of NH\(_{4}^{+}\) in the moss. Alternatively, it has been suggested that the build up or depletion of metabolites, among them amino acids such as asparagine or glutamine, produced in NH\(_{4}^{+}\) assimilation, may have a negative feedback on the regulation of NR (Filner 1966; Lee \textit{et al.} 1992).
Whatever the mechanism behind the declines in NR activity shown, this particular response is consistent through three contrasting moss species, shows a relatively rapid response within 18 hours (Figure 6.6) and is also sensitive to the concentration of NH$_4^+$ applied (Figure 6.7). There is also a slight indication within the results, that acidity, particularly in conjunction with NH$_4^+$ may further inhibit NR activity (Figures 6.3-6.5 & 6.7). The results suggest that this is not due to an enhanced NH$_4^+$ uptake, as the uptake of $^{15}$NH$_4^+$ is in fact reduced slightly at pH 3 (Figure 6.2). Increased inhibition of NR in the presence of both NH$_4^+$ and acidity, may be due to interactions of protons, possibly with cations such as K$^+$ that are used for NO$_3^-$ transport in plants (BenZioni et al., 1971).

Induction of NR activity, with NO$_3^-$ treatments also appears to be a consistent response. Repeated or continued exposure to high levels of NO$_3^-$ are known to cause a loss of induction capability and declines in NR activity (Morecroft et al., 1994). As for higher plants, induction or inhibition of NR will be influenced by the proportions of NH$_4^+$ and NO$_3^-$ present in precipitation. The potential for NR activity as an indicator of excess NH$_4^+$ and NO$_3^-$ in N deposition, may be easier to characterise in mosses than higher plants, due to the lack of influence from soil sources of N.

### 6.4.3 Organic acids, base cations and cell pH

The two terrestrial species showed variable responses of their base cation content with regard to the type of inorganic N treatment. Calcium had a tendency to be the most mobile, particularly in _R. loreus_ (Figure 6.3). It also had a general tendency to increase with NO$_3^-$ treatment, especially in _R. loreus_ and _R. lanuginosum_ (Figures 6.3 & 6.4). Where this Ca comes from is not clear; it could be due to
remobilisation from lower plant tissues, enhanced uptake from the surrounding substrate (especially more likely for R. loreus, growing in grassland), or to enhanced atmospheric deposition. 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. The response of Mg was extremely variable and interpretation of effects not possible. It is interesting to note that P. fontana had the highest overall cation content of the three species studied, most likely reflecting its semi-aquatic habitat and the availability of nutrients in run-off water, rather than the treatments applied. The method of cation analysis for all species, was for total cations and did not distinguish between external and internal cation contents. The possibility therefore remains that charged particles present in the misting solutions for example NH$_4^+$ and K, may cause immediate changes in the exchangeable cation pool, which in turn may influence cell physiology.

More distinct and consistent changes in the two organic acids are evident in all the species (Figures 6.3-6.5). Citrate and malate, decrease in concentration with NH$_4^+$ treatment; this is more noticeable for R. lanuginosum and P. fontana. Although, there is also some evidence that the converse can occur with NO$_3^-$ application, ie. an increase in organic acids, this is not as consistent as the above. These changes, at least a decline in organic acids with NH$_4^+$ treatments, are consistent with changes found in higher plants (Pearson and Stewart, 1993; Raven 1988; Chapter 3).

Raven (1988) states that the assimilation of inorganic NH$_4^+$ into organic form in plant tissue leads to a net increase in H$, while the assimilation of NO$_3^-$ generates OH$. The buffering of OH$^{-}$ generated by NO$_3^-$ reduction is less of a problem to plants, which can readily
neutralise OH⁻ with an increase in organic acids, possibly demonstrated with the increases in malate and citrate shown with NO₃⁻ treatments in this investigation. The neutralisation of acidity is more problematic for plants (Raven et al. 1992b; Pearson and Stewart 1993). Short-term changes in both cations and organic acids may provide one 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 hydrogen ion inputs (Soares et al., 1995). Previous experiments by Bates (1992) and Kooijman and Bakker (1994) have demonstrated the relative mobility of cations and high exchange capacities in moss tissues. The mobility of cations in mosses may, therefore, be of benefit in alleviating the effects of acidic pollutants. Soares et al. (1995) have also shown a strong positive correlation between the rate of NR activity, base cation content and subsequent buffering capacity against acidity for higher plants. Taken overall, the changes in both cation and organic acid concentrations in this study, suggest that these metabolites are used for short-term homeostasis of cell pH within bryophytes as well as higher plants.

6.5 Conclusions

This study has highlighted several potential mechanisms that help mosses regulate their metabolism when faced with atmospheric N uptake. In particular, the need to maintain cell pH homeostasis, as well as the regulation of metabolism in the presence of defined inorganic N sources. Several physiological responses also indicate that mosses do not differ significantly in their basic biochemical regulation when compared to higher plants. This similarity occurs,
despite mosses greater reliance on atmospheric rather than soil-derived N.

Long-term studies of N accumulation in mosses have revealed dramatic increases and provided evidence for increases in pollution of the environment. These studies however, are often limited by long periods in history, where no data is available. Thus an increase in N over 100 years for example, may be presented with a limited amount of data points at irregular intervals. Short-term studies on the other hand can reveal comparable uptake of N, at realistic concentrations of pollutant, over much shorter periods of time. This comparison outlines the importance of comparing both short and long-term studies, especially when considering the influence acute pollution episodes may have on historical comparisons.

The lack of a cuticular barrier to atmospheric inputs, the reliance of atmospherically derived sources of major nutrients and the increasing levels of pollutants in the environment, suggest that these mechanisms may only be of limited use for mosses, with most benefit gained where pollution episodes are sporadic. Although NH$_4^+$ is the predominant form of deposited N, mixed NO$_x$ / NH$_x$ deposition does occur and will inevitably make the interpretation of effects more complex. Long-term exposures to excess N may well break down the ability of mosses to physiologically buffer against acidity, leading to a decline or loss of more sensitive species.

The degree to which N content in mosses can reflect atmospheric concentrations of N, has led many workers to suggest that mosses in general, are particularly sensitive to atmospheric N pollution. Several studies, including this one have outlined the ability of mosses to accumulate N in response to N pollution, far in
excess of non-polluted individuals, over short and long-term investigations. Studies of herbarium collections of moss tissue have provided an interesting insight to changes in N pollution over long periods of time. The ability of mosses to rapidly accumulate N in their tissue, suggests that changes in N contents as revealed by herbarium studies may not have taken as long as is often suggested. The same species of moss can also show a large range of total N and $\delta^{15}$N contents, one example being the comparisons between mosses growing in urban and rural environments studied here. Such variability in N content of mosses is often in contrast to higher plants, that do not generally exhibit large variations in N content. Evidence exists of the disappearance of mosses from polluted environments over long-term studies. However, there is seldom evidence of visible damage where N has been applied experimentally, even at high concentrations. No doubt, there will be certain species of mosses that are more sensitive to N pollution than others; grouping all mosses as ‘sensitive species’ may be too much of a generalisation. Detrimental effects on mosses, where N has increased over a long period of time, or has been applied experimentally, may reflect nutrient imbalances and associated acidity either directly from acid deposition, or from the uptake and assimilation of N, rather than direct effects of N itself. In view of the capability of some mosses to rapidly accumulate and store N, with little evidence of harmful effects, they may prove to be useful as indicators of N pollution in urban and rural environments.
Concluding remarks

7.1 Physiological responses demonstrate foliar uptake of NH$_x$

The leaves of plants represent the most direct route for uptake of atmospheric pollutants, particularly in gaseous form and are therefore, the most direct route for potential damage. In the presence of excess NH$_x$, activities of GS, NR and ME, concentrations of organic acids, photosynthetic rate and stomatal conductance, have all shown consistent and reproducible responses to foliar uptake of NH$_x$, in a number of higher plants and mosses. Studies such as the above, help to characterise the mechanisms of physiological responses in the presence of excess NH$_x$, and allow responses to be directly associated with uptake of NH$_x$. The lack of visible injury in all the investigations, reveals that physiological perturbations are occurring well in advance of visible injury, thus facilitating more rapid assessment of potential damage.

The study of changes in PS confirmed previous reports of initial stimulation of PS with excess NH$_x$. Although concentrations of NH$_x$ in excess of environmental levels were required to illicit a stimulation of PS when measured by O$_2$ electrode, repeat treatments over longer periods with realistic levels of NH$_x$, also provided evidence of a stimulation of PS. A synchronous relationship between photosynthetic carbon (C) and N metabolism is well established in plants, however, PS is subject to many physiological and environmental influences, for example light, temperature and water availability. As a consequence, changes in PS may not prove to be a suitably specific means of assessing physiological damage or changes to atmospheric pollution.
Other parameters may also be too variable to use as physiological markers. These include cations, which show great variability, especially in mosses. It is likely that general or specific cation changes, may only be detected at sub-lethal concentrations of pollution, where visible damage is also apparent. A large volume of work on amino acid responses to changes in N metabolism exists. However, changes in amino acid concentrations between species, are often difficult to assign solely to the effects of pollution (Pearson and Soares, 1996). In contrast, changes in organic acids appear to be more consistent between species, with similar reductions found in organic acids for a wide range of contrasting plants. Thus, a measurement of changes in the pool of total organic acids in response to atmospheric NH₄, could be a more practical means of assessing potential damage.

A recent paper by Champigny (1995) has proposed several biochemical mechanisms by which C and N metabolism in plants are intimately linked. With particular reference to the physiological mechanisms studied here, is the proposed short-term modulation of PEP carboxylase, sucrose phosphate synthase and NR by the glutamine:glutamate ratio, in the presence of excess NO₃⁻. In this thesis, atmospheric applications of NHₓ have shown how foliar uptake may perturb the balance of C and N metabolism, particularly by 'short circuiting' assimilation of soil derived NO₃⁻. The evidence for this is provided by the marked inhibition of NR activity in a range of species. Therefore, foliar assimilation of NHₓ may have implications for shoot-root communication and exchange of organic N and C and subsequent translocation of cations (BenZioni et al., 1971). Indeed, this thesis has emphasised the differences between species that rely mainly on roots or shoots for primary NO₃⁻ assimilation (Chapter 4). Most evidence suggests that soil derived NH₄⁺ is assimilated directly at the site of uptake, ie. the root tissue. The effect of increasing atmospheric NHₓ.
and subsequent foliar uptake, is a metaphorical one of turning the plant upside down. Thus, leaves are likely to be experiencing much greater concentrations of primary NH$_x$; the implications of which are discussed in the following section.

7.2 Regulation of cellular pH is a major consideration

A major consideration of the physiological responses studied here, is the similarity between the effects of gaseous (NH$_3$) and wet applied (NH$_4^+$) ammonia on leaf metabolism. As far as we are aware, the data presented in this thesis, plus evidence presented in Pearson and Soares (1996), are the first such reports showing similarity in responses, for the two major forms of atmospheric NH$_x$. Since NH$_3$ is an alkaline gas and NH$_4^+$ is circum-neutral, the similarity in responses are probably due to the ready assimilation of NH$_x$ from either form. The high affinity of NH$_3$ for H$_2$O, leads to the rapid formation of NH$_4^+$, in either the cell apoplast or symplast. The net result of NH$_3$ and NH$_4^+$ assimilation are the same, leading to the generation of protons (H$^+$) (Raven, 1988). Photosynthetic responses such as stomatal opening, also suggest the involvement of acidity rather than excess N. The speed with which physiological responses occur following foliar applications of NH$_x$ and acidity, especially changes in the organic acids, suggest that only small changes in H$^+$ within the leaf are required, to cause significant physiological perturbation.

It has been demonstrated that differences in the ability to buffer against H$^+$ changes, occur between species, at the physiological level. Further levels of buffering may also be provided by the atmosphere and the soil. Gaseous components of the atmosphere, may provide minimal buffering against acidity, relying solely on physical processes of gaseous exchange between leaf and surrounding air. Rainwater is
likely to be a more efficient source of buffering for leaves, albeit dependant on rainfall events, due to basic cations present in rainwater and the physical effect of rainwater washing leaves. However, acidic compounds are often more abundant in rainfall. The soil has possibly more scope for buffering against acidity, from physical and biological processes such as cation exchange reservoirs and microbial breakdown of acidity. As a consequence, the foliar regions of plants rely mainly on the uptake of balancing ions from the soil and physiological mechanisms of pH regulation. Although this study centred on the effects of excess NH$_4^+$ on plants, the importance of pH regulation suggests that the physiological responses considered here, in particular organic acids and ME, may also be of use in assessing the responses of plants to other acidifying pollutants such as SO$_2$.

7.3 Species susceptibility reflects the ability to maintain pH homeostasis

Multivariate screening of physiological variables provided evidence as to why some plant species are reported as being more susceptible to NH$_4^+$ pollution than others. The importance of enzymes such as NR, and ME, in conjunction with organic acids and cations, are established as contributory factors to overall plant buffering capacities against acidic inputs (Soares et al., 1995; Pearson and Soares, 1995). Thus, the ability to buffer against increases in acidity, is possibly the deciding factor in species susceptibility. These consistent physiological responses to excess NH$_4^+$, coupled with intrinsic buffering abilities, could possibly be used in the construction of a pollution stress index for individual species (Pearson and Soares, 1996). In its broadest sense, the separation of pioneer and climax species as non-susceptible and susceptible vegetation respectively, represents the first step in the
construction of a stress index. The measurement of leaf buffering capacity index is possibly the second step, allowing differences between species to be quantified. The method for determining leaf buffering capacity is easy and rapid; these are essential features if physiologically based stress indices are to be practically applied. The measurement and addition of other physiological variables to a database of pre-existing measurements, may allow stress indices to be constructed, for plants subjected to a whole range of atmospheric pollutants. The construction of stress indices for plants may well be constrained in this instance, by the lack of long-term field evaluation. However, the lack of NR induction in leaves of many susceptible plants, coupled with the correlation of this enzyme with overall plant buffering capacity to acidity, suggests that stress indices based on plant buffering capacity, may be broadly applicable.

7.4 Short-term versus long-term monitoring

This study is a preliminary investigation of whether, differences in enzyme activities and metabolite concentrations can be detected in the short-term. It was understood, that the controlled laboratory and greenhouse conditions utilised, may not totally reflect environmental conditions. A variety of environmental and biological factors influence enzymatic activities, organic acid and nutrient concentrations. Possibly the most significant in terms of short-term responses, is seasonal fluctuations in enzyme activities (Deng et al., 1989; Stadler and Gebauer, 1992; Pearson and Stewart, 1993; Pearson and Ji, 1994; Pearson and Soares, 1996). Although diurnal and seasonal fluctuations in organic acids have not been considered in much detail, these will probably show a degree of change which may reflect changes in enzyme activities. Biotic factors such as the age of plant material
(Schjoerring et al., 1993b) and attack from pests also cause physiological perturbations. Despite these influences, changes in physiological responses after field applications of NH\textsubscript{x} were found to be consistent with greenhouse investigations, at least in the short-term. Long-term field investigations would be required to compare the effects of seasonal variations and excess NH\textsubscript{x} in combination with other atmospheric pollutants. The interaction of NH\textsubscript{x} with acidity suggests that short-term monitoring may give an indication of long-term responses, where acidity is the overriding problem.

Since increased NH\textsubscript{x} pollution is not a new phenomenon, it may be reasonable to assume that plants growing in habitats receiving large amounts of atmospheric NH\textsubscript{x} may already have an altered physiological status. Comparisons between the same species from habitats with different NH\textsubscript{x} deposition may help to clarify this. Alternatively, the use of transplanted individuals from one habitat to another, or placing plants previously grown under controlled conditions in polluted areas, may prove useful in field investigations. This kind of approach has provided comparative data in relation to N incorporation in Sphagnum species (Lee et al., 1988) and also, R. lanuginosum (Baddeley et al., 1994), where individuals have been transplanted from non-polluted to polluted habitats. Long-term studies of physiological responses will need to take into consideration the combined effects of foliar and soil uptake of NH\textsubscript{x}. Soil acidification and alterations in soil nutrient status by excess N deposition have been studied for longer than foliar responses. Therefore, the work on soil effects could be combined with the increasing awareness of foliar effects, to give a more complete picture.

The demand for early and specific indicators of damage to plants from atmospheric pollutants, has been clearly stated in the literature (Mathy, 1988b). Although none of the investigations in this thesis have
identified actual damage, metabolic changes that result in the alteration of a plant's physiological status, are considered to be potentially damaging, whether these changes are short-term or long-term responses. Monitoring the physiological responses of plants, is still preferable than waiting for visible damage and species losses to occur.

7.5 The contribution of physiological responses to critical loads and levels research

Monitoring the physiological responses of plants to excess NH_4^+, may contribute to the further understanding of critical levels and loads for plants and ecosystems. Anon (1993a) considered the addition of chemical and biological criteria to these concepts, as an important step towards a better understanding of the potential damage to soil/plant ecosystems. At that time, the main limiting factor was the lack of data. The data that was available, was not specific to N pollution and could be the result of other plant stresses, such as frost or pest damage. The need for the development of separate critical loads for NH_4^+ and NO_3^- was recently highlighted, since these N compounds can have different effects on plants (Bobbink and Roelofs, 1995). Thus, the specific nature of physiological responses to NH_4^+ and NO_3^-, may contribute towards determining critical loads for NH_4^+ and NO_3^- . One possible example of this, is the induction of NR with NO_3^- or inhibition with NH_4^+ application. However, considerations of diurnal and seasonal changes in enzyme activities would need to be incorporated, along with the influence of soil intercepted N compounds and subsequent root uptake (See 7.4).

The results of this study may be more immediately applicable to the evaluation of critical levels for individual species and vegetation types. The speed with which physiological responses can reflect the
presence of atmospheric NH₃, suggests that currently proposed critical levels for NH₃ (van der Eerden et al., 1993), may need to be lowered, since these levels were proposed mainly through visible assessment of damage. The distinction between physiological responses and overall physiological vitality, for susceptible and less susceptible species, also highlights the need for separate and possibly lowered critical levels for some habitats or species. A speculative reduction of critical levels for susceptible species based on results in this thesis, may be 50% or less than levels for non-susceptible species. It may ultimately be difficult to assign precise pollutant thresholds to vegetation, due to the large variety of vegetation types. However, the evidence in this thesis and the literature, indicates that critical levels should be set to account for the more susceptible species in a community.

7.6 Mosses as bio-monitors

Although the physiological responses of mosses to atmospheric NH₄⁺ and NO₃⁻ were not considered in as much detail as for higher plants, mosses may still prove to be more use as indicators of pollution. The lack of any direct soil contact removes the problems of distinguishing between the effects of soil and foliar uptake of excess N. The influence of soil processes such as nitrification and ammonification, which may alter N compounds before uptake by plants, can be largely discounted. Thus, perturbations of NR and organic acids in the presence of NH₄⁺ and NO₃⁻ can be directly related to the predominant N form in atmospheric deposition. The lack of a cuticular barrier confers a more immediate sink for excess NH₃ deposition in mosses, in comparison to higher plants. This feature may account for the large variations in total N content, found in some mosses. However, increased sensitivity to acidity, will no doubt be a feature that has to be considered where a
cuticle is not present. The lack of a cuticular barrier leading to increased uptake of $H^+$ ions, is possibly one reason why certain moss species have declined in polluted areas. Despite this, it would appear that some mosses demonstrate similar characteristics of susceptibility to higher plants. Thus, mosses with higher levels of organic acids, cations and NR activity, which may generate higher buffering capacity, may prove to be better short and long-term monitors of $NH_3$ and acid deposition.

The preliminary study of total $N$ and $\delta^{15}N$ contents of rural and urban lithophytic mosses, found that the total $N$ contents of some mosses, do not always reflect expected $N$ deposition rates for the site of collection. This relationship is more consistent with samples collected from nearby sites, even though traffic exposure estimates might suggest different pollutant levels, in particular $NO_x$. Since actual $NO_x$ levels were not measured, any inconsistencies between total $N$ content and $N$ deposition in this instance, cannot be clearly defined. However, other workers have also found inconsistencies in total $N$ content of mosses in relation to measured $N$ deposition. Woolgrove and Woodin (1996) found a high degree of variability of total $N$ content, which on occasion did not reflect $N$ deposition, in the snowbed moss *Kiaeria starkei*. The differences here were thought to be due to wind blown redistribution of $N$, intercepted by snow. Baddeley *et al.*, (1994) also demonstrated a high degree of total $N$ variation in *R. lanuginosum*, collected from nearby sites in Scotland. The variations were considered to be caused by altitudinal differences at the collection sites. Growth dilution of $N$, as opposed to accumulation, especially in areas where other nutrients are available to counter nutrient imbalances may be a significant factor when comparing total $N$ content to $N$ deposition. Likewise at high altitudes, the growing season is shorter, thus, $N$ accumulation in mosses is generally greater than at lower altitudes, where the $N$ can be utilised
for longer. The occurrence of wide ranging N contents in mosses of the same species, may also make the time of sampling crucial in relation to acute pollution episodes.

$\delta^{15}N$ values appear to be more consistent with estimated atmospheric N levels between nearby areas and more significantly, the predominant type of atmospheric N present. There is some evidence of differential $\delta^{15}N$ values for N compounds in the literature, however, a more detailed study is possibly called for. The results suggest that atmospheric N compounds may have different $\delta^{15}N$ signatures, which can be reflected in biological material. Recent observations by Soares and Pearson (unpublished data), have also shown a similar response of $\delta^{15}N$ values to N deposition in higher plants. With higher plants, soil contact has to be considered, as there is some evidence of microbial discrimination of $^{15}N$ with N assimilation (Linda Handley, SCRI, personal communication). This may affect the $^{15}N$ signature of N in higher plants via mycorrhizal associations, N$_2$ fixing bacteria and free-living bacteria releasing N compounds into the soil. There is very little evidence of enzymatic discrimination of $^{15}N$ by NR or GS in higher plants or mosses. The current state of knowledge suggests that NR does not discriminate in favour or against $^{15}N$, although GS may to some extent discriminate against $^{15}N$ (Linda Handley, SCRI, personal communication). If this is the case, the contrast between NO$_x$ with a more positive $\delta^{15}N$ signature and NH$_x$ with a more negative signature still remains. As a result, the relationship between N deposition rates, N compounds and $\delta^{15}N$ signatures, at least for lithophytic mosses, could make a significant and directly quantifiable contribution towards monitoring N deposition.
CHAPTER 8

References


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Rabe R & Kreeb K H (1979). Enzyme activities and chlorophyll and protein content in plants as indicators of air pollution. *Environmental Pollution* 19 119-137.


Appendix 1

Data set used for cluster and ordination analysis in Chapter 4

Values for each variable are averaged; \((n = 3)\) individuals for each species.

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<thead>
<tr>
<th>Plant Species</th>
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<th>NADP ME activity</th>
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<td>(\mu)mol h(^{-1}) g(^{1}) fwt</td>
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Appendix 1

Data set used for cluster and ordination analysis in Chapter 4
Values for each variable are averaged; (n = 3) individuals for each species.

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Appendix 1

Data set used for cluster and ordination analysis in Chapter 4

Values for each variable are averaged; (n = 3) individuals for each species.

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