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**Bioindicator and biomonitoring methods
for assessing the effects of atmospheric nitrogen
on statutory nature conservation sites**

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EXECUTIVE SUMMARY

- Bioindicators provide a range of techniques to assess the impacts of air pollution from reactive nitrogen (N) compounds on statutory nature conservation sites. They complement physical monitoring of atmospheric concentrations and deposition and risk assessment based on the critical loads approach by providing site-based information on atmospheric N concentrations, N deposition and/or ecological impacts.
- Appropriate bioindicators for N may be applied by sampling at one time to compare results between different locations. In particular, local-scale transects can help identify the impacts of a nearby point source of reactive N emissions to the atmosphere.
- The repeated application of bioindicator methods over time provides the basis for biomonitoring. In general, biomonitoring reflects changes over periods of several years, although short-term changes can also be monitored (over several weeks and months).
- This report reviews the wide range of bioindicator and biomonitoring methods for N and incorporates the results of a field test of several of the methods. In addition, datasheets are provided that summarize the key characteristics, advantages and limitations of the different methods.
- Bioindicator methods can be grouped into several contrasting approaches: *Biochemical methods* (based on an accumulation of N or a chemical/physiological response to N), *Species composition methods* (based on previously characterized species preferences) and *Transplant methods* (based on the response following transplanting of either locally native species or standardized plants).
- *Nitrogen accumulation methods* include measurement of plant tissue N concentration, amino acids, substrate N and foliar ammonium. The accumulation methods provide the closest link to atmospheric N deposition. Results show that the smaller and more available the chemical pool, the larger the magnitude of response, with increasing responses from: total N < substrate N < foliar ammonium.
- *Biochemical response methods* include analysis of enzymes such as nitrate reductase and emissions of nitrous oxide from soils. These methods are useful to demonstrate physiological effects, but tend to be less well correlated with atmospheric N deposition due to interactions with environmental conditions.
- *Species composition methods* are of particular interest to the statutory conservation agencies since they relate directly to changes in plant communities due to excess atmospheric N. ‘Ellenberg’ N preference scores for higher plant and bryophyte species can be used to score the overall community for nitrogen. The limitation of this approach is that a wide range of other factors may also affect species composition.
- Lichens are particularly sensitive to atmospheric reactive N, particularly ammonia. Detailed approaches are available to score lichen responses to N, but require more development for UK conditions. There is also the potential to refine simple methods that can be applied by non-experts.
- The use of *standardized grass plants* has been shown to provide a robust method for monitoring the deposition and effects of N. The method can be applied in situations of complex terrain where physical estimates of deposition are difficult and as a graphic demonstration of impacts to stakeholders. It has a key advantage that exposure periods of only a few weeks are necessary.

- *Transplanting native species* between sites is useful to demonstrate impacts at polluted sites and conversely the benefits of clean conditions. These methods have been shown to work well for lower plants, and have the benefit of being able to demonstrate recovery following a reduction in deposition where this occurs.
- Overall, recognizing the limitations and benefits of the different methods, it is concluded that bioindicators provide a practical site-based approach for assessing N concentrations, deposition and impacts. Each of the above mentioned approaches are have merits, with different techniques matching to the range of questions being addressed. The most robust results are to be obtained by implementing several complementary techniques simultaneously, where possible in combination with low-cost physical monitoring of atmospheric concentrations.

TECHNICAL SUMMARY

Background and structure of the report

1. Atmospheric nitrogen deposition represents a threat to naturally nutrient-poor plant communities, leading to a potential loss of conservation value for many statutory nature conservation sites. Until now, the regulatory assessment of these impacts has been focused on the critical loads approach, where estimated atmospheric deposition loads are compared with ‘critical loads’ to estimate the occurrence and extent of ‘critical load exceedance’. Critical loads represent the deposition below which environmental effects do not occur, according to present knowledge. Hence, where exceedance occurs on nature conservation sites, ecological changes are expected.
2. In parallel with the critical loads approach, the direct effects of air pollutant concentrations, including NO_x, NH₃ and ions in cloud water, have been assessed by comparison with ‘critical levels’, which represent threshold mean concentrations over defined time periods below which effects do not occur according to present knowledge.
3. Under domestic and European legislation, the UK has a duty to protect designated statutory nature conservation sites, including Sites of Special Scientific Interest (SSSIs), Special Areas of Conservation (SACs) and Special Protection Areas (SPAs) and maintain (or restore) these sites to favourable condition.
4. The critical loads and levels approaches provide a risk assessment that can indicate the likelihood of future change. However, the extent of exceedance does not indicate whether changes are already occurring on a site or provide a means to monitor the extent of actual change. There are a number of other limitations of applying the critical loads and levels approach to site-based assessment, for example, the spatial resolution of the model estimates.
5. The use of biomonitoring for nitrogen complements the assessment of critical loads exceedance in that it considers current biological responses at actual sites. A large number of bioindicator and biomonitoring approaches have been suggested for nitrogen. However, it has been recognized by the conservation agencies that the uncertainties currently prevent the widespread application of these techniques.
6. This report describes work commissioned by JNCC, English Nature and the Countryside Council for Wales to review and further develop bioindicator and biomonitoring methods for the assessment of atmospheric nitrogen deposition, air concentrations and ecological impacts.
7. The report is structured in three parts.
 - a. The main body of the report reviews in detail the different bioindicator approaches for nitrogen and their application for biomonitoring by the conservation agencies. This draws on available international literature, as well as a field test of a number of the newer methods from a transect with distance from a poultry farm.
 - b. Appendix I reports in detail the results of the field test, and a comparison with other bioindicator data already available at the same site.

- c. Finally, Appendix II, summarizes the basis of the methods, practical advantages and limitations in a series of easily accessible datasheets. These datasheets are also available on the Air Pollution Information System.

Purpose, types and application of different N bioindicator methods

8. There are many different types of biomonitoring and it is possible to become confused by the semantics of definitions, for example between bioindicators, biomonitors, biosensors, bioassays and biomarkers etc. In order to maintain simplicity in the present report, bioindicators are taken here to represent the basic application of a method, while biomonitoring is taken to represent the application of bioindicator techniques over time at given locations.
9. The relevant timescales for biomonitoring vary according to methods, but potentially range from several weeks to many years. Bioindicators may be applied using sampling at one time, with comparisons made between the observations and reference estimates or spatially, such as along potential pollution gradients.
10. Bioindicators for nitrogen can be used for one or more purposes, to estimate:
 - a. atmospheric N deposition loads,
 - b. atmospheric N concentrations (e.g. NO_x or NH₃) and/or
 - c. ecological effects of N, including physiological and species/community changes.
11. Given the interests a. and b. above, it is important to consider the application of biomonitoring in conjunction with the use of classical physico-chemical methods for monitoring air concentrations and deposition. In many cases, a much stronger picture may be derived by implementing a combination of biomonitoring and physico-chemical monitoring. In situations where costs are limiting, the physical monitoring contribution should focus on species with a high spatial variability (e.g. NH₃, NO_x and in hill areas also wet deposition) using low frequency time-integrated sampling.
12. In the case of N deposition, biomonitors have an advantage over physical sampling in that they provide a means of dealing with situations of complex terrain (e.g. forest edges, near sources etc), where the physical determination of deposition is extremely difficult. Conversely, for estimation of air concentrations, physical monitoring is more direct and will often be more appropriate than biomonitoring.
13. Bioindicators for nitrogen may be broadly categorized into three types. Methods in each of these categories may be variously suited to indicating atmospheric deposition, concentrations or ecological effects:
 - a. **Biochemical methods**, which sample a component of a species or species-group that occurs in the habitat. These include both parameters reflecting an *accumulation of nitrogen* in the system and *chemical/physiological responses* to nitrogen exposure.
 - b. **Species composition methods**, which characterize the presence and extent of occurrence of certain species, each of which have been previously categorized as having different nitrogen preferences.
 - c. **Transplant methods**, where either locally native species or standardized plants are exposed to a range of nitrogen deposition/concentrations and their responses assessed.

14. In relation to the bioindication of ecological effects, it is sometimes suggested that it is impossible to indicate species changes on the basis of a chemical parameter, or to indicate changes in one group of species based on changes in another group of species. The extent to which such connections can be made depends on the existence of functional relationships on a case-by-case basis, and in many situations a chemical change can be accepted as providing an ‘early warning’ of likely future species change.
15. Even where there is uncertainty regarding the extent to which a particular bioindicator response can *indicate* some other ecological effect, use of that bioindicator method may still support a site-level risk assessment through the critical loads/levels approach. This is possible where the bioindicator technique is suitable to estimate either atmospheric N deposition or concentrations. The derived site-level estimate of exposure can then be compared with the appropriate critical limit.
16. The various bioindicator and biomonitoring approaches range from rather complex tools revealing detailed information about specific processes to simpler empirical reference parameters. The full range of these types is considered in this report, although it is recognized that simplicity, practical robustness and availability of reference estimates will be of particular importance to allow application by the conservation agencies.
17. With each of the bioindicator approaches, biomonitoring programmes may be implemented by repeating standardized assessments at given locations at annual to decadal intervals. This provides the facility to observe the degradation or recovery over time, which may be related to changes in atmospheric nitrogen concentrations and deposition.
18. Based on the review, two approaches are used here to identify the most suitable methods for the conservation agencies:
 - a. A broad empirical scoring of the methods, which considers issues of robustness vs. ease of application. This identifies the potential of different methods. Subsequently, the extent to which the method has been well characterized is considered, which allows a scoring of the readiness of the method for practical application.
 - b. A decision tree approach, where the characteristics of each method are used to match the appropriate methods to the specific purpose of interest in each situation.

Biochemical bioindicators: N accumulation methods

19. Biochemical methods may be loosely categorized into those representing *accumulation of nitrogen* and those representing a *response to excess nitrogen*. The former provide a means of both assessing the atmospheric N deposition and a biological perturbation of the system. The latter represent different biological impacts, but are much less well suited to estimating deposition, particularly where there is cross sensitivity to other environmental changes. Nitrogen accumulation methods match logically to the accumulated nitrogen inputs and have therefore not usually been used to indicate *concentrations* of N pollutants in the atmosphere.
20. The most well characterized nitrogen accumulation method is the measurement of total foliar nitrogen concentration, typically expressed as a percentage of dry weight. There are substantial differences between species, which partly reflect the preferred

habitats and nitrogen preferences of the species and therefore the analysis has focused on selected species and species groups. The higher plant for which most data are available is heather (*Calluna vulgaris*), which reflects a particular concern of N deposition effects on heathland ecosystems. Attention has also focused on bryophytes (particularly pleurocarpous mosses), since these receive most of their nutrients from the atmosphere.

21. A substantial body of information is available for tissue N responses to atmospheric N deposition. Although various authors have found different responses, much of this appears to be related to the need for carefully standardized sampling protocols that account for within-plant, between-species and seasonal variation, and to variable quality in the site-level reference estimates of atmospheric N deposition.
22. Using standardized sampling procedures, atmospheric N deposition may be estimated using established regression relationships, with the uncertainties of the regression allowing the estimation of confidence limits for nitrogen deposition. While further work is required on the statistical assessment, it is now possible to use this approach to estimate whether the atmospheric deposition at a site is significantly greater or less than the critical load for the habitat in question.
23. The increase in total foliar N reflects changes in component nitrogen compounds, and these also have potential as biochemical nitrogen bioindicators. In particular, soluble nitrogen compounds may be expected to have a shorter response time than total nitrogen and change more substantially in response to excess nitrogen. The most well-studied of these methods is the measurement of foliar amino acid concentrations. In particular, arginine and asparagine tend to accumulate in large quantities. A disadvantage of the method, however, is the difficulty to generalize results, since different species accumulate different amino acids.
24. The measurement of “substrate nitrogen” provides a simple bulk term to assess the nitrogen available for growth, without going into the detail of which nitrogen compounds are accumulated. This may be approximated by measurement of soluble foliar nitrogen. Similarly, foliar ammonium levels represent a precursor (and recycling) pool for the synthesis of amino acids and other nitrogen compounds, and have also been shown to respond to atmospheric nitrogen deposition. New measurements for pleurocarpous mosses made as part of this study (Appendix I), show that the smaller N pools accumulate over a larger range, with observed increases in total N, soluble N and foliar ammonium in response to elevated N deposition by factors of 3, 5 and 20, respectively.
25. Of soluble N and foliar ammonium, the available data suggest that the latter is most sensitive, with the moss data permitting the estimation of atmospheric N deposition (with confidence limits). A transect application of the foliar ammonium bioindicator method in mosses also provided an independent means to estimate background atmospheric deposition of N and cuticular saturation of NH₃ deposition at very large NH₃ concentrations.
26. The measurement of foliar N:P ratios is a further technique to investigate the effects of atmospheric N deposition. While this increases analytical costs compared with analysis of just N, the method can provide information on the extent to which N or P is limiting at a site, and therefore help gauge the likelihood for ecological effects of N at a site.

27. Nitrogen exists naturally in both ^{14}N and ^{15}N forms, but the proportion of the two compared with fixed standard (expressed as $\delta^{15}\text{N}$) varies according to the source of the fixed nitrogen. Ammonia from volatilisation processes tends to be $\delta^{15}\text{N}$ negative, while nitrogen oxides (and ammonia) from vehicle combustion slightly $\delta^{15}\text{N}$ positive. Hence the measurement of $\delta^{15}\text{N}$ can potentially provide information on the source attribution of N deposition. Such isotopic analysis is a highly powerful technique, but its sound application is extremely complex because of the multitude of isotopically fractionating processes. As a result, great care and specialist input are required to avoid obtaining misleading results.

Biochemical bioindicators: chemical and physiological response methods

28. Biochemical methods representing responses to additional nitrogen include enzyme activities, emissions of N compounds from soils and a range of other bioassays.
29. The most well studied enzyme response to added nitrogen is that of nitrate reductase (NR). High nitrate loadings can lead to the suppression of NR activity in sensitive plant species, providing the basis for a bioassay for the impact of enhanced nitrate deposition. The method has been investigated for a range of plant species, with particular attention given to bryophytes. Nitrate reduction is an energy consuming process, and is therefore increased at high light levels, when higher levels of substrate carbon are available, and this illustrates the requirement to standardize the light levels for the bioassay. However, these interactions point to additional complexities, such as that high foliar ammonium levels and poor growing conditions (also associated with a shortage of substrate C), may be related to suppression of NR activity. In this context, it is possible that reduced NR activity may also respond to increased reduced nitrogen deposition and other stresses. As a consequence of these interactions, NR activity may provide a useful indicator of N (and other plant) stress, but is not well suited to estimate atmospheric N deposition. Such limitations also apply to the measurement of phosphomonoesterase, which can potentially give an indication of induced P limitation due to excess N deposition.
30. The limitations relevant to NR activity as a N bioindicator also apply to other physiological response based methods, but these are in general even less specific to nitrogen. Methods include the assessment of chlorophyll fluorescence, frost hardiness, ergosterol activity and the BIOLOG bioassay. In each case, these provide useful indications of ecosystem response to a range of stresses, including nitrogen, but they have little utility to indicate N deposition or provide specific markers of N response.
31. Excess nitrogen deposition leads to increases in available nitrogen in both plants and soils and this, in turn, affects the potential for emission of trace gases from the ecosystem. Most attention has been given to the emission of nitrous oxide (N_2O) and nitric oxide (NO) from nitrogen rich soils, with the former predominating in anaerobic (wet conditions) and the latter in aerobic conditions. These emissions represent a combined response from nitrification and denitrification, and are therefore also highly dependent on soil moisture and temperature. Existing data have shown clear responses of N_2O and NO emissions to N deposition, but do indicate a high variability, with a consequently high uncertainty in any derived deposition estimates. Because soil ammonium and nitrate represent primary drivers, it may be that these represent simpler estimates of enhanced N input than soil gas emissions.
32. The increase in emissions of N_2O and NO in response to increased *soil* N is paralleled by the increase in the potential to emit NH_3 in response to increased *foliar* N. In the

latter case, the potential to emit NH_3 is expressed by the ‘compensation point’ of the foliage, which is a function of temperature and the ratio of $[\text{NH}_4^+]/[\text{H}^+]$ (referred to as Γ) in the intercellular fluids (apoplast) of leaves. While NH_3 fluxes may be measured and bioassays to derive Γ are available, these measurements are rather complex, and the measurement of total foliar ammonium provides a more practical bioindicator.

33. In general, biochemical bioindicators that represent responses to added nitrogen may be useful for assessing specific processes associated with biological effects. However, they are themselves driven by the accumulation of available nitrogen (either in plant or soil components), so that for general bioindication of N deposition it is often simpler and more sensitive to apply direct N accumulation based methods.

Species composition based bioindicators for nitrogen: higher plants and bryophytes

34. Species composition changes represent the end-point of N deposition effects that are most of interest to the conservation agencies. Statutory nature conservation sites are designated on the basis of their habitats and species, so any losses of designated features constitutes an unambiguous negative impact on site condition.
35. Species-based bioindicator methods rely on the preclassification of different species preferences to a range of nitrogen exposures. In approaches that derive from the Ellenberg method, each species is allocated a characteristic score, so that an average Ellenberg score for a site may be derived from the list of species present. This score may also be weighted for the frequency or cover of the different species present. In other approaches, species are assigned as either favouring or avoiding a high nitrogen supply, and the species score in each group used to rate the site.
36. The advantage of species-based methods (that they are of direct conservation interest), also underlies the limitation of these methods, that species communities are affected by many factors other than nitrogen. As a consequence, particular care is needed in interpreting results, and considering interactions with other factors, such as light availability, soil drainage, site management and disturbance etc.
37. In the context of the Ellenberg index and atmospheric N deposition, particular attention has been given to the ground flora of deciduous and coniferous woodlands. Ground flora species composition appears to be much more sensitive to N deposition than tree health, although a modified composition of tree species may eventually occur in naturally regenerating woodlands. Ellenberg scores are available for both higher plants and bryophytes, and these have been demonstrated to vary with nitrogen deposition both on a local level (near farms) and at a regional scale across the UK.

Species composition based bioindicators for nitrogen: lichens

38. The occurrence of bark-growing (epiphytic) lichens provides the basis for several species-based approaches that are particularly sensitive to nitrogen. There is a very wide range of lichen species with characteristic bark preferences, and this allows these methods to be implemented with some robustness. By focusing on epiphytic lichens, the interactions of soil management and site disturbance become much less important than for the application of Ellenberg methods for higher plants.
39. The available data show a clear relationship between epiphytic lichen species and atmospheric NH_3 concentrations, but the responses to wet deposited N or NO_x are much less certain. This difference appears to relate to the importance of bark pH in selecting for different lichen species. The presence of ammonia raises bark pH (indicating that the basic nature of ammonia has a larger affect than any nitrification

of the deposited ammonia), while wet deposited N and oxidized N would be expected to reduce bark pH. Hence while the effects of NH₃ on lichens may be partly attributable to increased N availability, they are to a large extent mediated through bark pH.

40. Given the established relationship between ammonia and increased bark pH, it is not surprising that other factors that raise bark pH mimic the effects of atmospheric ammonia. Firstly, the deposition of calcareous dusts represents a potential artefact to the interpretation in relation to atmospheric NH₃. Secondly, the bark pH of twigs appears to be naturally higher than that of trunks, so that lichens on twigs are even more sensitive to NH₃ than lichens on trunks.
41. Several methods are available for assessing lichen biodiversity, including the European and the German (VDI) approaches. These, however, are not well suited to monitoring the effects of nitrogen, since total lichen biodiversity is a complex function of nitrogen, which favours some species and limits others.
42. The most sensitive and detailed scoring system for assessing the effect of atmospheric N on lichens is that developed by van Herk for the Netherlands, and this has also been tested in the UK. The scoring system of van Herk identifies two groups of lichens occurring on trunks that either favour low N conditions and naturally acidic bark (“Acidophytes”) or favour ammonia rich conditions with high bark pH (“Nitrophytes”). This method is time consuming to apply, but has been shown in the UK studies to respond to NH₃ concentrations less than the critical level of 8 µg m⁻³.
43. Modification of the van Herk method for application to sampling on twigs provides a simpler and more sensitive method for assessing the impacts of NH₃. For example, in the sampling for this study and at other UK sites, the results are consistent with a near complete loss of twig acidophyte species at NH₃ concentrations above 2 µg m⁻³. Since the response appears to be related to NH₃ concentration (as mediated by bark pH) rather than N deposition, these data point to the need to revise the critical level for atmospheric NH₃.
44. The data collected also demonstrate the interpretative value of measuring bark pH alongside lichen species composition. At present there is a need for more studies that provide data on NH₃ and other N pollutant concentrations, bark pH and lichen species occurrence on twigs and trunks.
45. An application of the Ellenberg N method for epiphytic lichens has also been developed by Wirth and has been applied with some success for the UK data. Although the method is less sensitive than that of van Herk, it is much easier to apply.
46. A key limitation of the lichen approaches is the requirement for specialist lichenologists to identify many species. This study, however, has shown that, by using a modest subset of species, a ‘simplified Ellenberg’ lichen approach can be applied by non-specialists to assess the occurrence of NH₃ impacts. This simplified approach has particular potential for application by conservation agency staff and is being further developed to allow more rapid assessments and encourage demonstration to stakeholders.

Other species composition based bioindicators for nitrogen

47. A wide range of other species responses to atmospheric nitrogen could potentially be applied as bioindicators. In particular, mycorrhizas have been considered as sensitive to nitrogen, which may lead to a reduction in their fruiting bodies (mushrooms,

toadstools etc), while invertebrate populations may also change. While it is very useful to monitor these changes, particularly where they represent a key conservation concern, there are many causes of change other than nitrogen, so there is limited use of these measures as specific nitrogen responses.

Transplant based bioindicator methods

48. The two basic approaches available are the application of native reciprocal transplants and standardized model plants.
49. Transplanting of native species provides the ability to contrast known polluted and clean sites. Specimens of the target species are taken from the sites of interest and their locations exchanged, with subsequent monitoring of their performance. This approach works particularly well for epiphytic bryophytes, as the transplanting process does not appear to impair the plants substantially. Conversely, while the method has been applied for lichens, these appear to be much more sensitive to subtle microclimate changes, while transplanting of higher plants needs to consider the effect of co-transplanting associated soil.
50. A key to the success of native reciprocal transplants is the use of sites with similar climatological conditions (temperature, precipitation, windiness). If enhanced N deposition is having an effect at the polluted site, transfer to a clean site may be expected to lead to improved growth rates, and *vice versa*. Similarly, foliar N concentrations may be expected to decrease on transfer to a clean site.
51. Reciprocal transplanting has the benefit of being suitable to show both damage due to high pollution loads and recovery on transplanting to clean conditions. This may be particularly useful for stakeholders to demonstrate the benefits of pollution abatement. In the case of a test with Atlantic bryophytes in the UK, the extent of recovery on transfer to a clean site after 1 year was found to be less than the extent of damage on transfer to a polluted site. This supports the expectation that rates of recovery are slower than damage.
52. The use of standardized model plants provides a means to estimate both nitrogen deposition in complex terrain and to demonstrate visible impacts of nitrogen deposition for stakeholders.
53. The most common standard species to be used are *Lolium multiflorum* or *L. perenne* and these are grown from seed in pots under standard conditions. Replicated samples are then placed in the field for several weeks under a range of N exposure conditions, before being harvested for total above (and sometimes below) ground biomass and for total foliar N concentration.
54. Results in the present study (Appendix I) show that, close to a farm, the above ground biomass was twice that at 300 m from the farm. This represents a highly graphic demonstration of the effects of atmospheric N for stakeholders. Tissue nitrogen concentration also decreased with distance from the farm. However, growth was less and tissue N more in sites (under a woodland canopy) with low light availability.
55. Measurement of the total N inventory of the plants largely cancelled out the interaction caused by variable shading and provided the best relationship to atmospheric N deposition. This parameter provides the basis for an independent assessment of nitrogen deposition in situations of complex terrain. In the situation where NH₃ concentrations are also measured, the method also provided the facility to

calculate the saturation of dry deposition velocities at very large concentrations immediately adjacent to the source.

56. Further work is required to assess the response of model plant transplants to wet N deposition and to dry NO_y deposition. A key limitation for the assessment of wet deposition N effects is that precipitation events are highly sporadic. For this purpose, work is needed to test the potential of using slower growing grasses, such as *Deschampsia flexuosa* and *Nardus stricta*.

Conclusions and recommendations

57. It is clear that there are limitations to each of the approaches for bioindication and biomonitoring of reactive atmospheric nitrogen. The methods vary in their response to different N forms, and are variously suited to indicating atmospheric N deposition, N concentrations and/or ecological impacts over different timescales.
58. Bioindicators are also applicable as complementary methods to indicate atmospheric concentrations and deposition of nitrogen. The most robust site assessments result from the joint application of physical concentration monitoring and biomonitoring, which may, for example, help refine local estimates of atmospheric deposition and critical loads exceedance.
59. The present study has identified a range of the most promising bioindicator methods suitable for general application by the conservation agencies and for specific process studies. Although further method development is needed, bioindicators provide a practical approach to estimate nitrogen exposure and impacts relevant for monitoring the condition of statutory nature conservation sites.

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1. INTRODUCTION: BIOINDICATORS AND BIOMONITORING FOR ATMOSPHERIC NITROGEN

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1.1. Atmospheric nitrogen deposition and critical loads

Over recent years, substantial progress has been made in the UK and Europe in developing the critical loads approach for investigating the acidification and eutrophication effects of atmospheric nitrogen deposition (e.g. Grennfelt and Thörnelof 1992, Hornung *et al.* 1995, UBA 1996, NEG-TAP 2001, Achermann and Bobbink 2002). In parallel, the critical levels approach has been developed to address the direct impacts of concentrations of nitrogen components in the atmosphere (e.g. UBA 1988, Ashmore and Wilson 1994, CLAG 1996). Although nitrogen is the main constituent of the atmosphere in inert form (N₂), the concern centres on the concentrations and deposition of reactive nitrogen (N) forms, which derive from the emissions of ammonia (NH₃) and nitrogen oxides (NO_x). As a result of these emissions, nitric acid (HNO₃), ammonium (NH₄⁺) and other nitrates (NO₃⁻) are also formed in the atmosphere, with the different species being deposited to ecosystems directly as gases and particles (dry deposition) and in precipitation (wet deposition) (NEG-TAP 2001). By comparing estimated critical loads with reactive total nitrogen deposition, and estimated critical levels with atmospheric concentrations of the different chemical species, the critical loads/levels approach provides a means of assessing the risk of change to ecosystems resulting from reactive atmospheric nitrogen. The approach was originally developed for regional scale risk assessment, for example, using deposition maps at 5 km resolution and critical loads estimates at 1 km resolution. However, it is also now being used as a tool to assess the risk to statutory nature conservation sites (Hall *et al.* 2002).

The European Habitats Directive requires that Member States prevent harm and deterioration to Special Areas of Conservation and Special Protection Areas. In addition, the UK Government is committed to achieve favourable condition on 95% of SSSIs in England, by area, by 2010. To fulfil these duties, robust monitoring of the condition of sites is required. The impacts from nitrogen pollutants is one of the many threats to the condition and integrity of sites. However, current approaches for monitoring the condition of sites is not aimed at consideration of air pollution effects. Subtle changes due to chronic air pollution stress may not be picked up until there is more substantial change to vegetation communities. Even then it is frequently difficult to relate an effect on a site to air pollution as a causal factor, given the effects of site management, or interactions with other abiotic and biotic factors, and the uncertainty of atmospheric pollution deposition estimates to sites.

The critical loads/levels approach provides a useful tool to help the conservation agencies to assess nitrogen impacts, and advise on the protection statutory nature conservation sites

(hereafter referred to as ‘protected sites’) from damage due to atmospheric nitrogen. It must be recognized, however, that there are many uncertainties in the approach. Fundamentally, the assessment of exceedance of critical loads only indicates a level of risk, rather than providing information on actual site condition: if the critical load is exceeded, then damage is eventually expected (e.g. after two to three decades), but it does not mean that damage has already occurred. In addition, there are many quantitative uncertainties in the approach, particularly when it is applied at the level of an individual protected site. These include spatial uncertainties in atmospheric concentrations/ deposition, soil types and specific habitat sensitivity, with the robustness of assessments depending on the extent of reliance on national datasets versus on-site measurements.

1.2. Bioindicators for atmospheric nitrogen levels and responses

The use of bioindicators and biomonitors for nitrogen therefore provides an approach that complements the assessment of nitrogen impacts on sites using critical loads and levels exceedance. There is a wide range of possible bioindicator methods for nitrogen, and these include:

- measurement of chemical parameters of components in the habitat,
- assessment of different aspects of species composition, or
- use of biological transplants.

As such, bioindication provides a measurement-based approach that is directly linked to biological and ecological conditions at actual sites. From the perspective of nature conservation, the changes in species communities represent the issue of ultimate interest. Where high concentrations or deposition of atmospheric nitrogen have selected against certain species for which a protected site has been designated, there is a clear case that the site is not in favourable condition. The same applies for use of transplants where native species relevant to the designation are used. However, biomonitoring methods based on changes in species composition are generally also dependent on factors other than nitrogen, such as land use history and climatic differences. The complementary measurement of chemical components or the use of transplants therefore allows a much more robust picture to be developed of the level of nitrogen exposure and its impacts on a site.

Where the bio-indicator response to nitrogen has been well characterized from prior studies, depending on the method used, a site-level indication may be obtained of:

- nitrogen deposition,
- atmospheric concentrations of specific nitrogen components, and/or
- ecological effects of nitrogen concentrations or deposition on the site features.

The quality of the estimates obtained for these different parameters is highly variable, depending on both the in-principle suitability of the different methods and the (significant) current uncertainties concerning the application of the methods.

The recording of bioindicators at a site provides a ‘snapshot’ in time of some feature of the system, with each method having different characteristic response times. For example, measurement of a plant nitrogen pool may reflect the prior conditions over a shorter period than measurement of plant species composition. The repeated application of bioindicator approaches over time provides the basis for biomonitoring. Classically, this might consist of sampling at a site using standardized methods every few years, but responses within a year

may also be expected for some parameters, particularly where a significant change in nitrogen exposure occurs.

As with the critical levels/loads approach, there are also significant limitations to the use of bio-indicators for nitrogen. The different limitations are method specific and relate to the specific purpose for which the method is being applied. A key limitation with many of the methods is the extent to which responses may be caused or modified by factors other than atmospheric reactive nitrogen. In addition, the extent to which the measurement of one bio-indicator parameter can actually *indicate* other information about the habitat or species for which a site is designated is highly dependent on the existence of a direct or indirect process link. For example, a chemical bioindicator method might provide a good indication of atmospheric nitrogen deposition in certain contexts, for example representing an accumulation of nitrogen, but a much less certain indication that species changes are currently occurring. Despite these limitations, a well-established chemical or transplant bioindicator response might still be accepted as providing a site-based “early warning” of future species composition changes.

1.3. Objectives of the report

The objective of this study was to review the suitability of existing bio-indicator techniques, which provide an early indication of N effect or N exposure. Application of suitable methods will help assessment of N effects or exposure in relation to site condition assessment, wider surveillance or informing the conservation agencies’ statutory advice on the impacts of ‘plans and projects’ on the integrity of European sites.

Specific objectives of the work are:

- 1) To review existing bio-monitoring techniques in the context of estimating nutrient nitrogen loading, or reduced and oxidised nitrogen concentrations, to protected sites and assess the applicability to the statutory conservation agencies’ monitoring requirements (i.e. scientifically robust, economic and relatively straightforward).
- 2) To assess the existing techniques in relation to their application as an indicator of ecological impact on a site.
- 3) To make recommendations on the most appropriate techniques for the purpose and propose a methodology for a wider national survey programme to validate these at a larger spatial resolution.

1.4. Structure of the report

This report reviews the wide range of different bio-indicator methods for atmospheric nitrogen impacts on terrestrial habitats, relevant for the statutory conservation agencies. The relevance of different approaches is considered together with an assessment of the method ‘time constants’, information on cross-sensitivity, and suitability to address different questions. A key question for each method is the applicability for use by the nature conservation agencies, and therefore practical questions such as specific skills and resources required are also relevant. As many of the methods that need to be considered are only at an early stage of development, a field test of a range of methods was conducted at a site where much information on bioindicators was already available (Pitcairn *et al.* 1998, 2002, 2003). This field test is reported in Appendix I of the present report (Pitcairn *et al.* 2004a), with the key findings incorporated into the main review section. In addition, to distil the reviewed information on the different methods into an easily accessible form, datasheets have been constructed that summarise the basis, suitability and constraints for each method. These datasheets are presented in Appendix II (Pitcairn *et al.* 2004b), and have also been

incorporated into the Air Pollution Information System (APIS) of the UK conservation agencies and environment agencies (APIS 2003, Bealey *et al.* 2003). Finally, on the basis of the detailed scientific review, an empirical rating is made of the different methods in considering their applicability for use by the conservation agencies. This is used to highlight a short-list of methods that are best suited to practical application in the field or warrant further development. Using the best methods identified a decision tree is constructed to aid the selection of appropriate nitrogen bioindicator methods in different contexts.

2. PHYSICAL METHODS FOR MEASURING ATMOSPHERIC NITROGEN CONCENTRATIONS AND DEPOSITION

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2.1. Comparing physical- and bio-monitoring

It is recognized that different applications of bioindicators for nitrogen include the estimation of atmospheric deposition and concentrations, as well as of ecological effects. It is therefore necessary to consider how bioindicators and biomonitoring fit together with classical physical monitoring of atmospheric concentrations and deposition for nitrogen species.

Two key interests of bioindicators and biomonitoring are to provide:

- a) direct, site-based evidence of situations where high exposure to and impacts of atmospheric reactive nitrogen occur,
- b) practical methods to estimate pollutant exposure that can be implemented easily and with lower cost than physical monitoring techniques.

As with the comparison with the critical loads approach, bioindicators may be seen as providing complementary information to that derived from physical monitoring of atmospheric concentrations and deposition. One key difference is that bioindicator measurements can in certain circumstances provide useful information from measurements made only at one time, while atmospheric measurements generally require regular repeated monitoring over several months to years. The reason for this difference is that bioindicators represent the accumulated response on the component being studied, which may have a characteristic integration time of up to several years, depending on the method used.

Conversely, concentrations and deposition of atmospheric nitrogen compounds vary substantially over daily, monthly and seasonal scales, indicating the requirement for measurements over many months. This temporal variability in deposition is particularly apparent for wet deposition, where a significant fraction of annual N may occur in a few events, while values may also vary substantially depending on wet or dry years and the trajectory of air mass origin during wet periods. The high temporal variability in atmospheric concentrations is illustrated in Figure 2.1, which shows the monitored concentrations of ammonia (NH_3), nitric acid (HNO_3), aerosol ammonium (NH_4^+) and aerosol nitrate (NO_3^-) at four sites of the UK monitoring network (Sutton *et al.* 2001). It is clear that for each of the components there is a substantial seasonal variation, and that there are clear relationships between the concentrations of the different species.

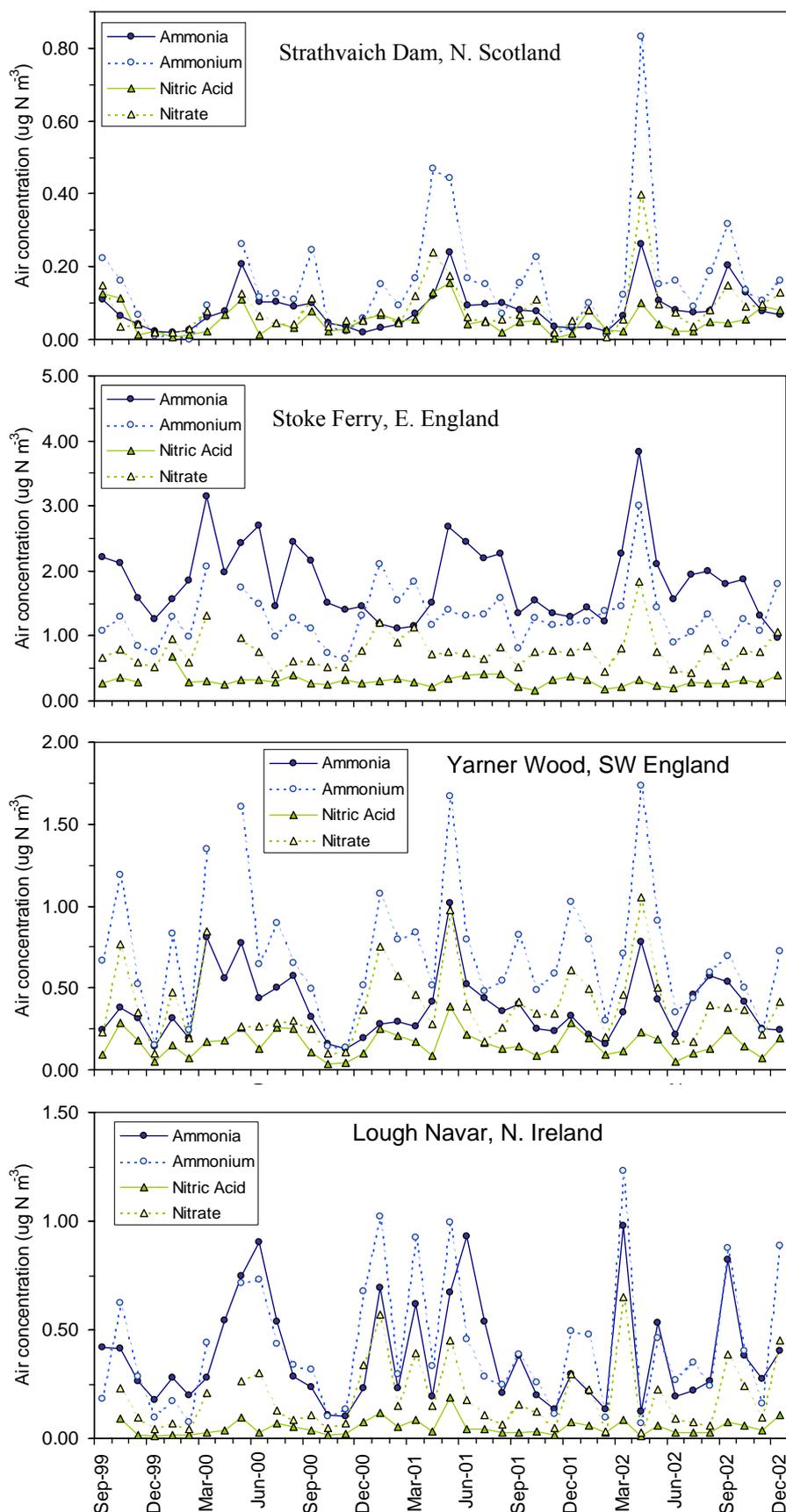


Figure 2.1: Temporal variation in gaseous NH₃ and HNO₃ and aerosol NH₄⁺ and NO₃⁻ at four sites in the UK measured using the DELTA approach (Sutton *et al.* 2001). Clear seasonal variability and correlations between components can be seen. Note the high concentrations recorded in April 2002 for each site except Lough Navar.

2.2. Monitoring Strategies

A wide range of physical methods exists for monitoring atmospheric nitrogen compounds, and this range partly reflects the different purposes for which the monitoring data are used. In the context of developing an atmospheric monitoring strategy under the European Monitoring and Evaluation Program (EMEP), three levels of air monitoring were proposed by Sutton (2001) to reflect the different purposes:

Level 1: Low-cost, simple techniques that meet the objective of providing long-term data and high spatial coverage for the assessment of temporal trends and spatial patterns.

Level 2: More detailed, but still basic, techniques that allow some indication of processes at a few sites, while still allowing for the assessment of long-term trends.

Level 3: Highly complex, time-resolved (e.g. hourly) measurements that focus on the investigation of processes to allow the refinement and testing of atmospheric models, including transboundary transport.

While there has subsequently been a significant debate on the details of European air monitoring for transboundary air pollution, with different views on the levels approach being proposed for a future EMEP strategy (e.g. Torseth and Hov 2004), it is agreed that a levels approach is robust and reflects the fact that different monitoring methods are more cost effective for different purposes.

From the perspective of assessing air pollution impacts on protected sites, the physical monitoring requirement most closely reflects the Level 1 techniques as listed above. There are a large number of protected sites where measurements might be conducted and the requirement is for average concentrations and deposition, and in some cases long-term trends, using the minimum resources. The remoteness of many of the sites, and common lack of mains electricity also requires a focus on the simpler techniques. In terms of sampling frequency, monthly sampling is generally sufficient, and in some cases an even lower frequency may be adequate. Conversely, because of the different effects of each of the component nitrogen forms, and their different contributions to nitrogen deposition, it is vital that the sampling techniques speciate between gaseous forms (NO_x , HNO_3 , NH_3), aerosol (NH_4^+ , NO_3^-) and wet deposited components (NH_4^+ , NO_3^-).

Substantial cost savings can be made in the assessment of atmospheric concentrations at a site level by the recognition that certain components vary substantially on a local level (e.g. NH_3 , NO_x , wet deposition in hill areas), while others vary significantly only at a regional scale (HNO_3 , aerosol NH_4^+ , NO_3^- , wet deposition in low altitude areas). The consequence is that actual air monitoring at the sites of interest only needs to be made for the components with significant spatial variation, and national network data and maps can often be used to estimate the other components with reasonable accuracy (Sutton *et al.* 2003b).

The scale of spatial variation in the different components of dry and wet deposition can be seen in Figure 2.2. In the regions of highest spatial variability it is expected that such maps provide a rather uncertain estimate of atmospheric deposition at individual sites. Conversely, in areas where the estimates mapped at a 5 km level show little variation between squares, it such mapped estimates will often be adequate for site assessment.

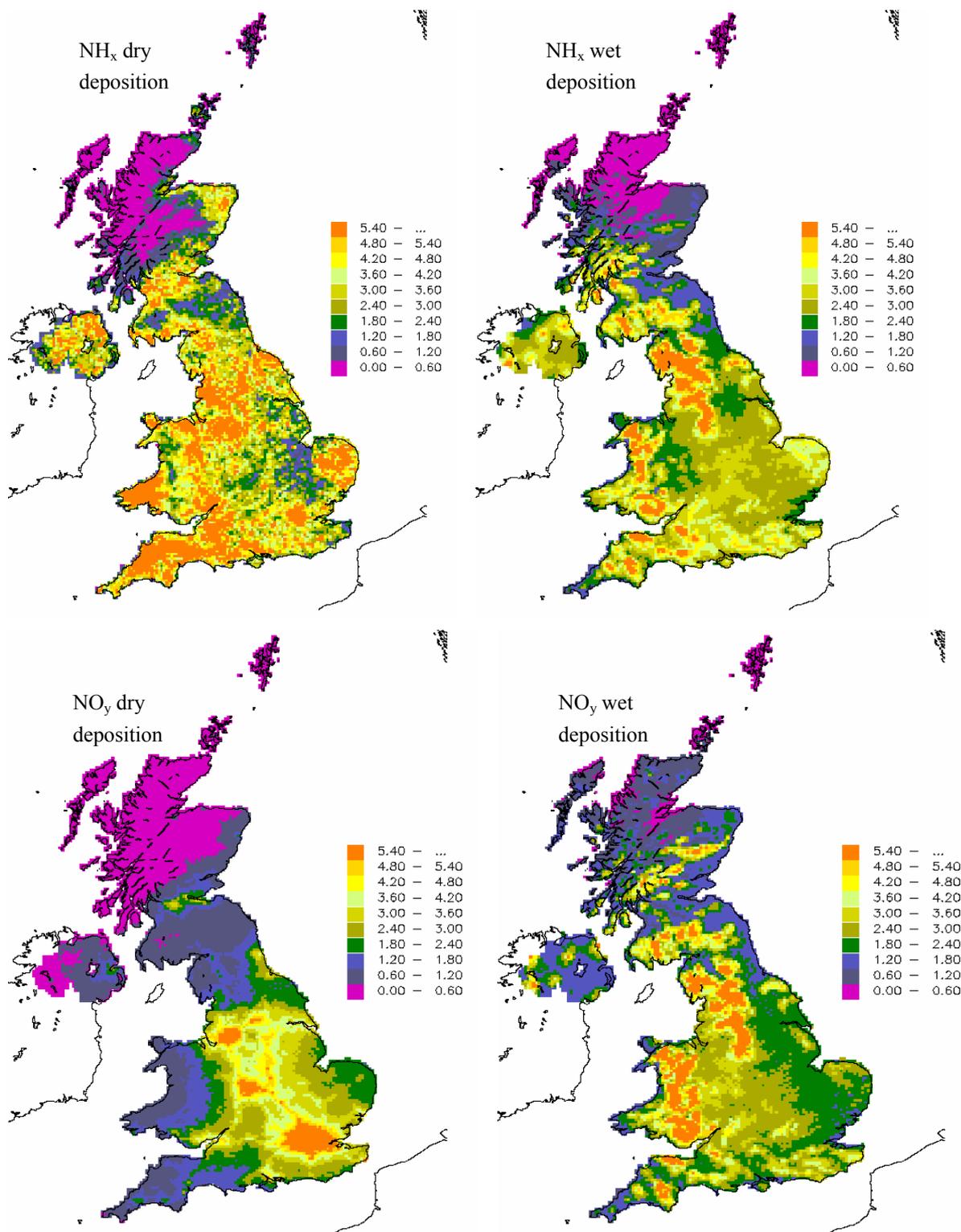


Figure 2.2. Modelled spatial pattern of reduced (NO_y) and reduced nitrogen (NH_x) deposition to the UK according to the FRAME model for 2000 (FRAME 4.21). The dry deposition figures shown represent the average deposition across each 5 km grid square according to the land uses present. Larger rates of deposition than those shown apply to specific habitats such as forest and moorland vegetation. The maps demonstrate the high spatial variability of NH_x dry deposition and of wet NH_x and NO_y deposition in hill areas. NO_y dry deposition is largest near major conurbations although significant enhancements are also predicted adjacent to trunk roads.

It should be noted that to keep the maps in Figure 2.2 comparable in scale, these show the average deposition to each 5 km square of the UK. Because of the different rates of dry deposition between habitats (particularly for NH_3), the deposition received by low-semi natural vegetation and woodland will differ and be larger than shown in Figure 2.2. There are several estimates of mapped atmospheric deposition for the UK, and Figure 2.2. shows results from the FRAME atmospheric transport model (Fournier *et al.* 2002, 2004). This model is driven by 5 km emissions estimates and therefore highlights for the present purpose the link between the location of emissions and the magnitude of dry deposition. The other main approach used to model nitrogen deposition in the UK is to infer this from the results of measured wet deposition and air concentrations from monitoring networks. This is the approach taken by the National Expert Group on Transboundary Air Pollution (NEG-TAP 2001), the estimates from which (updated to 1998-2000) are available on-line through the Air Pollution Information System (APIS, www.apis.ac.uk).

2.3. Air concentration and deposition measurement methods relevant for nitrogen impacts on statutory nature conservation sites

Although there is a wide range of possible measurement methods for measuring atmospheric concentrations and deposition, the priority for simple low cost, long-term methods at protected sites substantially simplifies the selection of suitable methods.

For concentration measurements, all of the methods should be listed as Level 1, with monthly sampling frequency being fully adequate for the assessment of effects on protected sites. Only in the case of highly intermittent emissions near a protected site would there be an interest in shorter-term measurements (e.g. daily), but since the concentrations in these cases would be very large, again the Level 1 measurement methods would be adequate.

Table 2.1 lists a range of different Level 1 atmospheric concentration monitoring approaches for nitrogen species, noting methods used in the UK.

In addition to the Level 1 techniques, in some instances, Level 2 and Level 3 techniques may be applicable for protected sites. The main Level 2 technique of relevance is long-term dry deposition monitoring. Classically, the measurement of dry deposition rates is a highly expensive and complex task focused on data of sufficient quality to develop new models. Recently, however, progress has been made in the development of time integrated (e.g. two weekly) measurements to enable seasonal and annual estimates of dry deposition to be made. An approach described by Fowler *et al.* (2001) measures the time average vertical profiles of pollutant concentrations using sampling conditional on current meteorological conditions. This method incorporates the DELTA low cost denuder method as the basis for the concentration measurements (Sutton *et al.* 2001). While such measurements can be made remotely using wind/solar power, there is still a site requirement for an extensive uniform area of land (e.g. >200 m) in the main wind directions around the sampling station.

Continuous time resolved sampling methods are included in Level 3, and these without exception require significant capital expenditure. While chemiluminescent sampling of NO_x is widely established, several instruments are now available for continuous sampling of NH_3 . Continuous sampling of the other components (HNO_3 and aerosol) remains a research challenge.

Table 2.1: Methods suitable for physical monitoring of the concentrations of reactive atmospheric nitrogen compounds at statutory nature conservation sites. The methods listed focus on low cost ‘Level 1’ techniques (see text) that are in use in the UK

Atmospheric Component	Level 1 approach	Example UK implementation	Reference	Notes
NH ₃	Passive sampling	1. Diffusion tubes	Thijsse <i>et al.</i> (1998) Sutton <i>et al.</i> (2001a)	No electricity needed QA possible but difficult. Replication and permanent calibration essential Only for high concentration areas.
		2. ALPHA samplers	Tang <i>et al.</i> (2001)	No electricity needed Replication and permanent calibration essential High sensitivity for low concentrations.
NO _x	Passive sampling	Diffusion tubes	Downing <i>et al.</i> (1994) Tang <i>et al.</i> (2001)	No electricity needed Some sampling artefacts, plus limitation to shorter sampling periods (e.g. <2-3 months).
Wet deposition of NH ₄ ⁺ and NO ₃ ⁻	Bulk precipitation collection	Open funnels to collect precipitation chemistry, plus precipitation amounts from standard meteorological collector	RGAR (1997) Cape and Leith (2002) Sutton <i>et al.</i> (2003b)	Near sources dry deposition to open collectors can be significant Care needed to avoid under sampling of precipitation amounts by some collectors Increased deposition in hill areas due to orographic effects and cloud deposition need more detailed consideration.
NH ₃ and aerosol NH ₄ ⁺	Low cost denuder system	DELTA system	Sutton <i>et al.</i> (2001b)	Typically monthly measurements Mains or wind/solar power required Robust QA/QC, speciating gas and aerosol for typically monthly sampling.
HNO ₃ and aerosol NO ₃ ⁻	Low cost denuder system	DELTA system	Based on Sutton <i>et al.</i> (2001b) Sutton <i>et al.</i> (2001d)	Extension to DELTA for NH ₃ /NH ₄ ⁺ including additional denuders. Technique can also sample SO ₂ , SO ₄ ²⁻ , HCl, Cl ⁻ and base cations.

The only context in which such measurement techniques would be justifiable for application at a protected site is for campaign measurements of the continuous concentration record coupled with site level meteorological in order to help identify the location of nearby sources and assess whether pollution events are detectable at the site.

Other issues regarding the estimation of atmospheric nitrogen deposition to sensitive terrestrial ecosystems have been recently reviewed by Sutton *et al.* (2003b).

2.4. Relative benefits of physical- and bio-monitoring for atmospheric concentrations and deposition

Given the availability of relatively cheap and robust methods for measuring atmospheric concentrations of nitrogen species, physical monitoring provides a more direct and reliable approach to estimate air concentrations than use of bioindicators and biomonitors. However, the negative aspect to physical measurements is the need for ongoing repeated (e.g. monthly) measurements. Therefore if a quick indication of atmospheric levels is required, application of bioindicators may provide a useful alternative.

A further limitation of bioindicators and biomonitors is that they respond to some extent to several different forms of nitrogen. While one method may be more sensitive to a particular N compound than another, the consequence is that it is generally difficult to specify from bioindicator measurements alone which source and chemical form of nitrogen is dominating inputs.

Conversely, while physical monitoring of atmospheric *concentrations* is reasonably straightforward, monitoring of wet and dry *deposition* is more involved. This is particularly the case in situations of ‘complex terrain’ where local heterogeneity and closeness to source make estimation of dry deposition complex. This might occur for example in the close vicinity of a road or agricultural source. Similarly, in hill areas the existence of orographic and cloud deposition make estimation of wet deposition complex. In these cases, bioindicators and biomonitors have a more important role to play in the estimation of atmospheric inputs.

In considering the suitability of bioindicators and biomonitoring to estimate air concentrations and deposition, the objective is frequently different to that of physical monitoring. In the case of the latter, a precise quantitative estimate of exposure is required. However, with biomonitoring the target is often more simply to give a broad indication of whether the air concentrations and deposition are at acceptable or unsustainably high levels.

3. BIOINDICATOR METHODS BASED ON FOLIAR NITROGEN ACCUMULATION: TOTAL TISSUE NITROGEN

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3.1. Background

In assessing impacts of N deposition from point and diffuse sources on vegetation, it is not always easy to detect changes in species composition or possible to measure atmospheric N deposition. Plant chemical bioindicators may provide an early indication of enhanced N deposition and its effects on vegetation. This section considers approaches that measure accumulated nitrogen in a plant component. Bioindicators that reflect other chemical responses to excess nitrogen are considered in Section 4.

Tissue N content of certain plant species represents the most well studied bioindicator of atmospheric nitrogen deposition (e.g. Bobbink *et al.*, 1993; Baddeley *et al.* 1994; Hyvarinen & Crittenden, 1998; Pitcairn *et al.*, 1995, 1998). The foliar N content of trees (van Dobben, 1993; Pitcairn *et al.* 1998) ericaceous shrubs (Pitcairn *et al.*, 1995, 2001; Hicks *et al.*, 2000; Leith *et al.*, 2001), herbs (Pitcairn *et al.* 2002), mosses (Baddeley *et al.* 1994; Pitcairn *et al.* 1995, 1998, 2002; Woolgrove and Woodin 1996) and lichens (Hyvarinen & Crittenden, 1998; Gaio-Oliveira *et al.* 2001) have been shown to be related to atmospheric N inputs, suggesting that tissue N could be a useful indicator of N deposition in a wide range of habitats. What is the background to this evidence and how well do we believe it?

3.2. Evidence for links between tissue N and atmospheric N deposition

3.2.1. Evidence from transplants experiments

Some of the earliest work showing that tissue N in plants can respond to N from the atmosphere came from John Lee and colleagues working with *Sphagnum* species in the southern Pennines. For example, when *Sphagnum cuspidatum* was transplanted from Migneint, North Wales (a 'clean site'), to bog pools at Holme Moss in the polluted Pennines and the Berwyn Mountains, a clean site in North Wales, tissue N concentrations increased from 1.2% N, to 1.8% N and 1.35%, respectively, after 6 months. After 12 months, the tissue N concentrations had further changed to 2.5% N and 1.31% N, respectively (Press *et al.* 1986). Although, atmospheric pollutant monitoring was limited at that time, deposition estimates based on bulk precipitation measurements and diffusion tube measurements of NO₂ concentrations provided strong evidence of differences in the N pollution climate between sites.

In recent transplant experiments, Mitchell *et al.* (see section 9.1) monitored N deposition to epiphytic communities in Atlantic woodlands in northern Britain for 12 months. Plants transplanted from a clean site in northern Scotland to a more polluted site in Cumbria showed an increase in tissue N, whereas some species transplanted from the 'polluted' to the 'clean'

site, showed a decline in tissue N concentrations. Wet deposition, air concentrations and stemflow were all correlated, and it was not certain whether the tissue N was responding most to the total atmospheric N deposition and stemflow flux or to the concentrations of ammonium and nitrate in stemflow.

3.2.2. Evidence from simulation experiments

A considerable body of experimental evidence has built up over the last 20 years of increased tissue N following treatment with wet deposited and gaseous N compounds in field and chamber experiments. In the UK for example, long-term addition of NH_4NO_3 (40-120 kg N $\text{ha}^{-1} \text{y}^{-1}$) to *Calluna* moorland resulted in significant and sustained increases in N content from a control value of 1.4% N to 1.73-1.92% (Carroll *et al.* 1999) and a similar range of treatments applied to calcareous grasslands increased the foliar N content of a range of herbaceous species (Moorcroft *et al.* 1994). In open-top chamber experiments, exposure of ombrotrophic mire vegetation to a range of concentrations of either NH_3 or NH_4^+ , produced a significant linear dose response relationship ($p < 0.05$) between foliar N content of all species present and $\text{NH}_4\text{-N}$ applied in both 1999 and 2000 (Leith *et al.* 2001). The N concentrations in *C. vulgaris* ranged from 1.70% to 2.25% for 1999 and 1.4% to 1.93% for 2000. In the NH_3 treatment, only *C. vulgaris*, and *Polytrichum commune* showed a significant response to increasing N additions, whereas in the $\text{NH}_4\text{-N}$ treatments, all the species (*Eriophorum vaginatum*, *Narthecium ossifragum*, *Potentilla erecta*, *Molinia caerulea*, *Deschampsia flexuosa*, *Calluna vulgaris* and *Polytrichum commune*) showed a significant response to increasing N additions. The form of N applied is also important, with the tissue N responses being greater in NH_3 treatments compared with $\text{NH}_4\text{-N}$ treatments on a per unit N basis (Leith *et al.* 2001).

3.2.3. Evidence from field studies

Following the attribution of vegetation changes in heathlands and other ecosystems in the Netherlands to N deposition, a countrywide survey in 1989 provided evidence of similar changes in the UK (Pitcairn *et al.* 1991). This study showed a relationship between tissue N content of certain species (*Calluna vulgaris* and a range of pleurocarpous moss species) and atmospheric N deposition across the UK. Around the same time studies by Baddeley *et al.* (1994) linked the deterioration of *Racomitrium* heath in Britain during the last 50 years with increased atmospheric N deposition by showing a relationship between deposition and changes in tissue N content of *Racomitrium lanuginosum*.

In Norway tissue N concentrations in the acrocarpous moss *Dicranum majus* were measured by Bakken (1995a,b), and plants from more polluted areas of southern Norway were shown to contain significantly larger tissue N concentrations than those from a 'cleaner' site in central Norway. In Finland, surveys of element concentrations in epiphytic lichens showed that tissue N in *Hypogymnia physodes* ranged from 0.75-2.56% with a mean value of 1.3%. The largest concentrations were found in southern Finland with a local peak in the far north. These are large concentrations considering the relatively small N deposition inputs in Finland and may partly reflect a response to large concentrations of N in snow during lengthy periods of snow cover rather than N deposition (Kulbin, 1990).

3.2.4. Evidence from altitude studies

Atmospheric N deposition increases with altitude due to 1) increased precipitation with altitude, 2) frequent cover by orographic cloud (containing 3-5 times the concentrations present in rain) and 3) orographically enhanced deposition according to the "seeder-feeder effect", Fowler *et al.* (1988). Baddeley *et al.* (1994) demonstrated the close link between altitude, atmospheric N deposition and tissue N by transplanting turfs from low altitude sites

to sites at increasing altitudes on four mountain ranges in North West Scotland. Pitcairn *et al.* (1995) also demonstrated altitude enhanced tissue N concentrations by sampling *Calluna vulgaris* along a transect of increasing altitude in North West Scotland. This relationship was confirmed and expanded to a range of species by Hicks *et al.* (2000) sampling along altitudinal transects at sites of known atmospheric N deposition in northern Britain. They found that for *Nardus stricta*, *Deschampsia flexuosa*, *Calluna vulgaris*, *Erica cinerea*, and *Hylocomium splendens*, measured % foliar N concentrations increased linearly with increasing altitude and estimated N deposition, by 0.07, 0.12, 0.15, 0.08 and 0.04 % respectively for each $1 \text{ kg ha}^{-1} \text{ y}^{-1}$ increase in estimated N deposition on Beinn an Fhurain, a mountain in a relatively unpolluted area of north-west Scotland. These independent studies of altitude-related increases in foliar N in a range of species provide strong evidence that foliar N concentrations can be indicative of levels of atmospheric N deposition.

3.2.5. Evidence from herbarium specimens

Herbarium samples can provide a record of the past pollution climate within their tissues. Baddeley *et al.* (1994) found that tissue N concentrations in samples of *R. lanuginosum*, collected from a range of locations between 1853 and 1899, were very much smaller than samples collected in 1989 at any altitude and that the tissue N of samples collected from Ingleborough summit increased 3-fold between 1879 and 1989. Increases in tissue N in certain pleurocarpous moss species have also occurred over the period of 15-35 years (1955-1989) at some sites in Scotland and Cumbria, although in north-west Scotland, where atmospheric nitrogen deposition remains small, tissue N concentrations did not change significantly (Pitcairn *et al.* 1995). Herbarium and more recent collections of the snowbed species, *Kiaeria starkei* from Bidean nan Bian and Lochnagar in Scotland have also shown increased tissue N over the past century (Woolgrove and Woodin, 1996). These studies clearly demonstrate an increase in tissue N in a range of species closely coupled to the atmosphere, and may also provide some indication of atmospheric N deposition during the past century.

3.2.6. Evidence from long-term studies

Evidence has also been provided through long-term studies. Such studies have been largely associated with assessments of forest health and included measurements of the nutrient status and health of a range of tree species. Hence increased deposition of N in the Netherlands over recent decades was reflected in increased N content of needles. In the Peel area of the Netherlands, in the period 1956-1988, needle N content of the most important tree species increased from about 1.3% to 1.8% (van der Eerden *et al.* 1997). Malmer (1990) found evidence of increased tissue N in Sphagnum species in Sweden over the same time period. Tree studies have also shown that ceilings for plant tissue N accumulation exist. For example, Emmett *et al.* (1995) found no significant change in current year foliage N of Sitka spruce in response to up to $75 \text{ kg N ha}^{-1} \text{ yr}^{-1}$.

A summary of foliar N content from both herbarium and long-term studies is shown in Figure 3.1. This shows a convincing demonstration of the increase in values both over the past century and between 1950 and 1990.

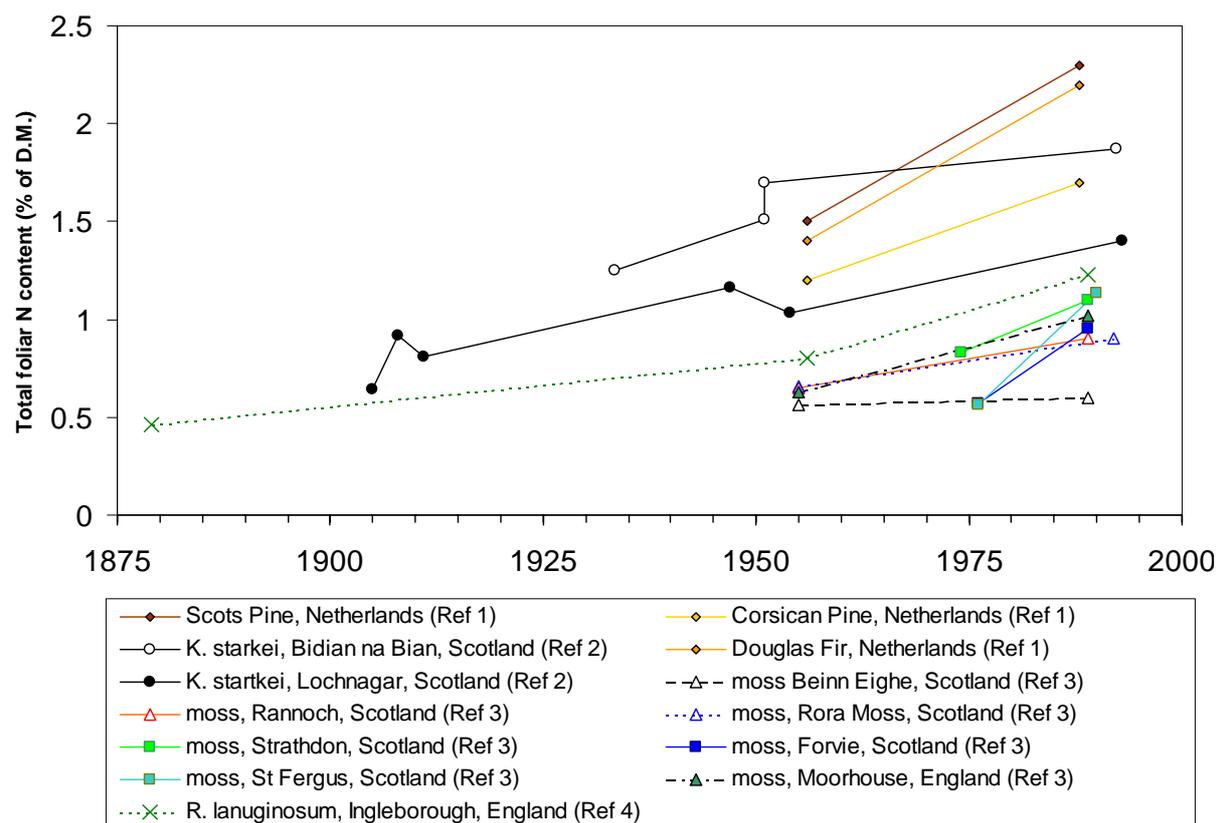


Figure 3.1. Compilation of data on foliar N content with time at sites from the UK and the Netherlands from herbarium and long term field studies. Ref. 1: Van der Eerden *et al.* (1997), Ref. 2: Woolgrove and Woodin (1996), Ref. 3: Pitcairn *et al.* (1995), Ref. 4: Baddeley *et al.* (1994).

3.2.7. Evidence from near-source transect studies

Species composition and tissue nitrogen content of a range of plant species were determined along a gradient of ammonia concentration and N deposition in woodland in the vicinity of four intensive livestock units in Scotland (Pitcairn *et al.* 1998). Foliar N concentrations decreased with distance from the livestock buildings and close relationships were shown between foliar N, atmospheric ammonia concentrations and estimated total N deposition at each distance for selected tree, herb and moss species. The accuracy of the ammonia measurements and careful N deposition estimates together with the broad range of species sampled provide excellent evidence of a robust relationship.

Measurements along a gradient of decreasing NO_x concentrations radiating from central London have also shown significant declines in tissue N concentrations. In this case Power and Collins (pers comm.) found a correlation between tissue N of *Calluna vulgaris*, traffic exposure (distance from major roads) and NO_x concentrations. Preliminary work in Scotland (C.E.R. Pitcairn, unpublished data) with road verge mosses has not shown a relationship between tissue N and distance from kerbside, but further work is in progress. However, it is recognized that vehicles fitted with catalytic converters emit significant quantities of ammonia (Sutton *et al.* 2000), so that impacts of roads on tissue N may have increased in recent years, and it becomes increasingly difficult to attribute tissue N responses near roads specifically to NO_x.

3.3. Inconsistencies

Not all studies have demonstrated a close link between tissue N and atmospheric N deposition. Woodin & Sullivan (2001) found a significant positive relationship between tissue N concentrations in *Hylocomium splendens* and N deposition for a range of sites in the UK. However, the degree of scatter in the data led the authors to question the suitability of tissue N as a bioindicator of N deposition (see comparison in Figure 3.2). The scatter may be to a large extent accounted for by the absence of site-specific N deposition estimates (estimates were obtained from the UK 5 km x 5 km maps), but also to a long collection period and a long period in cold storage. The authors point out that the collection sites covered a wide geographic area and many habitat types. However the sites do appear to be concentrated in Scotland and Wales with few sites in the more polluted area of northern England. Hence a fairly narrow range of N deposition (5-23 kg N was matched by relatively low maximum tissue N concentrations (<1.4%, majority 0.5-1.1%). The authors found a better relationship with NO_y deposition than with NH_x deposition.

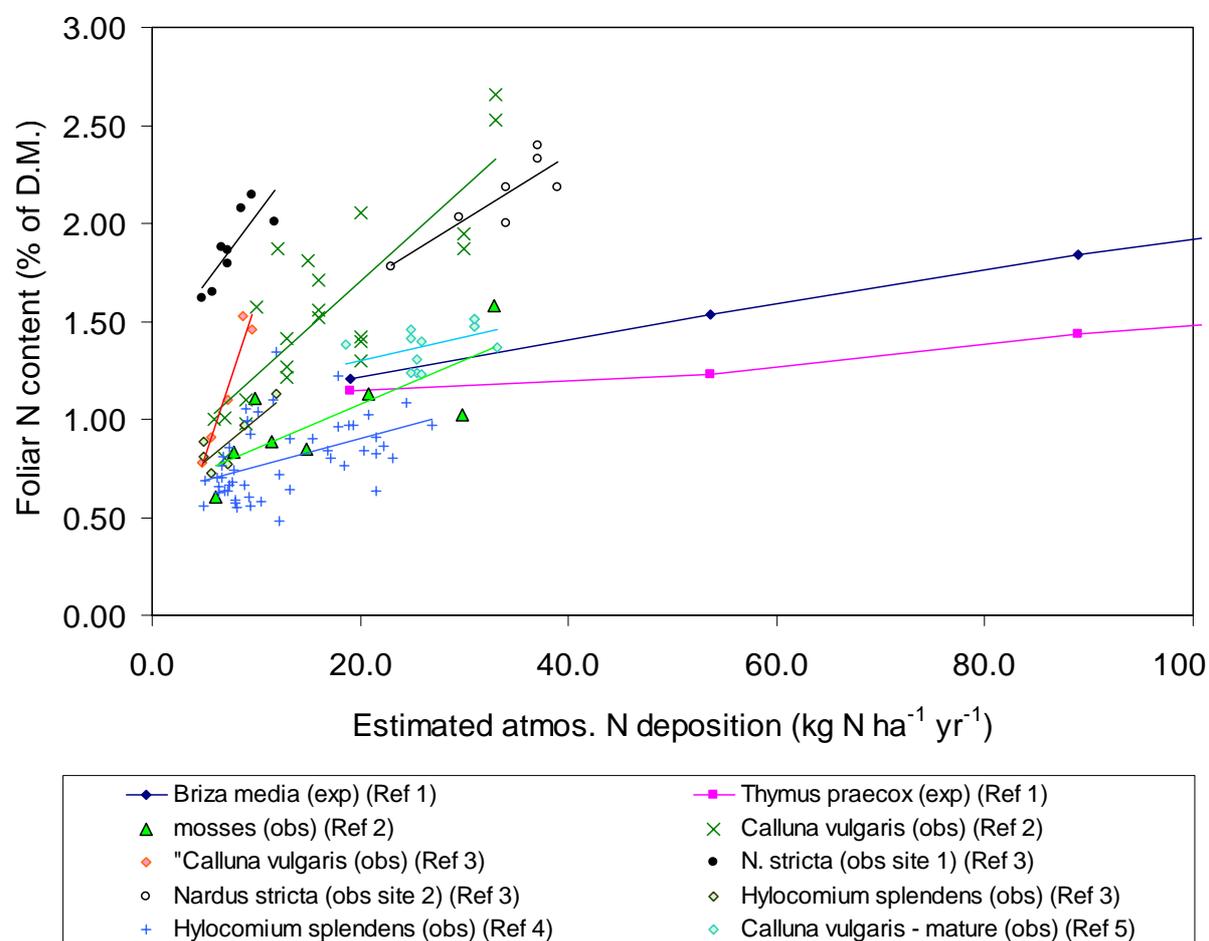


Figure 3.2. Summary of example datasets on the relationship between estimated total atmospheric nitrogen deposition and foliar nitrogen content. Results are shown from both observational field studies (obs) and from an experimental manipulation adding N for three years (exp). A consistent increase of foliar N with estimated atmospheric N deposition is seen, with the exact relationship depending on species and the quality of the deposition estimates used. Ref. 1: Moorcroft *et al.* (1994), Ref. 2: Pitcairn *et al.* (1995), Ref. 3: Hicks *et al.* (2000), Ref. 4: Woodin and Sullivan (2001), Ref. 5: Kircham (2001).

An extensive survey by Kirkham (2001) of tissue N in a range of upland species found little correlation with total N deposition, nor NH_x deposition but some relationship with NO_y deposition. The ranges of estimated deposition ($16\text{-}36 \text{ kg N ha}^{-1} \text{ y}^{-1}$) and tissue N ($1.2\text{-}1.5\%$) were fairly narrow. The samples were collected over 2 years, but no mention of season is made. Estimates of N deposition were obtained from mapped estimates on a $20 \text{ km} \times 20 \text{ km}$ grid and thus are unlikely to be accurate for all localities. NH_x in particular, tends to vary substantially over a much smaller area (NEG TAP 2001). However, relationships may be difficult to demonstrate where sites covering a small range in deposition are sampled. Although Pitcairn *et al.* (1995) sampled fewer sites, the range of pollution climates was larger and hence differences easier to demonstrate. Figure 3.2 shows good agreement for *Calluna* between the study of Pitcairn *et al.* (1995) and Hicks *et al.* (2000), while the values of Kirkham (2001) are lower, which may reflect an underestimation of site specific N deposition in that study. Figure 3.2 also shows the experimental increase in foliar N observed for two grassland species (*Briza media* and *Thymus praecox*) by Moorcroft *et al.* (1994). Although the values for background atmospheric deposition (estimated at $19 \text{ kg N ha}^{-1} \text{ yr}^{-1}$) are within the range of the other species, the response to N is less, and this may be expected given that the data represent results after only three years exposure to elevated N deposition.

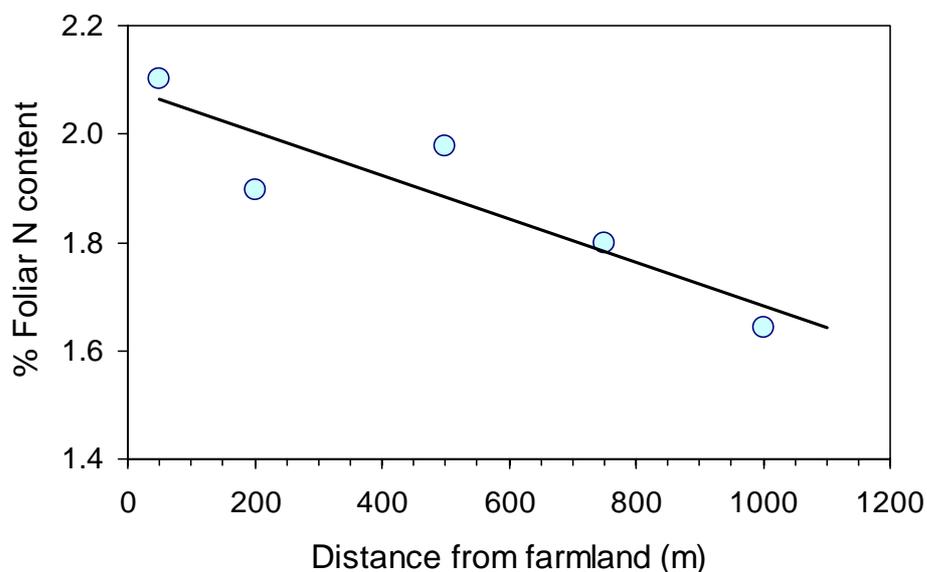


Figure 3.3: Local variability in the plant tissue N concentrations in *Calluna* across a dry heathland nature reserve in the Netherlands (Leende Heide) (from Sutton and Fowler 1995; Pitcairn *et al.* 2001). The decrease in plant tissue N with increasing distance from agricultural land is consistent with the expected decrease in atmospheric NH_3 deposition in this agricultural area.

Despite the large deposition of N recorded in the Netherlands, foliar N concentrations in *Calluna* from Dutch heathlands are not always as large as expected. While concentrations of 1.8% N were found in Leinde Heide (Pitcairn *et al.* 2001), on Asselse Heide where N deposition was estimated to be $30\text{-}45 \text{ kg N ha}^{-1} \text{ y}^{-1}$, foliar N content of *Calluna* was between 0.9% and 1.4 % of dry weight. These differences may again reflect the substantial local variability in NH_3 emissions and concentrations that is characteristic of agricultural areas in the Netherlands (Sutton *et al.* 2003a). This is illustrated by the tissue N results for *Calluna* at Leende Heide, which were found to decrease with increasing distance from the nearest agricultural land (Figure 3.3). These Dutch heathlands also provided evidence that high levels of atmospheric N input could raise the amount of NH_x in the plant tissues, thereby increasing the NH_3 ‘compensation point’ (that might parallel the increase in tissue N concentration),

which would provide a feedback tending to limit net nitrogen deposition (Sutton *et al.* 1993, 1995, Sutton and Fowler 1995). Combined with other feedbacks, this could provide a limit on the maximum value of plant tissue N concentrations.

In summary, there are several possible explanations for situations where inconsistencies have been observed between plant tissue N and atmospheric N deposition. These include:

- 1) poor estimates of site-specific N deposition, either through the use of low resolution map estimates (e.g. 20 km) or the existence of substantial local variability (50 m - 1 km) in source areas;
- 2) the occurrence of a maximum tissue N concentration, that might be both species and climate specific;
- 3) unsuitable site selection, where the existence of other disturbance factors becomes important;
- 4) lack of firm a protocol for sampling, which would be important because of variability between different plant parts and temporal variability through the year;
- 5) the filtering effect of overstorey co-occurring competitive vegetation where mixed species stands are sampled. This is particularly relevant where ground flora components are sampled under a fast growing woodland canopy, as deposition to the ground layer will be substantially less than the total atmospheric N deposition (Sutton *et al.* 2003b).

3.4. Species Differences in Tissue N Content

3.4.1. Between-group differences in Tissue N content

Across the plant kingdom there is a very large range of characteristic tissue N concentrations. There are clearly differences in the range of tissue N concentrations found in different plant groups and in the quantitative response to N deposition. In the poultry farm studies of Pitcairn *et al.* (1998), foliar N concentrations were determined for four tree species and ectohydric mosses. The slope of the regression lines for the tree species, especially beech, was very shallow compared with that for ectohydric mosses (Figure 3.4) and demonstrated the buffering effect of stored N on changes in foliar N in large trees relative to the ectohydric mosses, many of which rely primarily on the atmosphere for the N supply. Conversely, the very steep slope of the regression line for ectohydric mosses emphasised the close link between foliar N concentrations in ectohydric mosses and atmospheric N inputs providing further support for the use of such mosses as indicators of NH₃ concentrations and N deposition.

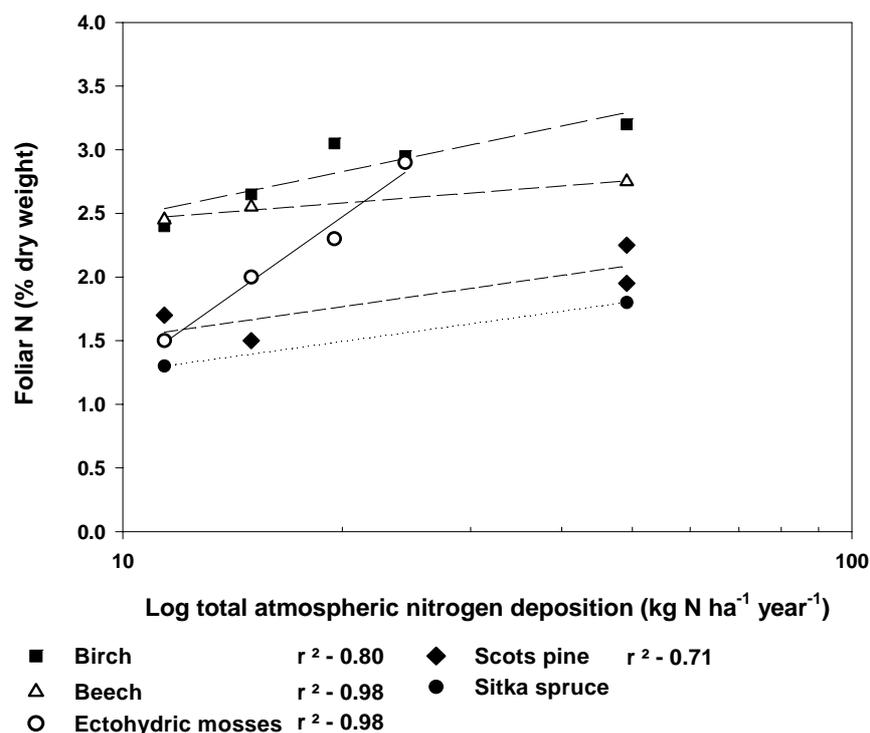


Figure 3.4: Relationship between foliar N and estimated total atmospheric N deposition for 4 tree species and ectohydric mosses from a transect of sites in the vicinity of a poultry farm (from Pitcairn *et al.* 1998, 2004a, see Appendix 1).

Many ericaceous shrubs, particularly *Calluna* grow in nutrient poor soils and are also partly dependant on the atmosphere for their N supply. At least 40% of the N demand can be satisfied by foliar uptake in *Calluna* (Skiba *et al.* 1986). Many of the studies noted above have shown relationships between tissue N of *Calluna* and atmospheric N deposition. Although not so closely linked to the atmosphere as bryophytes, foliar N concentration in *Calluna* may integrate over a longer period and thus provide a better indicator of annual N deposition estimates, although seasonal and interclonal differences exist.

3.4.2. Within-group differences in Tissue N content

If the close link between foliar N concentrations within plant groups and atmospheric N inputs is to be exploited, we clearly need to know whether plants in the group respond in the same way and if seasonal differences in tissue N concentrations occur. A good example of this is the ectohydric mosses, which in Figure 3.4 incorporated data from both *Rhytidiadelphus triquetrus* and *R. squarrosus*.

Ferguson and Lee (1984) showed difference in tissue N content in five species of *Sphagnum*. The differences were largely related to the niche occupied (pool or hummock) and form adopted (cushion or lax). Pitcairn *et al.* (2004c) sampled five moss species at two-monthly intervals from ‘clean’ drained blanket bog and a polluted woodland for one year. The data showed a small seasonal trend in tissue N concentrations, but, more importantly, consistent differences between the species sampled. Of the five species recorded at the blanket bog, *Pleurozium schreberi* had the lowest tissue N levels (0.75-0.85%) and *Rhytidiadelphus squarrosus* the largest (0.88-1.37%); at the woodland site 100 m from the poultry farm, tissue N concentrations in *Eurynchium praelongum* (2.4-3.9%) were slightly larger than those of *Rhytidiadelphus squarrosus* and *Mnium hornum* (2.1-3.8%). While part of these differences undoubtedly reflects different species characteristics, they may also reflect local variability in

nitrogen supply either due to locally variable atmospheric N deposition across the wood or heterogeneity of nitrogen cycling at the moorland.

Similar species differences have been found within lichens. The lichen *Xanthoria parietina*, that is indicative of nutrient rich habitats (see Section 5.3), was shown to contain almost twice as much N as other species, when collected from the same sites in Portugal. Gaió-Oliveira *et al.* (2001) found concentrations as high as 2.5 % in *X. parietina* at one site compared with 1.1-1.37% in other non-N fixing lichen species. This high concentration resembles values found in N fixing lichens although *X. parietina* is not a N-fixer. Gaió-Oliveira *et al.* (2001) suggest that of the species studied, *X. parietina* is the best bioindicator of high N deposition, particularly of ammonia deposition. To date, however, there has been little comparative assessment of the tissue N response of lichens to atmospheric N deposition in relation to bryophytes and higher plants.

3.5. Models for tissue N versus N deposition

The above results indicate the existence within many studies of a link between atmospheric N deposition and plant tissue nitrogen, as illustrated in Figure 3.5. As discussed in Sections 3.3. and 3.4., many of the inconsistencies can be explained by species differences, uncertainties in atmospheric deposition, sampling protocols etc. Caution is therefore needed in the application of tissue N as a bioindicator of atmospheric N deposition. The extent to which relationships can be established that infer atmospheric deposition from plant tissue nitrogen depends on the ability to deal with these inconsistencies in the primary studies. Because of this, the development of simple regression models to predict atmospheric nitrogen deposition needs to consider different species groups separately, follow standardized protocols and be based as far as possible on robust site-based estimates of atmospheric N deposition.

3.5.1. *Calluna vulgaris* and other ericaceous shrubs

The relationship of tissue N of *Calluna vulgaris* and other ericaceous shrubs to atmospheric N deposition has been characterized at several spatial scales, from European transects, national regional variability and from local variability in relation to atmospheric deposition. Measurements along trans-European transects from northern Finland to southern Norway (2000 km) and from central Sweden to Stockholm, south east Sweden (330 km) showed a roughly linear relationship in the range 0.8 to 1.4% N with a slope of 0.04% N/kg N ha⁻¹ yr⁻¹, and an intercept at zero atmospheric deposition of 0.79% N (Pitcairn *et al.* 2001). This may be compared with extensive data from UK and Netherlands sites (Pitcairn *et al.*, 1995), Scottish sites (Iason & Hester, 1993; Hicks *et al.*, 2000) and other Scandinavian sites (Karlsson, 1987), which gives a relationship with a slope of 0.036 %N/ kg N ha⁻¹ yr⁻¹, (the intercept was 0.87% N), which is remarkably close to that of the Fennoscandia data, even though it is obtained using different N deposition data sources. The robustness of the relationship between foliar N and N deposition for this species group supports the use of this simple bioassay for identifying areas of excess N deposition.

3.5.2. Bryophyte tissue N model

Using data from tissue of bryophytes near poultry farms and earlier regional studies, a model relating atmospheric N deposition to tissue N in mosses was derived (Pitcairn *et al.* 1998). The curve (described by $L_N = 3.81(1 - e^{-0.04 F_N})$ where L_N = foliar N % dry weight and F_N = N deposition in kg ha⁻¹ year⁻¹) showed that the relationship was not linear and that foliar N increased rapidly with increasing inputs of atmospheric N up to 20 kg year⁻¹. At larger

inputs the foliar N increases became progressively smaller, suggesting that physiological saturation may have occurred, including, for example, the potential for a larger compensation point with NH_3 emission during clean periods. Deposition of $20 \text{ kg N ha}^{-1} \text{ y}^{-1}$ resulted in a moss tissue N content of 2.0%. A relationship between foliar N and N deposition in Europe has also been derived for bryophytes from data compiled from the literature (C Pitcairn pers comm.). Several moss species have been included in this model, which may have introduced inter specific variations in N content (as discussed in section 3.4.1.).

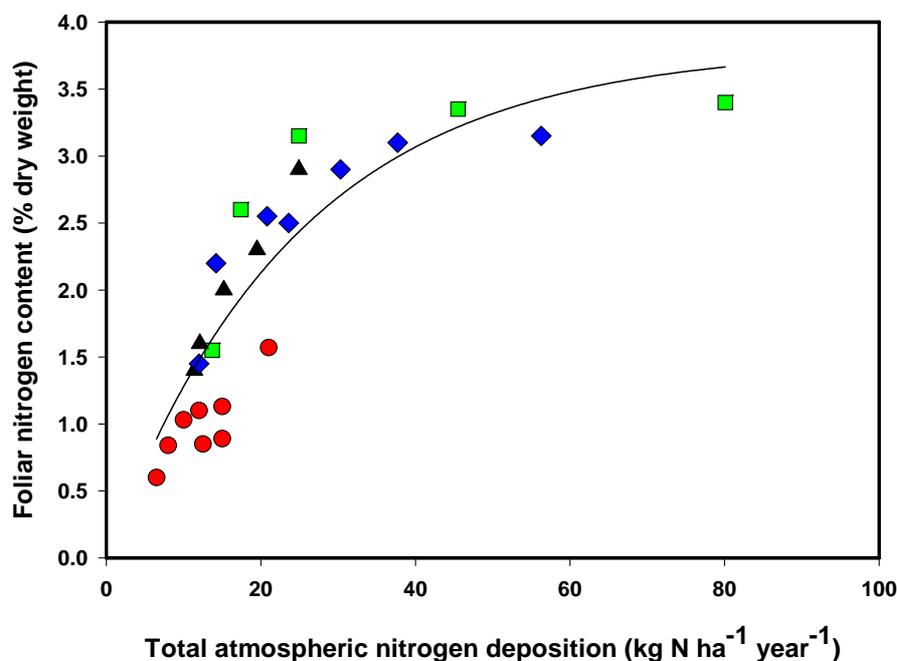


Figure 3.5: Foliar nitrogen content of pleurocarpous mosses in response to nitrogen deposition (Pitcairn *et al.* 1998). a) circles: data across UK from Pitcairn *et al.* (1995); b) triangles: data from the vicinity of a pig farm; c) diamonds: data from the vicinity of a poultry farm (farm E, Pitcairn *et al.* 2004a, see Appendix 1); squares: data from a second poultry farm (farm L). The regression is given by $L_N = 3.81(1 - e^{-0.04 F_N})$, where L_N is foliar N as % of dry weight and F_N is total N deposition ($\text{kg N ha}^{-1} \text{ yr}^{-1}$).

3.5.3. Estimating atmospheric deposition based on foliar tissue N

The relationship between atmospheric deposition and foliar N content shows the strong potential for this parameter as an indicator of enhanced N deposition. For example, based on the emerging regression relationships (e.g. Figure 3.5) it is possible to estimate the atmospheric deposition that would be necessary to give rise to a certain tissue N content. In this case tissue N can be used to support a site-based risk assessment using the critical loads approach, by assessing whether the atmospheric N deposition inferred from foliar N values is larger or smaller than an empirically estimated critical load (Achermann and Bobbink 2003) for the ecosystem concerned.

It is of particular interest to consider the statistics of the N deposition to foliar N relationship, for this provides the potential to estimate whether a given foliar N concentration at a site equates to deposition significantly above or below the critical load. A first attempt to demonstrate this for foliar N is shown in Figure 3.6. Since the purpose is to estimate N deposition from foliar N, and recognizing the uncertainty in deposition estimates, the former is taken as the dependent variable. It is not a straightforward matter to provide confidence limits for the complex regression of Figure 3.5, and in Figure 3.6 a simpler power function is applied to demonstrate the approach. For example, in deciduous woodland the empirical critical load for ground flora change is currently estimated as $10\text{-}15 \text{ kg N ha}^{-1} \text{ yr}^{-1}$

(Achermann and Bobbink 2003). Applying the mid point critical load of $12.5 \text{ kg N ha}^{-1} \text{ yr}^{-1}$, using Figure 3.6 would imply that measurement of foliar N concentration above 2.3% equates to atmospheric N inputs significantly greater (95% confidence) than the critical load.

While this provides a first approach to estimate the uncertainty limits in deposition based on bioindicator data, it is clear that both caution and further development are required. Given the potential for wide ranging values as indicated in Figure 3.5, bryophyte samples need to be taken following standardized protocols (e.g. Pitcairn *et al.* 1998) and with appropriate replication. Refinement of the approach needs to consider the collation of further datasets and the calculation of confidence limits for more complex functions to better fit the data. The non-optimal fit of the simple regression in Figure 3.6 indicates that the confidence limits are overly precautionary.

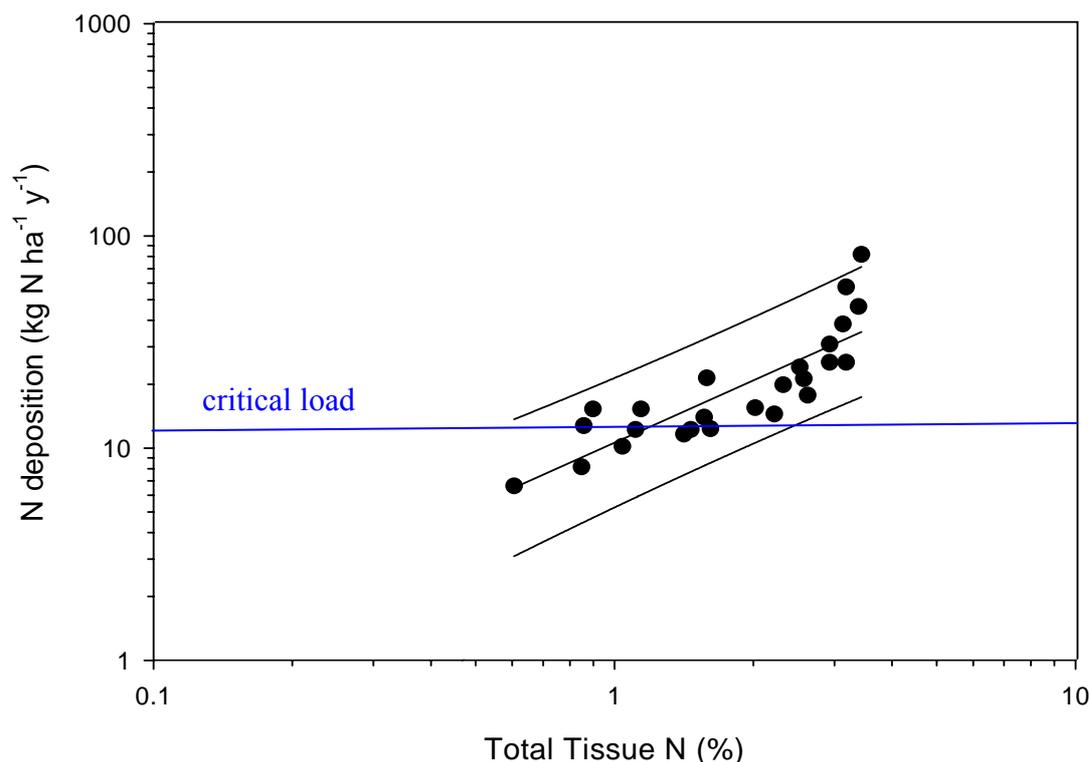


Figure 3.6: Plot showing the application of the relationship between foliar N and total N deposition for bryophytes (data from Figure 3.5) to estimate N deposition based on measurement of foliar N content at a site. To allow 95% confidence limits of the population to be calculated a simple power regression of the form $F_N = a L_N^b$ is applied here. Supposing an empirical critical load for a habitat of $12.5 \text{ kg N ha}^{-1} \text{ yr}^{-1}$, this relationship would imply the critical load to be exceeded with $L_N > 1.2\%$, while $L_N > 2.3\%$ would indicate deposition significantly (95% confidence) above the critical load. Refinement of the N response function to that used here would be expected to reduced the confidence limits for the same dataset.

3.5.4. Refinements of the UK model

The UK model is being further developed in order to answer important questions:

- Does tissue N respond to dry and wet deposited nitrogen and to oxidized (NO_y) and reduced nitrogen (NH_x) in the same way?

Studies in progress under the GANE Thematic Programme suggest that tissue N does respond differently to different forms of atmospheric N (Pitcairn *et al.*, unpublished data). Mosses were collected along a gradient of wet deposited N (NH_4^+ and NO_3^-) at Great Dun Fell in the northern Pennines in November 2000 and May 2002. On both occasions, tissue N, averaged for four species, ranged from 1.0 to 1.35 %N for an estimated N deposition range of 20-50 kg

$\text{N ha}^{-1} \text{ yr}^{-1}$. By contrast, the tissue N of mosses collected along a gradient of NH_3 concentration downwind of a poultry farm ranged from 2.0-4.8 %N for the same N deposition range (Pitcairn *et al.* in press) although other factors such as species, growth rates and rainfall were not equal.

These initial findings are supported by results from open-top chamber experiments where ombrotrophic mire vegetation was exposed to either a range of NH_3 concentrations or to NH_4^+ (applied as NH_4Cl solutions). *C. vulgaris*, and *P. commune* showed a significant tissue N response to increasing N additions and also to the form of N applied (Leith *et al.* 2001). Both species exhibited greater N uptake when N was supplied as NH_3 rather than as NH_4^+ on a per unit N basis. This is consistent with the findings of van der Eerden *et al.* (1990), who suggest that NH_3 taken up through the foliage is assimilated more quickly than NH_4^+ or NO_3^- absorbed via the cuticle or roots. Dueck *et al.* (1991) found little or no NH_3 adsorbed to the leaf surface of either graminoid or ericaceous species, and hence it was suspected (at the high concentrations of that study) that the uptake pathway for NH_3 is through the stomata and not through the leaf surface. By contrast, it is possible that uptake also occurs via the leaf surface, but that because the transport into the plant in the aqueous phase (either through cuticular channels or through water microfilms on the surface of stomatal guard cells) is very effective so that the amount stored on the surface is small compared with the total NH_3 deposited (Sutton *et al.* 1995).

Interestingly, workers from southern Europe (Gaio-Oliveira *et al.* 2001) also found that N concentrations in the lichens studied were more closely related to dry deposition than wet deposition and more closely influenced by reduced N than oxidised N. This is in contrast to some lichen studies from northern Europe, where closer links were found with NO_y concentrations and wet deposition (Hyvarinen and Crittenden 1998). (See section 5 for further discussion).

- What is the characteristic response time (+ve and -ve) for foliar N content?

The episodic exposure of plants, mosses in particular, to wet or dry deposited N may lead to fluctuations in tissue N content if the characteristic response time of the plants is shorter than the temporal variability of exposure. The poultry farm studies, while always showing a gradient in tissue N in relation to N deposition, do not always show the same absolute values of tissue N. Depending on the stages in the poultry cycle, wind direction etc, ammonia concentrations will vary considerably and it is possible that tissue N values integrate over a shorter period than one year. Experiments carried out in Open-Top Chambers where plants received dry deposited NH_3 , showed that tissue N concentrations of *Sphagnum capillifolium* responded to added NH_3 (at above $90 \mu\text{g m}^{-3}$) in less than 10 days. After four weeks of exposure to N, the moss mats were allowed to recover in a chamber receiving natural rainfall (containing small amounts of N) and filtered air. Tissue N immediately declined but then increased again, suggesting recycling within the moss mat (Pitcairn *et al.* in press). Control plants which did not receive NH_3 showed a temporary increase in N on receiving rainfall in the recovery chamber. Although seasonal variability in tissue N is well known (with lower values during the main growing season), these results suggest that temporal variability in exposure may also have a significant effect on the values of tissue N recorded.

- In high rainfall areas, does tissue N respond to concentration or deposition?

It is clearly difficult to separate the effects of concentration and deposition. Studies in progress in the field and laboratory under two GANE projects aim to address the above question. This requires an assessment of the importance of episodicity of exposure, for situations of the same nitrogen deposition. An increase in response for more episodic

exposure (i.e. a few high concentration events), compared with continuous exposure to low concentrations, may point to the importance of concentrations as well as deposition.

3.6. Evidence for links between tissue N and effects on plants

Extensive forest studies in the Netherlands found a close link between tree damage and tissue N content of the needles in areas with high atmospheric N deposition particularly as NH_3 . Tissue N concentrations exceeded optimal levels for tree growth (van Dijk and Roelofs 1988) and the highest concentrations were found in damaged trees with yellowing needles. High N deposition frequently results in large nutrient imbalances in trees (van Breemen and van Dijk 1988). Similar connections between N deposition and tissue N have been made for bryophyte species. For example, Baddeley *et al.* (1994) linked deposition related changes in tissue N content of *Racomitrium* with the deterioration of *Racomitrium* heath in Britain during the last 50 years.

Transplantation studies (Mitchell *et al.* –see section 9) of epiphytic bryophytes between Atlantic oak woods on the west coast of Britain, have also indicated a detrimental impact (reduced growth) of increased N deposition on the bryophyte species studied, that was coupled with an increase in tissue N content. In addition, the reverse transplantation from polluted to clean conditions, both showed decreased tissue N content and for one species an improvement in growth rate. The recovery effects (growth and reduced tissue N) were less than the responses to increased exposure within the one year of observation, suggesting that recovery is slower than damage (Mitchell *et al.*, in press).

While these examples serve to show a link between the tissue N response to atmospheric N deposition and plant effects, the potential to use tissue N as a bioindicator of effects depends on quantifying the relationships with different effects. Much more work is required in this area, which requires the parallel assessment of tissue N and ecological responses. A key target of scientific studies linked to critical loads has been to identify the atmospheric deposition rate at which ecological changes are observed (Bobbink *et al.* 2003; Sutton *et al.* 2003a). The need to improve bioindicators for nitrogen extends this to identify the tissue N concentrations at which other changes are observed.

3.7. Foliar tissue N: Conclusions and application

Foliar tissue nitrogen represents perhaps the most studied bioindicator for atmospheric nitrogen deposition. While the literature is extensive and there are clearly established relationships, there is still debate as to the quality of the indication of either atmospheric deposition or ecological effects. Part of this may be addressed through the use of standardized protocols, understanding of species differences and the use of more robust site-specific deposition estimates. At the same time the further use of statistical uncertainty assessment is needed, so that a quantitative estimate of deposition ranges and comparison with estimated critical loads can be made.

In terms of practical application of the method, particular consideration needs to be given to the choice of species, timing of sampling, and standardization of the plant parts to be sampled. The key points are summarized below, with further information provided in the method data tables (Appendix II of this report).

3.7.1. Choice of species

- Species selected for monitoring must demonstrate a wide range of tissue N concentrations and be able to respond to small changes in N deposition at least within one growing season.
- Foliar N contents of tree species are well buffered from change by recycling and storage processes and unlikely to respond rapidly to changes in deposition. However trees may provide useful biomonitors of long-term change. Further work is required to characterize the response time for tree foliage and differentiate between deciduous and evergreen species.
- Herbaceous perennial plants may also be unsuitable due to recycling and storage in underground organs, but fast growing annual species may be suitable biomonitors particularly if grown from seed under uniform conditions (see section 10).
- Ericaceous shrubs such as *Calluna* tend to have low N requirements and the tissue N of these plants has been shown to respond to N additions. There is also a large body of data available on tissue N in *Calluna* published in grazing and heathland conservation studies, as well as gathered during the GB ‘Countryside Survey 2000’.
- Ectohydric mosses are thought to be the most sensitive and the most suitable group for biomonitoring the plant tissue N response to atmospheric N deposition. This is both because of the absence of a cuticle and small plant size (leading to shorter characteristic response time) and in most cases the dependence on the atmosphere rather than the soil for nutrients.
- Non-nitrogen fixing lichens are also largely dependent on atmospheric N for nutrients. As with bryophytes, the tissue N of lichens shows a clear response to atmospheric N deposition, although there are less data available. However, because of the habit of many species, being closely appressed to twigs or stones, sample preparation for analysis can be time-consuming and imprecise. There is, however, potential for further testing of fruticose lichens where these occur (including *Cladonia* species), which are free standing and easily collected.

3.7.2. When to sample

- The optimum time for sampling evergreen foliage from trees is after growth has stopped and carbohydrate accumulation is on the decrease i.e. between November and February. This is important because starch accumulation increases dry weight, effectively ‘diluting’ the nutrient concentrations (Linder 1995). Samples should not be taken too near bud burst from April onwards, as, at this time, much of the N has been remobilised to supply the developing bud. Deciduous trees should be sampled during the growing season and July is probably optimal.
- Sampling procedures for *Calluna* must be determined by its ontogeny. Tissue N concentrations of *Calluna* are largest in young plants, declining to a steady low level after about six years (Miller & Miles, 1969; Robertson & Davies, 1965). There is also seasonal variation, with N concentrations being largest in early summer and at a minimum in winter, although the difference is negligible in mature *Calluna* plants (Thomas, 1937; Brunsting & Heil, 1985). Differences also occur within the plant, the largest concentration being found in the young leaves (Robertson & Davies, 1965). Based on this information, sampling should be conducted during the winter months (later at high altitude exposed sites).

- UK studies have generally found that moss growth and nutrient uptake is greatest in the moist, cool conditions of spring and autumn. Studies in N. America on *Sphagnum* species suggest that the N retention is greatest in the warm, moist, conditions of early summer. In most areas of the UK, ideal sampling periods are likely to be in spring and autumn, although periods of drying winds in early spring and possible late drought in early autumn should be avoided.

3.7.3. What to sample

- For coniferous trees, samples should be removed from the upper crown from shoots growing in the light (shaded leaves have atypically high N to compensate for the lower light levels). For deciduous trees, some species grow in flushes e.g. Beech while others are more indeterminate e.g. Birch, hence fully expanded leaves should be removed at around 4 or 5 leaves behind the growing tip.
- Only mature *Calluna* plants should be sampled and when possible large composite samples should be collected at each site. Either the entire annual increment or a standardized fixed length of the annual increment should be selected for analysis.
- For mosses, actively growing green material should be sampled using a fixed length of shoot. For *Sphagnum* sampling, particular care is necessary: tissue N concentrations are much larger in the capitulum and fall off sharply down the shoot with distance from the apex (Aldous 2000). Sampling should include just the capitula, or a standardized fixed short length of shoot. Periods of drought should be avoided as N retention may be reduced under dry conditions (Aldous 2000).
- There is evidence that tissue N concentrations in shoot tips of some species (e.g. *Rhytidiadelphus triquetrus*) are influenced by the length of the shoot (D Brown pers comm) and hence care should be taken when selecting moss clumps for sampling.
- Lichen shoots have also been demonstrated to show a gradient in tissue N with distance from the growing apex. Hyvarinen and Crittenden (1998) found tissue N to be 2-5 times larger in the apices than in the basal strata of *Cladonia portentosa*. This again points to the need to use standardized sampling procedures.
- Shaded plants, particularly vascular plants, should be avoided. Shading tends to result in etiolation of shoots, lower shoot densities and cessation of flowering. More importantly it results in increased concentrations of foliar N. Iason & Hestor (1993) found an increase of more than 50% foliar N when *Calluna* moorland was shaded with netting. The netting reduced light levels to those measured under dense young birch stands. Sampling procedures for other ericaceous shrubs should follow similar lines.
- Tissue N concentration can be affected by the presence of fast growing competitors (Malcolm Cresser, pers. comm.), and hence it is preferable to collect samples from the centre of large clumps or mats of the selected species.

4. BIOINDICATOR METHODS BASED ON FOLIAR NITROGEN ACCUMULATION: OTHER NITROGEN PARAMETERS

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4.1. Introduction

While total foliar nitrogen concentration is by far the best studied accumulation-based bioindicator for nitrogen, there are a wide range of methods that consider other aspects of nitrogen accumulation. In addition to total N content of foliage, bioindicator approaches include the analysis of foliar amino acids, total soluble nitrogen compounds and foliar ammonium concentration. Nitrogen to phosphorus ratios have been used to indicate relative nutrient limitation by N or P, while the analysis of natural ^{15}N vs ^{14}N isotopic signatures provides a method that can help understand N cycling processes and identify the source of N deposition. Each of these methods is considered here in turn.

4.2. Free amino acids of higher plants and bryophytes

4.2.1. Background

When N availability increases, and particularly when other nutrients are in short supply, N may be taken up by plants in surplus and accumulated rather than used in biomass production. At a cellular level, if protein is not synthesized, NH_4^+ is assimilated into specific N metabolites of which free amino acids are particularly important. Thus accumulation of amino acids could potentially be used to monitor N impacts on vegetation. Only a few amino acids are important in most species and these include the amides, asparagine and glutamine, and guanidine compounds such as arginine. The N:C ratio is greatest in arginine making it the most C cost effective amino acid for storage of excess N. Arginine is the major storage amino acid in many coniferous tissues during most of the year, but proline or glutamine may periodically become dominant (Näsholm & Ericsson 1990). In general, species typical of N-rich habitats tend to accumulate asparagine whereas those more typical of N poor habitats accumulate arginine. For example, in a study of nine boreal ground flora species, Nordin and Nasholm (1997) found that arginine dominated the amino acid pool in six species, arginine and asparagine were co-dominant in two species and glutamine alone dominated the pool in one species. Concentrations of free amino acids may be affected by several environmental factors including nutrient deficiencies and form of nitrogen available and the sites of storage also vary between species. For example, working with *Picea glauca* and *Pinus banksiana*, Durzan and Steward (1983) report that low K levels led to accumulation of glutamine, whereas P deficiency in spruce resulted in storage of arginine. Thus amino acid accumulation might be regarded as a non-specific indicator of perturbation of nutrient availability.

4.2.2. Evidence for links between amino acid accumulation and elevated N deposition

Examples of amino acid accumulation include early studies of N saturation and forest decline in the Netherlands, where damaged trees were found to contain high concentration of the free amino acid arginine (van Dijk and Roelofs 1988). Several experiments have confirmed the link between enhanced N deposition and arginine concentrations. For example, when N deposition to the forest floor was reduced to an estimated 1-2 kg N ha⁻¹ y⁻¹ in the NITREX manipulation experiment in Speuld forest in the Netherlands, arginine concentrations in coniferous needles declined 3-fold within four years (Boxman *et al.* 1995). Another example is the very large reduction in arginine concentrations with increasing distance from a fur farm in Finland and a poultry farm in Scotland (Pietila *et al.* 1991, 2003). Extensive studies in Sweden have demonstrated that excess N availability either from N fertilization or atmospheric deposition leads to accumulation of N in free amino acids in a range of species and that large concentrations of amino acids may indicate high N availability. For example, it has long been known that forest trees accumulate amino acids in response to increased N inputs (Näsholm and Ericsson, 1990). Näsholm *et al.* (1994) found that exposure to large concentrations of N increased the accumulation of amino acid N (as arginine, asparagine, glutamine and others) in a range of forest plants including bryophytes, and suggested that concentrations of specific amino acids in a range of plants may be used to indicate atmospheric N deposition. Swedish studies on a similar range of forest plants also showed that fertilisation with NH₄⁺-N resulted in larger increases in amino acid N (and also total N) than with NO₃⁻ (Ohlson *et al.* 1995). More recent work by Nordin *et al.* (1998) showed that changes in the composition of the amino acid pool of certain species may also indicate a response to increased N supply.

A response to concentration of applied NH₄⁺ rather than deposition was indicated by the work of Baxter *et al.* (1992). They measured a rapid response in amino acid concentrations in two populations of *Sphagnum cuspidatum* following addition of 0.1 and 1.0 mM NH₄⁺ in the laboratory. The population, which was taken from a relatively clean, remote site in Migneint, N Wales, showed a dramatic increase in glutamine (3-fold), arginine (19-fold) and asparagine (4-fold) after 20 days exposure and a reduction in growth. A contrasting population from Holme Moss, a polluted site in the southern Pennines of northern England, showed very much smaller changes in amino acid concentrations, but a growth stimulation following addition of both 0.1 and 1.0 mM NH₄⁺.

More extensive measurements of amino acid response to enhanced N deposition were carried out downwind of a poultry farm in southern Scotland (Pitcairn *et al.* 2003). Free amino acid concentrations were determined in three moss species (*Rhytidiadelphus triquetrus*, *Brachythecium rutabulum* and *Pseudoscleropodium purum*) in late autumn (initial pilot experiments carried out in July having confirmed the seasonality of amino acid accumulation in plants) and showed a strong relationship with distance from the poultry buildings and hence with NH₃ concentrations. Arginine was the dominant amino acid at high concentrations close to the buildings in the three species, but was especially dominant in *R. triquetrus*, representing 40% of total free amino acids (Figure 4.1). The linear regression of arginine concentration with log distance from the poultry farm gave values for r² of > 0.95 for the three species demonstrating the potential for arginine accumulation in moss as an indicator of enhanced N deposition.

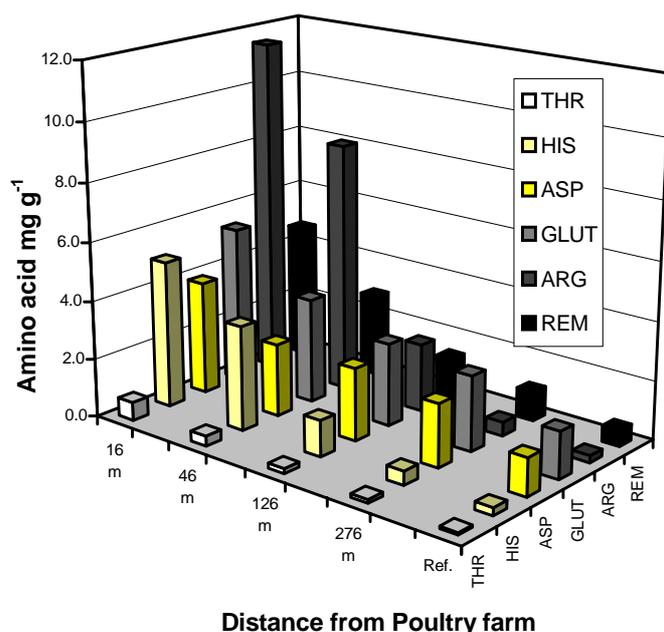


Figure 4.1: Free amino acid concentrations in *Rhytidiadelphus triquetrus* at 4 distances downwind of the poultry farm and a ‘clean’ reference site, November 2001. (THR-threonine, HIS-histidine, ASP-aspartic acid, GLUT-glutamic acid, ARG-arginine, REM- remaining amino acids) (From Pitcairn *et al.* 2003)

4.2.3. Composition of the amino acid pool

On the basis of accumulation of particular amino acids, the relative abundance of different amino acids may be used to provide an indication of enhanced nitrogen deposition. The potential advantage of this measure is that information on the absolute amounts of amino acids is not necessary.

Changes in the composition of the amino acid pool in response to changes in ammonia concentrations downwind of the poultry farm examined by Pitcairn *et al.* (2003) are shown in Table 4.1. The composition of the amino acid pool at 276 m downwind of the farm, where NH₃ concentrations are similar to local background levels, showed a co-dominance of aspartic acid and glutamic acid in the three moss species examined, with arginine comprising < 8%. Closer to the farm buildings (46 m), the composition of the amino acid pool changed from aspartic acid and glutamic acid dominance to arginine dominance in *R. triquetrus* and *P. purum*, whereas in *B. rutabulum*, aspartic acid was still dominant. At a distance of 16 m from the poultry buildings, where measured NH₃ concentrations were largest, the amino acid pool was dominated by arginine, especially in *R. triquetrus*. Clearly a change in dominance from aspartic acid and glutamic acid to arginine would denote a change from low to high N deposition and hence may be indicative of enhanced N deposition for these species. The different uptake strategy of *B. rutabulum*, compared with those of *P. purum* and *R. triquetrus*, and the arginine dominance of the amino acid pool only close to the poultry buildings, may partly explain its frequency close to sources of N and its ability to accumulate tissue N.

The significance for biomonitoring of aspartic acid and arginine in the amino acid pool of mosses of boreal forest ground flora was demonstrated by Nordin *et al.* (1998). They found that simulated N deposition (added as NH₄NO₃) significantly increased the three dominant amino acids, asparagine, glutamine and arginine in the bryophytes *Pleurozium schreberi* and *Dicranum scoparium*. Arginine and asparagine together dominated the pool. A similar pattern was found in the grass, *Deschampsia flexuosa*. However, in response to increased N

supply, the composition of the amino acid pool in *D. flexuosa* showed a shift from arginine dominance to asparagine dominance, typical of nitrophilic species. Nordin *et al.* (1998) hypothesized that changes in the amino acid pool composition of the grass *D. flexuosa* in response to N addition could explain the competitive success of this species following enhanced N deposition and that changes in the composition of the amino acid pool may indicate increased N deposition at a site before any one amino acid showed a large increase and before impacts to vegetation could be seen. These studies in boreal forest under-storey vegetation using intermittent N fertilizer additions have been broadly confirmed by the studies of Pitcairn *et al.* (2003) for a mixed-woodland where NH₃ deposition was semi-continuous.

Table 4.1: Percentage contribution of 3 key amino acids to the amino acid pool of example mosses at 3 distances downwind of a poultry farm. (Asp-aspartic acid; Glu – glutamic acid; Arg – arginine; Oth – other amino acids) (Pitcairn *et al.* 2003)

Moss species	% contribution of the noted amino acid to the total amino acid pool for each species at the sampling point											
	276 m				46 m				16 m			
	Asp	Glu	Arg	Oth	Asp	Glu	Arg	Oth	Asp	Glu	Arg	Oth
<i>Rhytidiadelphus triquetrus</i>	31.6	37.7	7.1	23.6	11.6	16.8	40.0	31.6	12.5	17.3	37.1	33.1
<i>Pseudoscleropodium purum</i>	36.9	34.7	4.5	23.9	14.8	15.3	29.6	40.3	14.5	17.3	23.7	44.5
<i>Brachythecium rutabulum</i>	34.5	23.5	7.3	34.7	23.0	14.6	16.0	46.4	13.8	13.5	23.6	49.1

4.2.4. Evidence for links between amino acid accumulation and impacts on vegetation

Dutch studies showed that elevated arginine concentrations were not only linked to large N inputs, but also to visible tree damage and pathogen attack (see review of Sheppard and Wallender 2004). A similar link, between pest and pathogen attack and amino acid concentrations, was demonstrated by Nordin *et al.* (1998) for *Vaccinium myrtillus*. Damage to current annual shoots by lepidoptera larvae and the incidence of the parasitic fungi, *Podosphaera* and *Valdensia* on leaves increased, following simulated N deposition to boreal coniferous forests. The authors attribute the increased susceptibility of the plants to raised levels of amino acids in leaves and shoots following the N additions.

4.3. Substrate nitrogen and foliar ammonium

4.3.1. Background

Substrate nitrogen and foliar ammonium represent newly recognized parameters in the context of the bioindication of atmospheric nitrogen concentrations and deposition. The interest in these parameters originates from the analysis of plant carbon and nitrogen dynamics in ecosystem models in relation to the potential for plants to absorb or emit ammonia from the atmosphere, as regulated by the ammonia ‘compensation point’.

Nitrogen in plant tissues may be distinguished into two functional pools: substrate N and structural N (Riedo *et al.* 1997, 2002): substrate N is the nitrogen available for plant growth,

existing in the form of a range of soluble compounds, such as amino acids, ammonium, amines, soluble proteins (and to a much lesser extent nitrate). Following the photosynthetic production of substrate C (i.e. sugars etc), substrate C and N are used to build the plant structure, consisting of insoluble compounds. In parallel with the model development by Riedo *et al.* (2002), field studies have provided the first protocols to estimate 'substrate N' – as total soluble plant N (Hill 1999, Loubet *et al.* 2002), with studies at the same time determining the total foliar ammonium concentration as an indicator of substrate N. The latter was also investigated to assess its relationship to the concentration of ammonium in the apoplast, which, with apoplastic pH, allows calculation of $\Gamma = [\text{NH}_4^+]/[\text{H}^+]$, which is the temperature normalized scalar for the ammonia compensation point (Sutton *et al.* 2001). Given the complexity of determining Γ from bioassays (Husted and Schjoerring 1996, Hill *et al.* 2001), there is significant interest in using bulk foliar NH_4^+ as a bioindicator of the compensation point.

It is well established that the ammonia compensation point is a function of both agricultural nitrogen inputs (Loubet *et al.* 2002, Riedo *et al.* 2002, Mattsson and Schjoerring 2002) and, for semi-natural communities, the atmospheric N inputs (Sutton *et al.* 1995, Schjoerring *et al.* 1998). This points to the existence of a negative feedback on atmospheric ammonia inputs, where enhanced nitrogen deposition leads to an increase in apoplastic ammonium, raising the ammonia compensation point and providing a limitation to further nitrogen deposition (Sutton *et al.* 1993, 1995). In this context, the rates of atmospheric ammonia deposition, and hence total N deposition, depend partly on the extent to which the ecosystem has already responded to N deposition. A reduced rate of measured NH_3 deposition may therefore be an indication that the critical load is already exceeded. As a consequence of these interactions, the level of substrate N or foliar NH_4^+ may provide a convenient indicator of accumulated nitrogen deposition and ecosystem response.

The same principles that lead to accumulation of total tissue N and amino acids apply to substrate N. The substrate N method is expected to be more sensitive than total tissue N, since it is the substrate N pool that undergoes the primary variation with N supply. Secondly, by considering all forms of soluble N, the substrate N approach is more general than the analysis of amino acids, since different plant species accumulate different amino acids. The linkage to free foliar ammonium is expected since ammonium is a basic precursor for amino acid production.

4.3.2. Evidence for links between substrate N, foliar ammonium and elevated nitrogen deposition

The first measurements of free foliar ammonium were made in the context of providing a simple bioindicator of apoplastic ammonium. Hill *et al.* (2001) demonstrated a positive correlation between the two parameters in wood rush (*Luzula sylvatica*), with both parameters increasing with elevated N supply (Hill 1999). These relationships were subsequently shown to hold for a fertilized *Lolium perenne* sward in the field (Loubet *et al.* 2002). However, in this case the evidence pointed to apoplastic ammonium being a more quickly varying parameter in response to N deposition events, which is consistent with its smaller pool size than foliar ammonium or total substrate N (Reido *et al.* 2002). Responses have also been observed for standardized *L. perenne* biomonitor plants set out in a transect away from a poultry farm (Picairn *et al.* 2004a, see Appendix I), where foliar ammonium increased in the range 30-80 $\mu\text{g/g}$ FW linearly to the log of NH_3 concentration.

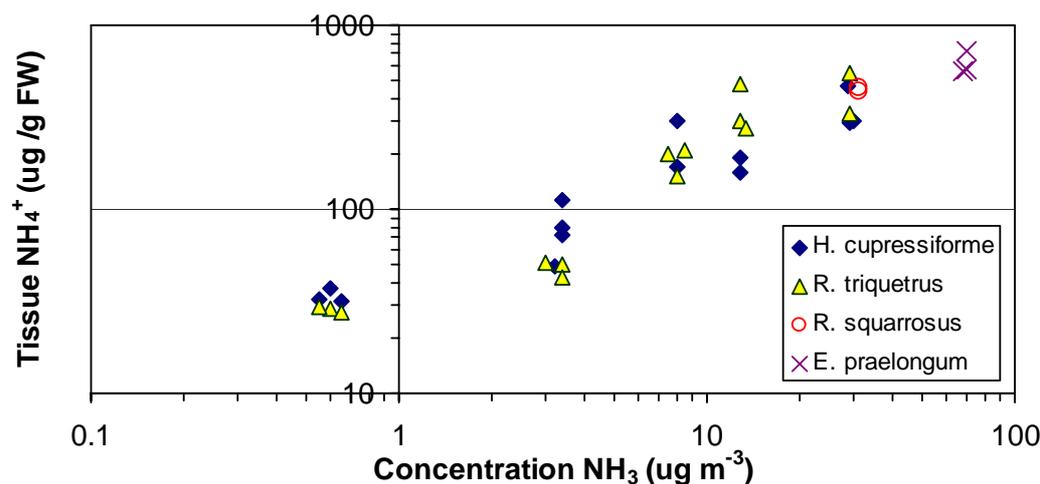


Figure 4.2: Relationship between measured foliar NH_4^+ concentration of pleurocarpous mosses and NH_3 concentration from a woodland in the vicinity of a poultry farm (Picairn *et al.* 2004a, see Appendix I). *Hypnum cupressiforme* and *Rhytidadelphus triquetrus* were selected to investigate changes along the transect. At sites where these species were in short supply or absent, *R. squarrosus* or *E. praelongum* were also sampled.

The largest observed responses of foliar ammonium and soluble nitrogen, however, have been recorded in pleurocarpous mosses downwind of the poultry farm assessed by Picairn *et al.* (2004a, see Appendix I). Foliar ammonium showed an increase from values at 20-40 $\mu\text{g/g}$ FW at background NH_3 concentrations up to values of >500 $\mu\text{g/g}$ FW immediately adjacent to the farm. Although the different moss species were associated with different characteristic foliar NH_4^+ values, these differences were consistent with the different N supply at the locations where the species were sampled. This is illustrated in Figure 4.2, which shows, for example, that *Eurynchium praelongum* was only sampled at the location with the largest atmospheric NH_3 concentration (because of the absence of the other species) and had the highest foliar NH_4^+ concentration.

The relative performance of foliar NH_4^+ as a bioindicator is compared with the estimate of substrate nitrogen and total tissue nitrogen in Figure 4.3. In all three foliar parameters, the higher NH_3 concentrations resulted in higher N levels in the plants. What is of particular interest is that the lower the concentration of the parameter, the larger was its response to NH_3 . At 1 $\mu\text{g m}^{-3}$ of NH_3 the values for foliar NH_4^+ , soluble N and total foliar N were 35, 900 and 11000 $\mu\text{g g}^{-1}$, while the response across the full range of NH_3 exposure was a change by a factor of 21, 5.5 and 3.5, respectively. These differences are consistent with the idea that a smaller pool size is likely to show larger response to perturbation in nitrogen input (Riedo *et al.* 2002). Of the different parameters observed by Picairn *et al.* (2004a, Appendix I) the correlation was better and the response larger for foliar NH_4^+ than substrate N.

4.3.3. Timescales of plant response to N and seasonality

As recently recognized parameters, there is much less experimental data at present on the time-scale of responses to N input and the seasonality in substrate nitrogen and foliar NH_4^+ than for total foliar N. Available laboratory work shows that foliar NH_4^+ may be expected to respond within a few days to a sudden increase in nitrogen supply (Mattsson and Schjoerring 2002) and this is consistent with the field measurements of Loubet *et al.* (2002). In the context of biomonitoring over short periods, such as following the establishment of a new N source, this may make foliar NH_4^+ of particular interest in order to detect changes rapidly.

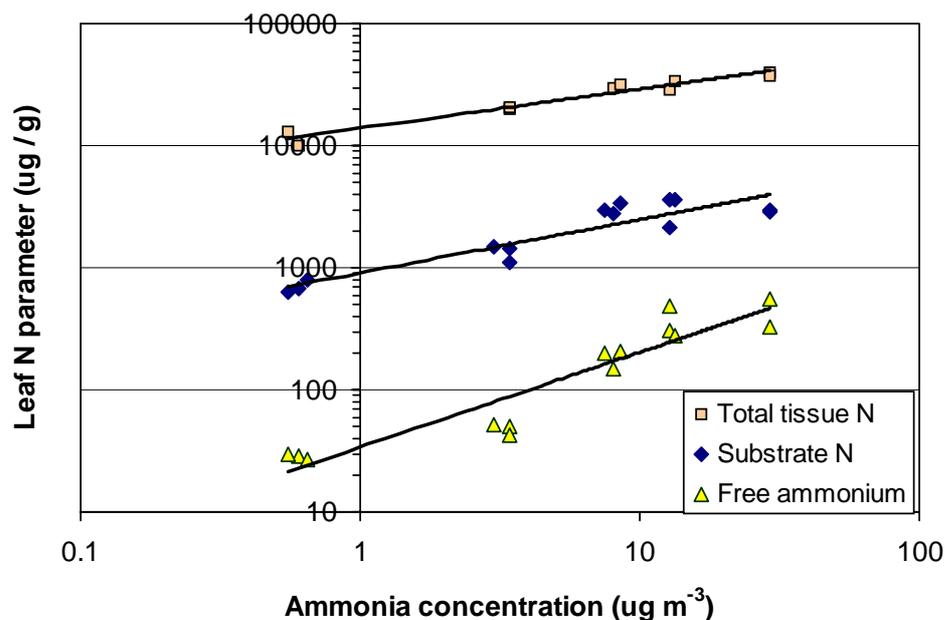


Figure 4.3: Comparison of three bioindicator methods for nitrogen for the moss *Rhytidiadelphus triquetrus*: total tissue nitrogen, substrate N and foliar NH_4^+ (Picairn *et al.* 2004a, see Appendix I). The largest relative response to NH_3 is shown for foliar NH_4^+ , which is consistent with it representing the smallest nitrogen pool of the three measures.

The concomitant of a high temporal sensitivity is that there may be a large seasonal variability in substrate N and foliar NH_4^+ concentrations. Such seasonality has been demonstrated in relation to field fertilization events in grassland (Loubet *et al.* 2002, Riedo *et al.* 2002), but this situation is very different from the situation of long-term atmospheric inputs to forests and semi-natural ecosystems. At present the available data for bryophytes are for autumn, and further measurements are required to investigate the seasonality of these parameters in situations where atmospheric inputs remain approximately constant throughout the year.

4.3.4. Estimating atmospheric N deposition and effects from foliar NH_4^+

As with total tissue N, substrate N and foliar NH_4^+ can be used as both indicators of atmospheric N deposition and ecological impacts. The limited available data suggest that the relationship to atmospheric N deposition is more precise (or can be measured more precisely) for foliar NH_4^+ than for substrate N (e.g. Figure 4.3). At present there is a requirement for more data to make the link between these parameters and plant community changes. However, estimates of atmospheric N deposition may be made and these compared with current nitrogen critical loads (Achermann and Bobbink 2003), as seen for total foliar N (Figure 3.6).

Figures 4.2 and 4.3 show that the log-log plots of NH_3 concentration versus foliar NH_4^+ is not completely linear. While it is feasible that this is a natural plant response, it can easily be explained by the non-linear relationship between NH_3 concentrations and total N deposition, with the biological response being proportional to N deposition. Firstly, background N deposition needs to be considered in the relationship, which accounts for the non-linear response at low NH_3 concentrations. Secondly, at high NH_3 concentrations there is a tendency for the deposition velocity to reduce due to a partial saturation of cuticular uptake (Sutton *et al.* 1993, Fowler *et al.* 1998), explaining the non-linearity at high NH_3 concentrations.

Two approaches can be taken to address this phenomenon. One is to estimate independently the background N deposition and the cuticular saturation and account for these in estimating N deposition. An alternative, however, is to identify the atmospheric N deposition and cuticular saturation rate that would best linearize the relationship of Figure 4.3 at the low and high ends, respectively (Pitcairn *et al.* 2004a, see Appendix I). Firstly, the value of background atmospheric N deposition is identified that would linearize the response to N deposition at low input rates. Secondly, a saturation parameter to modify the canopy resistance (R_c) is identified that linearizes the response to N deposition at very high input rates. By applying this linearization approach, the background (non-NH₃) deposition to the woodland site of Pitcairn *et al.* (2004a, see Appendix I) was fitted as 11 kg N ha⁻¹ yr⁻¹, while a saturation parameter (S) was fitted to be a value of 1, where canopy resistance (R_c) in s m⁻¹ is calculated as $10 + \chi_a^S$ and R_a+R_b are taken as 20 s m⁻¹. These values turn out to be in reasonable agreement with the estimated background deposition to the site (approximately 10-15 kg N ha⁻¹ yr⁻¹) and the observations of cuticular saturation in open top chamber studies by Leith *et al.* (unpublished data).

Based on these estimates, the relationship between foliar NH₄⁺ and atmospheric N deposition can be used to apply foliar NH₄⁺ of bryophytes as a bioindicator of N deposition. As with the similar procedure for total foliar N (Figure 3.6), by setting N deposition as the dependent variable, confidence limits of atmospheric N deposition may be determined from observations of foliar NH₄⁺ in pleurocarpous mosses.

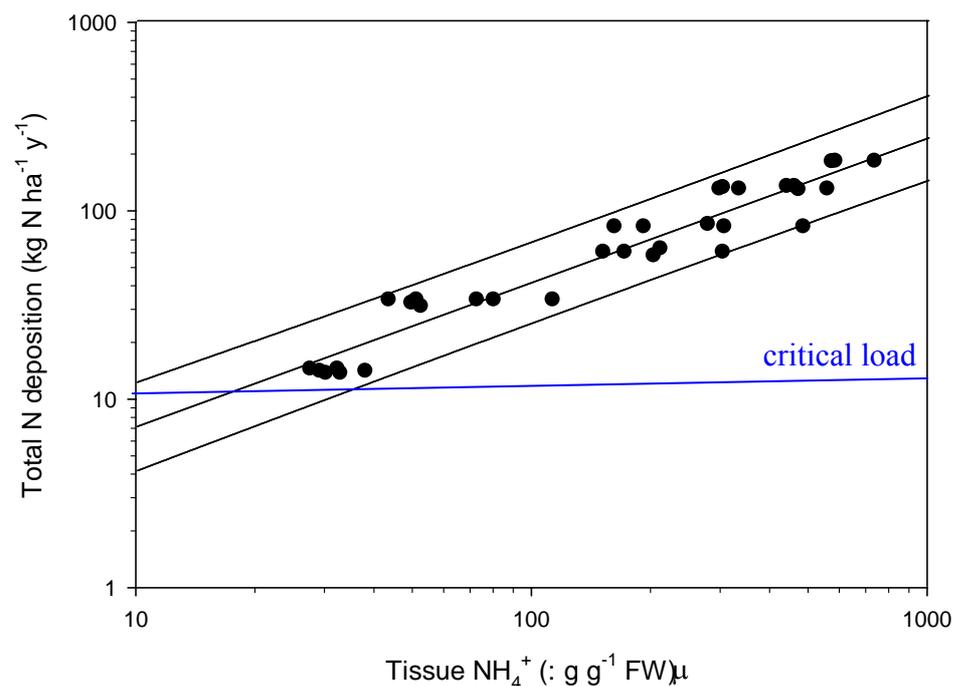


Figure 4.4: Relationship between estimated total N deposition and measured foliar ammonium of several pleurocarpous moss species under a woodland canopy in the vicinity of a poultry farm (Pitcairn *et al.* 2004a, see Appendix I). 95% confidence limits of the population are shown, and demonstrate how a measured estimate of moss foliar ammonium can be used to estimate whether the atmospheric deposition at a site is significantly greater than the critical load. The estimate of total N deposition includes a tendency for the deposition velocity (V_d) to reduce at large NH₃ air concentrations as calculated by Pitcairn *et al.* (2004a, see Appendix I). The regression estimates total N deposition as $F_N = 1.5106 (L_{NH_4^+})^{0.7398}$; $R^2 = 0.922$.

This is illustrated in Figure 4.4, which also compares the results to an empirical critical load for nitrogen, set here at an indicative value of 12.5, which is the mid point of the empirical critical load for deciduous woodland of 10-15 kg N ha⁻¹ yr⁻¹ (Achermann and Bobbink 2003).

In this example, a foliar NH_4^+ concentration in pleurocarpous mosses of $>40 \mu\text{g g}^{-1}$ FW would be equivalent to atmospheric N deposition significantly larger than the critical load (95% confidence limits).

4.4. Nitrogen to Phosphorus ratio of plant tissues

4.4.1. Background

Where plant growth may be limited by P availability or when enhanced N deposition may lead to reduced P availability and/or uptake, measurements of the N:P ratios in selected species may provide a useful indication of N saturation. Optimum N:P ratios for plant growth range from 10-14 (van den Driesshe, 1974; Ingestad, 1979). Low ratios (<10) indicate N limited growth and high ratios (>14) indicate P limitation (Koerselma & Meuleman, 1996).

4.4.2. Evidence for links between N:P and N deposition

In areas of southern Sweden with high inputs of atmospheric N, *Sphagnum* growth was found to be P limited and N:P ratios of around 34 were measured in *Sphagnum capitula* (Aerts *et al.* 1992). Conversely in northern Sweden where N inputs are small, *Sphagnum* growth was limited by N availability and N:P ratios were very small at around 6.

Long-term increases in atmospheric N deposition has led to increased foliar N levels in both coniferous and deciduous tree species. In addition to enhanced foliar N, Duquesnay *et al.* (2000) found reduced concentrations of P, K and Mg in beech stands in north-eastern France, leading to increases in ratios of N:P, N:K and N:Mg of 42, 19 and 77, respectively. High N deposition may affect P availability in soils through acidification and perturbations of mycorrhizal activity. Plants showing nutrient imbalance and large N:P and N:Mg ratios exhibited a greater sensitivity to damage by parasites, frost and water stress.

By contrast, the decrease in foliar N of *Calluna* with increasing distance and decreasing NO_x concentrations from the centre of London (Power and Collins, *pers comm.*) was not matched with a decrease in N:P ratio. In fact N:P ratios increased significantly with increasing distance from London presumably due to the higher availability of P in urban areas. Ratios for the transect sites fell between 9 and 14 indicating that neither N nor P was limiting.

4.4.3. Evidence for links between N:P and impacts of N deposition

Biodiversity and species richness have also been related to N:P ratios (Ertsen *et al.*, 1998). In Dutch experiments using grassland and heathland sites, where N:P ratio in the vegetation exceeds 16, biodiversity was reduced, while a balanced nutrient supply ratio with co-limitation of N and P favours biodiversity (Roem & Berendse, 2000). Increased N:P and N:K ratios resulting from N deposition may therefore seriously threaten species richness in nutrient-poor grassland and in heathlands.

In the UK, most upland soils are P limited and calcareous soils are frequently limited by both P and N. Hence increased N deposition may increase the N:P ratio without growth increasing. Additions of NH_4NO_3 to calcareous grassland, caused P concentrations in calcicolous species to remain constant or decrease while N increased consistent with increasing P limitation (Moorcroft *et al.* 1994).

4.5. Stable isotope $\delta^{15}\text{N}$ signal of foliage

4.5.1. Background

Stable isotopes have been used extensively in relation to nitrogen processes in plants (Handley and Raven, 1992; Hogberg, 1997). Nitrogen exists as the two stable isotopes ^{14}N and ^{15}N , but only 0.3663% is present as the heavier ^{15}N as inert N_2 in the atmosphere. Isotopic composition is expressed in terms of δ

values – parts per thousand (per mil ‰) difference from this standard, with a positive $\delta^{15}\text{N}$ value indicating ^{15}N enrichment and a negative value indicating ^{15}N depletion:

$$\delta$$

$$\delta^{15}\text{N} = \left[\left(\frac{^{15}\text{N}/^{14}\text{N} \text{ sample}}{^{15}\text{N}/^{14}\text{N} \text{ standard}} - 1 \right) \right] * 10^3$$

Analysis of the natural ^{15}N abundance in different biological components can help to identify the source and fate of N added to the environment by anthropogenic activities.

There is evidence that the two major forms of atmospheric nitrogen: NO_y and NH_x have different δ

^{15}N signatures, the former tending to be positive, and the latter negative. This difference makes it possible to use the δ

^{15}N value as a means of monitoring the source of N from plant measurements. However, to interpret correctly δ

^{15}N in terms of sources and sinks, it is necessary to characterise both the source signal and the fractionation occurring between source and sink (Handley and Scrimgeour, 1996). Subsequent fractionation between ^{15}N and ^{14}N changes the δ

^{15}N values and can result from both physico-chemical reactions, such as gaseous loss of $\text{NH}_3\text{-N}$ where the lighter isotope is more readily lost, and also from biochemical processes such as de-nitrification. δ

^{15}N values have been examined for many plant and soil components. However, in the context of atmospheric inputs moss species have received special attention, because they

obtain most of their nutrients from the atmosphere.

4.5.2. Evidence

Studies in the UK and the Netherlands have shown a close correlation between $\delta^{15}\text{N}$ and traffic exposure for higher plants (A. Soares, pers comm., S. Power and T. Collins, pers. comm.) and mosses (Pearson *et al.*, 2000). Samples collected near motorways or busy roads by Pearson *et al.* showed mainly positive $\delta^{15}\text{N}$ values ranging from +6 to -1‰. Power and Collins demonstrated a significant relationship between $\delta^{15}\text{N}$ signature in *Calluna vulgaris*, distance from London and from nearest trunk roads and also background NO_x in London. Signatures were less negative in areas with high NO_x , ranging from just 0 to -9‰. By contrast, samples collected from rural areas with little traffic (low NO_x concentrations) tend to be more negative (from -2 to -12 ‰) reflecting the greater contribution of reduced nitrogen from agriculture in these areas (Pearson *et al.* 2000). Ammonia emitted from agricultural practices has very negative $\delta^{15}\text{N}$ values, as the ammonia volatilised is preferentially enriched with the lighter ^{14}N . $\delta^{15}\text{N}$ values decreased from -6.8 ‰ and -8.8‰ for *Rhytididelpus*

triquetrus and *Hypnum cuppressiforme* respectively, in a rural area upwind of the poultry buildings considered by Pitcairn *et al.* (2004a, see Appendix I) to -9.3‰ and -11.5‰ , respectively, immediately downwind of the poultry buildings (Harrison *et al.* 1999). At the measurement point 276 m downwind of the farm, $\delta^{15}\text{N}$ values were indistinguishable from those upwind. These examples, however, represent extreme cases, and in most situations nitrogen deposition includes significant contributions from both reduced and oxidized nitrogen. In addition, traffic now emits a significant amount of NH_3 , associated with the introduction of catalytic converters (Sutton *et al.* 2000), and it is most likely that this has a similar $\delta^{15}\text{N}$ signal to NO_x emissions (i.e. not as $\delta^{15}\text{N}$ negative as NH_3 derived from volatilisation processes).

A further complexity in the analysis is the frequent assumption that little or no fractionation occurs in the uptake of N by plants thought to be dependant on atmospheric N. Studies of vascular and bryophyte epiphytes in Costa Rica showed that variations in the isotopic signature reflected not only the N sources, but also discrimination during N acquisition and the partitioning changes in isotopic N within the plants (Wania *et al.* 2002).

In some of the above studies, tissue N was also measured. For example in urban studies, Power and Collins (pers. comm.) found that the relationships between total tissue N in *Calluna* and background NO_2 in London were also significant, whereas Pearson *et al.* (2000) did not find a correlation between total tissue N and traffic exposure in the moss samples collected. In the poultry farm studies, foliar N measured on the same samples gave a better relationship with distance from the poultry farm ammonia emissions than the stable isotope signature.

4.6. Other methods based on foliar N accumulation: Conclusions and Application

4.6.1. Free Amino Acids

- The total foliar content of free amino acids in a range of plant species responds positively to enhanced N deposition. This is particularly clear in response to NH_3 and NH_4^+ inputs, with available data suggesting smaller responses to NO_3^- inputs.
- The dominant amino-acid under conditions with a high nitrogen supply varies with plant species. For species typical of low N supply, the dominant amino acid to accumulate tends to be arginine, while for species typical of N-rich habitats, the dominant amino acid is asparagine.
- There is evidence for changes in the composition of the amino acid pool following exposure to enhanced N deposition. Consequently, changes in amino acid pool composition (i.e. ratios between different amino acids) could provide a better indication of N enrichment for some species.
- Concentrations of free amino acids may be affected by several environmental factors including nutrient deficiencies and form of nitrogen available, while the sites of storage also vary between species. Thus amino acid accumulation might be regarded as a non-specific indicator of perturbation of nutrient availability.
- Increases in amino acid concentrations give some indication of the site of perturbation in the plant, while changes in amino acid composition may provide an early indication of potential perturbation from enhanced N deposition.

- Amino acid analysis clearly provides useful additional information about the impact of N deposition on plants and therefore provides more physiological relevant information that can complement analysis of total foliar N content.

4.6.2. Substrate nitrogen and foliar ammonium

- Substrate nitrogen represents the pool of nitrogen available for growth and may be approximated in measurement by analysis of soluble N in fresh leaf tissue. The available evidence points to this parameter as having a larger response to nitrogen deposition than total foliar N.
- Foliar ammonium represents a small pool of nitrogen available for the production of different organic nitrogen compounds within the plant. It is closely coupled to rates of atmospheric and other nitrogen inputs and shows an even larger response to N than substrate N.
- These parameters have been measured in grass, rush and pleurocarpous mosses, but the largest and most clear responses are seen for the mosses.
- Substrate N and foliar NH_4^+ are parameters that reflect similar responses to amino acid accumulation, as the former includes amino acids and the latter is a precursor for amino acid synthesis. However, by being less species-specific than amino acids, substrate N and foliar NH_4^+ are more easily generalizable parameters.
- Compared with substrate N, foliar NH_4^+ appears at present to be the more robust bioindicator of nitrogen deposition, due to its larger response and the higher precision with which it can be measured.
- The available data for pleurocarpous mosses show that species have different characteristic substrate N and foliar NH_4^+ levels, but that the observed differences are consistent with the sampling of these species in situations with different rates of N supply. Overall, the current evidence supports a single response curve to each of atmospheric NH_3 concentrations and N deposition for the different moss species.
- Non-linearity in the log-log response of foliar NH_4^+ to NH_3 concentrations is consistent with the contribution of wet and oxidized N to total N deposition and the saturation of NH_3 deposition rates at high NH_3 concentrations. This non-linear response provides an example of how bio-indicator data can be used to quantify deposition rates in a complex environment. Accounting for these effects, the log-log response of foliar NH_4^+ to total N deposition is linear within the range studied.
- Clear relationships have been established between both substrate N and foliar NH_4^+ of pleurocarpous mosses and atmospheric N inputs, and this is most precise for foliar NH_4^+ . Based on a regression relationship with N deposition as the dependent variable, measured values of foliar NH_4^+ in pleurocarpous mosses can be used to estimate the atmospheric nitrogen deposition at a site and specify confidence limits to the atmospheric deposition estimates.
- Although the relationships appear promising, at present there are limited data on substrate N and foliar NH_4^+ for native plant species. To improve the quantitative relationships, there is a need for further measurements, especially in situations with very low atmospheric N inputs, and to explore the seasonal variability and effect of light environment.

4.6.3. N:P ratio of foliage

- Where P is not limiting, N deposition will lead to increased growth and tissue concentrations of N will not necessarily reflect N deposition. In such cases measurement of N:P ratios may be preferable.
- However, when P availability is actually larger in areas of high N deposition (such as urban areas) compared with areas of low deposition, remote from traffic exposure, determination of N:P ratios may give misleading results.
- In most cases in the UK, N:P ratios are unlikely to provide more information than foliar N levels, but when funds are not limited and analytical facilities available, measurement of P may provide useful additional information.
- Woodin and Sullivan (2001) found tissue N to be a better indicator of N deposition than N:P ratios in their study of the relationship between tissue N content of *Hylocomium splendens* and N deposition. However, tissue P content helped to explain some of the variation in the tissue N values and the authors encouraged the measurement of both N and P when applying foliar N as a bioindicator.

4.6.4. Natural abundance of $\delta^{15}\text{N}$

- The $\delta^{15}\text{N}$ range obtained in two urban studies demonstrates that relative rather than absolute $\delta^{15}\text{N}$ values are the most robust indicators.
- The technique is most suited to 1) deposition gradient studies or 2) comparisons between sites with contrasting nitrogen deposition as a means of investigating the source of elevated N inputs e.g. either from agriculture or transport sources.
- Although the evidence available is limited, the relationships demonstrated between $\delta^{15}\text{N}$ and N deposition are strong and relevant for a range of species. Insufficient information is available on practical aspects such as sensitivity to other factors, sampling season etc., but it is likely that constraints similar to those governing total tissue N would also apply here. In general the method has not been adequately used or tested to provide any uncertainty measure.
- Measurement of $\delta^{15}\text{N}$ requires specialist equipment and technicians, and costs per sample can be high. Also relative to other estimations, substantial sample quantities are needed. Although no expertise is required in the collection and transportation of samples to a laboratory for analysis (other than a knowledge of the species to be collected) interpretation of the results can be difficult.
- Stable isotope signatures cannot be correctly interpreted without a detailed knowledge of the N source, and of any fractionation between ^{14}N and ^{15}N , which may occur during deposition and canopy exchange, as well as in soil N processes and root uptake of N. In general, the approach is therefore unsuited to the provision of quick bioindicator results. Interpretation requires the detailed background work and knowledge that can only be provided by a specialist in this area.

5. BIOINDICATOR METHODS BASED ON BIOCHEMICAL RESPONSES TO NITROGEN

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5.1. Activities of plant and soil enzymes

Enzyme activity in both soil and plants has been used to indicate N deposition and may provide a basis for biomonitoring of deposition and ecosystem response. The activities of a range of enzymes involved in the assimilation of N and other nutrients have been shown to respond to experimental additions of nitrogen. For example, activity of two key enzymes of the photorespiratory N cycle, glutamine synthetase and glutamate synthase, and the enzyme glutamate dehydrogenase which is active in both amination and deamination reactions may reflect enhanced N deposition. However, it is the activity of the enzyme nitrate reductase (NR) that has received the most attention in relation to N deposition. In addition, by increasing the availability of N, enhanced N deposition will also increase the demand for other nutrients, albeit by proportionally smaller amounts. Thus excess N deposition can cause or enhance P limitation in natural and semi natural systems. Increased P mineralisation would therefore be expected in such soils and can therefore also provide an indirect indicator of enhanced N deposition. The dominant phosphatase enzyme that is responsible for converting organic to inorganic P in most soils is phosphomonoesterase, and this could therefore also serve as a bioindicator of enhanced nitrogen deposition.

5.1.1. Plant nitrate reductase activity

NR activity has been used as an indicator of N deposition since the early 1980s. Apart from foliar N, the inducibility of NR appears to be one of the most useful indicative responses of bryophytes, especially Sphagnum species, to anthropogenic N (Woodin et al. 1985, Press et al. 1986, Woodin & Lee 1987). Bryophytes respond to and assimilate nitrate very rapidly and efficiently in small quantities, but continuous exposure to large N inputs suppresses this response. Sphagnum is capable of assimilating NO_3^- immediately, showing no lag phase unlike higher plants. In mosses the ability to induce NR activity has thus been used to assess their nitrate exposure history with the inability to induce activity being seen to signify N saturation. The *in vivo* methodology was developed by Woodin & Lee (1987).

NR serves plant, algae and fungi as a central point for integration of metabolism by governing the flux of reduced N by several regulatory mechanisms (Campbell 1999). The enzyme is the focal point for integration of control of C and N metabolism. NR is unusual among oxidoreductases as it exists in NADH and NADPH specific forms as well as bispecific forms that accept electrons from either NADH or NADPH. The activity depends on a) availability of substrates in the cytoplasm (steady state concentrations of NR polypeptide and availability of cofactors and metal ions - FAD heme, Fe Mo-MPT and Mo) and b) the activity level of the functional NR. The NR protein represents a short, soluble electron transport chain localized in the cytosol which catalyses the transfer of two electrons from NAD(P)H to NO_3^- (+5), which is reduced to NO_2^- (+3) and further reduced to NH_4^+ (-3) in the plastids. NR under certain, poorly understood, circumstances may reduce nitrate to nitric

and nitrous oxides (NO, NO₂). Because NO and NO₂⁻ and aqueous NH₃ are toxic in large amounts (although see Section 4.3) the production of NO₂⁻ is rigidly controlled, via control of 1) NR expression, 2) NR catalytic activity and 3) NR protein degradation.

The enzyme is known to be inducible depending on the availability of the NO₃⁻ substrate and light (activity is light dependent *i.e.* 70-90% active in light *cf* 10-30% in the dark, Campbell 1999). Photosynthesis is also required for NR activation, and it is thought that assimilates exported out of the chloroplast function as signals. The activation state of NR is sensitive to metabolite concentration. This possibly reflects the need to co-ordinate C and N metabolism by synchronizing NO₃⁻ reduction with photosynthesis and carbohydrate availability, thereby avoiding energy waste and accumulation of toxic products. However, this makes NO₃⁻ reduction sensitive to stomatal resistance in higher plants, so that under drought conditions the closing of stomata will also restrict NR activity. NR has a short half-life of several hours, which is controlled by its rate of synthesis and degradation.

5.1.2. Phosphomonoesterase

Phosphomonoesterase (PME) is a relatively non-specific phosphohydrolase that breaks down relatively low molecular weight P compounds with a monoester bond. Phosphatase enzymes associated with the root surface of plants may originate from mycorrhizal or saprophytic fungi, bacteria or root exudates. Enzyme activity in peat soils is greatest in winter and smallest in spring and early summer, but no seasonal relationships have been observed for mineral soils. Thus further investigation would be required to characterize the optimum sampling times. As with many such soil based bioassays *e.g.* denitrification, seasonal trends appear to be strongly linked to moisture and temperature (Skiba and Smith, 2000). In a short-term (18 month) N manipulation, Johnson *et al.* (1999) found that soil NH₄⁺ extracted in 2M KCl accounted for 67% of the variation in PME activity of *Plantago lanceolata* in calcareous grassland and 86% of the variation in PME activity of *Agrostis capillaris* in acid grassland. Activity of PME was an order magnitude lower in *P. lanceolata* than in *A. capillaris*, possibly reflecting the difference in root surface area.

Assessments of PME involve sampling from the upper soil surface to provide a representative bulked core from the top 5 cm, removing all non-soil debris such as stones, litter, macrofauna and roots where possible. An aliquot of soil is incubated with a prescribed buffer for 30 min in a shaking bath, centrifuged and the absorbance of the supernatant containing the released *para*-nitrophenol read with a spectrophotometer (Turner *et al.* 2002). In general the specialist equipment required for this method is not very expensive relative to some bioassays and is multi-functional. Results can be expressed as activity per dry weight or volume of soil. Activity is highly soil dependent and is likely to differ between soil horizons, and is, for example, greatly enhanced by the inclusion of litter emphasising the importance of careful sampling. Turner *et al.* (2002) report a range of values for different soil types that can aid interpretation of data. As shown by the results of Johnson *et al.* (1999), different plants differ in their PME response and interpreting the values with respect to N deposition is therefore difficult. While PME showed a clear response to KCl extractable NH₄⁺, and is useful to explore N:P interactions, bulk plant and soil extracts of NH₄⁺ may provide easier and more general means to biomonitor N deposition.

5.2. Trace gas emissions from soils

5.2.1. Background

The gases NO and N₂O are produced in soils by nitrifying and denitrifying bacteria. The magnitude of the emissions is controlled by the availability of N as soil NH₄⁺ or NO₃⁻ and

also by certain climatic and soil properties that promote nitrification or denitrification, e.g. temperature, rainfall, organic matter content (Skiba & Smith 2000). Nitrification is an aerobic process whereby soil NH_4^+ is oxidised to NO_3^- . At sub-optimal oxygen concentrations, oxidation is incomplete, giving rise to N_2O and NO . Generally, this reaction is brought about by very specific autotrophic bacteria, which are able to grow at the expense of generating energy from nitrification e.g. obligate chemo-autotrophs (*Nitrosomonas* and *Nitrobacter* spp). A range of heterotrophic bacteria, fungi and algae also produce N_2O , but at relatively slow rates. Soil type influences the organisms that dominate. De-nitrification, the anaerobic reduction of NO_3^- to N_2O and N_2 , occurs in facultative anaerobes, which switch to using NO_3^- as their terminal electron acceptor when O_2 concentration in soil are depleted in wet conditions. Emissions of N_2O and NO depend on the presence of an active nitrifying and denitrifying population and optimal soil aeration, which is controlled by soil moisture and its relationship to the proportion of water filled pore spaces, and soil texture.

5.2.2. Evidence of links with N deposition

Studies in a range of semi-natural ecosystems, which have received various forms of N deposition, suggest that measurements of soil NO and N_2O emissions may be useful indicators of soils in which N supply exceeds the demands of the vegetation (Skiba *et al.* 1998). Skiba *et al.* showed that nitrogen additions by fertilisation, manure and atmospheric deposition all increased emission of N_2O . For example, fluxes of N_2O and NO were closely related to atmospheric N deposition in the vicinity of several livestock farms in southern Scotland. Emissions of N_2O and NO were largest close to poultry houses and decreased with increasing distance from the farms (Figure 5.1). Emissions represented up to 0.8% of the atmospheric N estimated to be deposited. Larger emissions of N_2O were measured in a sheep-grazed pasture (1.7% of N input), and in an experimental Sitka spruce plantation (3.7 % of N input) that had received acid mist containing N ($96 \text{ kg N ha}^{-1} \text{ y}^{-1}$).

The relationship between atmospheric N deposition, both dry and wet, is robust and can be used to estimate enhanced N deposition and indirectly the potential for critical loads exceedances. Because of the buffering effects of soils on changes to pH and N availability, measurements of trace gas fluxes are likely to integrate over a long period of several to many years. By contrast, measurements of N_2O fluxes after fertilizer treatments, indicate rapid loss of N_2O decreasing exponentially with time over several weeks. This method is thus more suitable for indicating long-term changes rather than short-term effects, but may be able to detect situations with a sudden increase in atmospheric deposition, such as may occur in the vicinity of a new development.

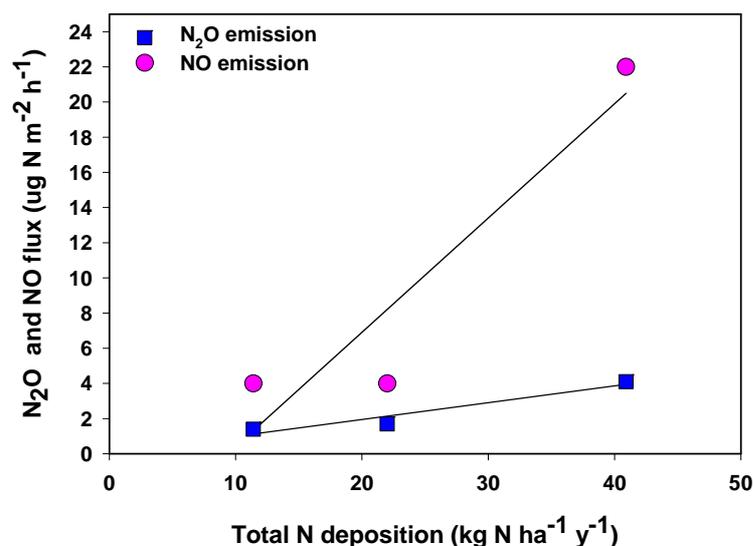


Figure 5.1: Emission of nitrous oxide (N₂O) and nitric oxide (NO) from woodland soils in response to atmospheric nitrogen deposition from measurements in the vicinity of a livestock farm (Picairn *et al.* 2004a, see Appendix I).

5.3. Other bioassays for indicating impacts of atmospheric nitrogen

5.3.1. Chlorophyll fluorescence of foliage

When plant leaves are illuminated, part of the light energy is trapped by the chlorophyll antennae, which become 'excited' and undergo a shift in energy states. The energised state is unstable and the energy is rapidly released (re-emitted) via three competing pathways. Chlorophyll fluorescence represents the energy that is re-emitted as a low energy, high wavelength red and far red light. The remaining energy is used to drive photosynthesis or dissipated as heat. Damage to photo-system II (PSII) and reductions in photosynthetic rate will reduce the amount of fluorescence released in response to light. Thus fluorescence measurements provide information on inhibition or damage to the transfer of electrons from PSII and photochemical quantum yield.

Reductions in fluorescence usually precede visible damage. Hence measurement of chlorophyll fluorescence has been used as an early indicator of physiological stress. Different parameters associated with fluorescence kinetics have been related to a range of stresses such as freezing, drought and high photo-illumination following cold nights. Changes in fluorescence kinetics may be linked to N through effects on water use and light harvesting. Such changes are considered to reflect increased sensitivity to environmental stress resulting from a range of perturbations including excess N, rather than as a direct effect of N deposition *per se*.

The required measurements are non-invasive, non-destructive and relatively quick, requiring a short period of time for dark adaptation (15-20 minutes for most species). During the dark period the majority of PSII reaction centres are open i.e. the primary electron acceptor Q_A is oxidised and photochemical quenching is maximal = 1 (F_o). Following the dark adaptation period, a white actinic light is applied to energise the chlorophyll molecules, resulting in a gradual increase in fluorescence to a maximum (F_m), equivalent to when the photochemical quenching of fluorescence is minimal = 0. F_v represents the distance F_o to F_m the point

where the pool of primary electron acceptors (Q_A) is reduced. The ratio F_v/F_m is typically in the range 0.75 – 0.85.

A decline in F_v/F_m is a good indicator of inhibitory damage *i.e.* when F_o is increased as PSII reaction centres are destroyed following chilling, freezing or drought. The area under the curve from F_o to F_m is proportional to the pool size of the electron acceptors. Inhibition of CO_2 fixation will reduce consumption of ATP and NADPH causing an increase in pH gradient and a more reduced electron transport chain, which will decrease F_m . Conversely, an increased level of reduced electron acceptors in PSII means that Q_A will increase F_m through a reduction in non-photochemical quenching (Q_{NP}). Excess light *i.e.* photo-inhibition causes proteolysis of the intrinsic PSII reaction centre protein (referred to as DI), possibly via a superoxide anion (active oxygen species) (Jensen 2002). Damage from high light levels is enhanced at low temperature because the slower rate of carbon metabolism increases the proportion of excess light energy available to PSII and the proportion of reduced Q_A in steady state.

Chlorophyll fluorescence can be measured using a simple fluorimeter photo-detector. However, more information is gained from studying the time dependent response of fluorescence to a high intensity light after dark adaptation – the response kinetics, described by the Kautsky curve. Over the last 20 years, equipment for measuring fluorescence has undergone significant development and improvement for field use. Today, relatively inexpensive (approx. £5000), lightweight (500-1000g) highly portable machines can be purchased for field use. These machines *e.g.* the Hansatech Handy Pea and the PAM 2000 (Walz Company, Germany) are relatively easy to use with rapid access to results. However, large numbers of samples need to be assessed to establish meaningful values and the output will vary depending on the choice of material. The significance of the data will be strongly coupled to the sampling strategy.

The main disadvantage of chlorophyll fluorescence as an indicator of nitrogen deposition effects is its lack of specificity. Excess nitrogen may be one source of stress to plants that is measurable by this technique, but similar responses may be obtained from many other stresses, such as drought, frost or exposure to other pollutants. It is therefore has limited usefulness for bioindication and biomonitoring the effects of atmospheric nitrogen deposition. The exception may be in situations where N deposition is already established as a cause of damage to vegetation. In this case a useful application of the technique may be establish whether there is further evidence of stress to other vegetation not yet showing visible injury.

5.3.2. Frost hardiness of foliage

Most of the studies addressing effects of enhanced N deposition on frost hardiness have investigated conifers, since the research interest primarily concerned the damage to commercial tree species. However, because of the importance of ‘berries’ for medical science, there have also been several studies on ericaceous species *e.g.* *Vaccinium* species, in Scandinavia, Eastern Europe and USA (Taulavuori *et al.* 2001). In the UK there has been very little evidence of N deposition increasing the risk of frost damage to tree species, except possibly as a consequence of changes in timing of budburst and budset. Planting of exotic species is often associated with increased risk of frost injury since their environmental receptors are out of synchrony with the environmental cues. The ability of plants to minimise the risk of freezing damage is conferred by synchronising their phenology with the growing environment. Generally indigenous flora has a good safety margin between its frost hardened status and minimum temperatures, unless the growth environment changes.

A negative link between enhanced N deposition and reduced frost hardiness was widely suspected to be a casual factor in the observed decline of red spruce in the nineteen eighties (Eagar & Adams 1992). Nihlgard (1985) postulated that increasing N deposition prolonged the growing season and led to carbon assimilate being diverted away from the production of cryoprotectants. Subsequent studies using N fertilizers, which significantly raised foliar N concentrations, failed to support this hypothesis showing that the addition of N to N-deficient trees improved frost hardiness (DeHayes *et al.* 1989, Klein *et al.* 1989). Nursery studies with conifers have also suggested improved frost hardiness with N fertilisation, although the effect was dependent on the timing of the fertilizer application (Benzian and Freeman 1967, Benzian *et al.* 1974). Shoots of spruce taken from three N manipulation experiments showed a positive, but non-significant, improvement in winter hardiness in response to N additions (Sheppard *et al.* submitted). When foliar N status is very significantly raised there does appear to be some loss of hardiness (Aronsson 1980).

Sugars, oligosaccharides are one of the main types of plant cryoprotectants. At high concentrations they act as anti-freeze agents, restricting intracellular ice crystallisation thereby minimising physical and chemical damage. Some novel proteins act in the same way (see Sheppard 1994). Maintaining membrane vitality and function by contrast relies more on proteins (N containing compounds) for augmentation and alignment and lipids (see Sheppard 1994; Sheppard & Pfanz 2001). The capacity to fix C and provide these assimilates is also positively linked to N through photosynthesis. Thus at low and moderate N supply frost hardiness should improve. When N uptake exceeds demand for growth and accumulates in foliage, there is the potential for increased frost sensitivity. This is because protein and associated N compounds have a relatively high rate of turnover, which increases carbon consumption potentially at the expense of cryoprotectants. In spring, it has been shown for evergreen conifers, that high foliar N concentrations can bring forward the date of bud burst (Oren *et al.* 1988). This is because the high N concentrations increase the capacity for C assimilation, which means the threshold carbon status needed to support the demands made by bud burst is reached earlier. Since the risk of frosts is greater earlier in the spring and newly flushed tissue has little capacity to withstand frosts, accelerating bud burst increases the risk of frost damage in spring.

Links between N and increased frost damage have been suggested for species of importance in conservation sites. In the UK, Netherlands and Denmark winter browning damage to *Calluna* is relatively common, even though minimum temperatures have usually not been sufficiently cold to cause freezing damage. The most likely cause of this browning is desiccation, in response to strong winds and frozen ground (Sheppard & Leith 2002). However, similar damage in a field manipulation experiment (Carroll *et al.* 1999) was largest in plots treated with high N (120 kg N ha^{-1}) suggesting that high N deposition can increase the risk of winter desiccation damage. Fumigation studies with NH_3 , where winter hardiness has been assessed, have been inconclusive except at very high NH_3 concentrations when hardiness was reduced. A recent study at CEH Edinburgh has shown reduced winter hardiness in *Calluna vulgaris* exposed in the field to NH_3 and NH_4Cl , but not to NaNO_3 (Sheppard *et al.*, unpublished results). Laboratory freezing of detached shoots received NaNO_3 were damaged least.

While there may be a link between N deposition and increased frost sensitivity, the evidence is often inconclusive, implying a large N excess may be a prerequisite. Some of the confusion may relate to the N form. When plants are exposed to NH_4NO_3 , damaging effects of reduced N may be counteracted by the presence of oxidised N. Such nitrogen excesses (Section 3) may be more meaningful to measure than frost hardiness itself. As a bioassay frost hardiness is not ideal. In conifers, it is accepted that shoots exhibit considerable

variation in the level of frost they can withstand, both within a population and even within the same tree. Microclimate can also contribute to heterogeneity in the population response so that establishing the underlying cause of frost damage in the field can be difficult and rarely conclusive. In a recent assessment of variation, in the absence of a known influence on frost hardiness in *Calluna* shoots collected over a 100 x 100 m² area, temperatures causing 50% shoot death varied by > 50% (Sheppard and Leith 2002). Assessments of frost hardiness in response to acid mist damage were used very successfully as a bioassay for acid mist effects on red spruce grown in open-top chambers (protected from rain washing), but results were less convincing for other spruces and where rain was allowed to wash the foliage.

One of the drawbacks of the frost hardiness bioassay arises out of the need to determine the effect (damage) of freezing. Establishing that damage has occurred often relies on a further measurement e.g. of visible injury, ion leakage which identifies damage to membranes (Sheppard *et al.* 1995), or chlorophyll fluorescence, which identifies damage to photosystem II (Wulff *et al.* 1994; Section 5.3.1). While protocols exist for frost testing *Calluna* shoots, there is a high uncertainty in the ion-leakage-rate method. Relative conductivity assessments, which require a measurement of ion conductivity after 24 h and again after autoclaving, are providing good data, but indicate the need to freeze at least 20 shoots for each collection site and use five test temperatures. Further development work is in progress to identify a working protocol for detecting frost damage in *Calluna* shoots.

5.3.3. Ergosterol

Plants obtain most of their nutrients from the soil often via the activity of mycorrhizal fungi. Excess atmospheric nitrogen deposition has been shown to upset this balance, resulting in reduced populations of both endo- and ecto-mycorrhizae (van der Eerden *et al.* 1998; Yesmin *et al.* 1996, Cairney and Meharg 1999). Ergosterol is a compound that is specific to fungi and might therefore be used to indicate the impacts of atmospheric nitrogen deposition on mycorrhizal biomass. Ergosterol is the principal sterol of membranes (Weete 1974) it is thus a more representative indicator of metabolically active fungal biomass than, for example, counts of mycorrhizal root tips.

The drawback in the use of ergosterol as a bioindicator for nitrogen effects on mycorrhizas is that it is not specific to mycorrhizal fungi, and is present in a wide range of soil fungi, including saprophytes, pathogens and free-living fungi. So, while the concept of N deposition causing reductions in mycorrhizal fungi and saprophytes is testable by measuring ergosterol, the results will be open to misinterpretation. For example, N additions might increase parasitic pathogenic fungi at the expense of the beneficial symbiotic mycorrhizal forms with no net change in ergosterol, but a distinctly different functional grouping. The rapid loss of ergosterol from dead roots when compared with chitin which is unaffected by vitality suggests a ratio of the two could provide an index of vitality.

The basic analytical procedures involve sample extraction, saponification, partitioning, purification of the sample followed by ergosterol determination with high performance liquid chromatography (HPLC). Freeze drying of samples is recommended prior to extraction, However, depending on the method applied this gives variable recovery rates (Nylund & Wallender 1992). At present there is a shortage of data of ergosterol response in relation to atmospheric nitrogen deposition, and further application of this approach would require more extensive characterization of the values expected in a range of clean to polluted sites.

5.3.4. BIOLOG

N deposition is well known to stimulate the activities of soil microbes. Large-scale changes in the composition and activities of soil bacterial communities can be assessed using the

BIOLOG micro-titre assay. The micro-titre assay plates provide community level physiological profiles (CLPP) for the rhizosphere and or non-rhizosphere microbial communities. They are thus reasonably characteristic of the species from which the rhizosphere community is taken, where N may be one of many drivers of change. This lack of specificity makes it less suitable for use as a N biomonitor. In soil, microbial growth is carbon limited so that in N enriched systems the demand for carbon is heightened. The BIOLOG technique measures utilisation of a variety of carbon compounds, although, opinions differ as to how representative the technique is of the whole community population. BIOLOG GN microplates (Biolog Inc. Hayward, CA, USA) contain 95 different carbon sources, a control well, with no carbon source, and a redox indicator dye (tetrazolium violet) (BIOLOG 1993). These represent approximately 100-125 wells in the plates, which are filled with different easily broken down carbon sources. It is expected that the ability to use N will also influence C utilization and that soil suspensions from N enriched sites will be distinguishable from control sites.

This methodology requires control sites, which will necessarily restrict its application for biomonitoring. Similarly, given the current lack of application for nitrogen responses, the method would require some development and optimisation work before it could be used in a robust way for biomonitoring of atmospheric nitrogen deposition and impacts. In addition, only a very small proportion < 5% of the microbial population may be assessed by this method, which requires skilled personnel for all aspects of the procedure through to interpretation of the results. Direct microscopic counts of micro-organisms require a fluorescence microscope for cell counting, a centrifuge (2000 rpm), incubator and microplate reader. Data analysis requires expertise in canonical variate analysis. The methodology is potentially costly unless the work can be outsourced to a laboratory that routinely runs and assesses such samples.

5.4. Biochemical methods based on N responses: Conclusions and Application.

5.4.1. Nitrate Reductase (NR) activity.

- Samples for enzyme analysis have to be collected following a specified protocol and the *in vivo* assay for NR must be undertaken under conditions that permit photosynthesis. In *Sphagnum*, NR activity is greatest in the capitulum decreasing down the stem. Below pH 3 the activity of NR is reduced. Specialized equipment is required for the analysis of NR.
- In the past, the activity of NR has been thought to be specific to oxidised N, however, it may also be sensitive to reduced N, and further work is required to elucidate relative responses to NH_x vs NO_y .
- The between species responses of NR to nitrogen input may represent a different relative tolerance of species to nitrogen, with NR activity decreasing in association with either reduced growth (and reduced substrate C availability) or limitation induced by associated high NH_4^+ in the symplast. Comparisons of NR activity may therefore indicate species-specific sensitivity to excess N.

5.4.2. Phosphomonoesterase (PME) activity

- PME has been shown to be induced by high soil ammonium availability demonstrating an interaction between N and P nutrient limitation. The equipment requirements for the PME assay are general and easily available.

- The PME response to N varies substantially with plant species, and with the limited available data there is a lack of the general relationships that are needed for practical application as a biomonitoring method.
- While enzyme responses to nitrogen may provide mechanistic insights and information relevant to different plant species sensitivities, more general assays of total N or available NH_4^+ may provide more suitable techniques for practical biomonitoring of nitrogen deposition.

5.4.3. Trace gas (N_2O and NO) emissions

- Nitrous oxide fluxes are measured using static chambers, which remain in situ throughout the measurement period. Chambers are sealed for approximately 1 hour when gas samples are withdrawn, stored in PTFE bags (for up to 2 weeks) and analysed by sophisticated gas chromatography equipment, using electron capture detection (ECD).
- The main advantage of N_2O measurement is that it is non-destructive and relatively rapid since the static chamber can be inserted and a gas measurement made the same day. However, there is debate over the impact of inserting the chambers, on the soil microbial activities activity of the soil.
- Soil temperature and gravimetric moisture, % water content must be measured as they influence emissions. KCl extractable NH_4^+ and NO_3^- in soil can also be measured but these concentrations are also sensitive to moisture and temperature, because their generation through mineralisation has a biological component.
- Laboratory extractions require rigorous handling of the sample and specialist equipment (usually autocolorimeters) for measuring soil NH_4^+ and NO_3^- .
- The measurement of nitric oxide (NO) fluxes requires more complex continuous analysers and must be measured on site. Methods are described in Skiba *et al.* (1993). As a practical bioindicator for application at remote sites, N_2O emission is preferable due to the simpler equipment required in the field.
- Freeze thaw cycles can influence the availability of soil mineral N and thus can potentially increase N_2O and NO emissions. This is because temperatures $<4^\circ\text{C}$ may kill (lyse) micro-organisms and release their cell contents increasing the availability of both C and N.
- Responses to N show a ‘memory effect’ and simulation studies have shown that the N history of a site will strongly modify its capacity for N_2O emissions (Skiba *et al.* 1998). In addition, time is needed to build up a denitrifying population following enhanced deposition of N.
- Measurements of N_2O emissions indicate that amounts are strongly dependent on temporal inputs and thus may be governed by the continuity of deposition, as well as the amount.
- The evidence suggests that emission levels of NO and N_2O provide a good indication of the N status of the soil and the N demands of the plant species which can be related to enhanced atmospheric N deposition.
- The impacts of deposition on soils can be well buffered and changes in soil N leading to gas emissions may take time. Hence measurement of emissions of these gases is more suitable for the indication of long-term change.

5.4.4. Chlorophyll fluorescence

- The measurement of chlorophyll fluorescence is non-invasive, non-destructive and rapid – many measurements can be undertaken on a single day, providing that sufficient leaf clips for prior dark adaptation of sampling material are available.
- The equipment to determine F_v/F_m is portable, relatively inexpensive and provides rapid identification of photosynthetic malfunction before visible symptoms.
- Measurement of F_v/F_m provides a general indicator of plant stress. While excess nitrogen may be one source of such stress, the method is not specific to nitrogen, and the data may be difficult to interpret in the context of biomonitoring for nitrogen impacts.
- F_v/F_m is strongly affected by moisture content of the plant, and this is particularly relevant for mosses, lichens and other cryptogams in which photosynthetic activity is determined by water availability. For example, in several species of the lichen *Parmelia*, F_v/F_m was approximately 6.5 when the water content was > 30%, but only ~ 0.1, when the plant was very dry (Lang *et al.* 2001). Similar observations were recorded for mosses (Proctor & Smirnoff, 2000).
- The method is not very sensitive to temperature within normal UK conditions (Wulff *et al.* 1994).

5.4.5. Frost hardiness

- High rates of atmospheric deposition have been shown, in certain instances, to lead to a reduced frost hardiness of vegetation, with resulting damage to cell membranes and photosynthetic processes. Measurement of frost hardiness therefore provides a potential bioindicator of the impacts of enhanced nitrogen deposition.
- Frost hardiness may be measured by placing plant shoots under a controlled temperature cycle to different levels of freezing, with subsequent determination of damage, e.g. via membrane leakage or using chlorophyll fluorescence. This procedure requires specialist equipment and trained, experienced personnel.
- The frost hardiness analysis is destructive and requires large numbers of samples, especially as assessments may need to be made at different times – early autumn, winter and spring.
- The responses between plant populations vary widely, so that the method is best suited to situations with known different rates of N input, where results can be compared with low-N control conditions at the same site. The high degree of plant and temporal variability means that only the largest impacts are detectable.
- Assessments of frost hardiness are of particular interest where a signal of frost damage is implicated in deterioration of a species or habitat due to atmospheric nitrogen deposition. However, given the expense, specialist staff and equipment required and the high variability, the method is unsuited as a general biomonitoring tool for the assessment of N deposition impacts on conservation sites.

5.4.6. Ergosterol

- Ergosterol is a compound that is specific to fungi and might therefore be used to indicate the impacts of atmospheric nitrogen deposition on mycorrhizal biomass. It

provides a more representative indicator of metabolically active fungal biomass than mycorrhizal root counts.

- The drawback in the use of ergosterol as a bioindicator for nitrogen effects on mycorrhizae is that it is not specific to mycorrhizal fungi, and is present in a wide range of soil fungi.
- The rapid loss of ergosterol from dead roots when compared with chitin which is unaffected by vitality suggests a ratio of the two could provide an index of vitality.

5.4.7. BIOLOG

- BIOLOG micro-titre assay plates provide community level physiological profiles (CLPP) for the rhizosphere and or non-rhizosphere microbial communities.
- In soil, microbial growth is carbon limited so that in N enriched systems the demand for carbon is heightened. The BIOLOG technique measures utilisation of a variety of carbon compounds which might be affected by the carbon-nitrogen interaction.
- The method however, is not specific to impacts of nitrogen making it less suitable for use as a N biomonitor.

6. BIOINDICATOR METHODS FOR NITROGEN BASED ON COMMUNITY SPECIES COMPOSITION: HIGHER PLANTS AND BRYOPHYTES

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6.1. Introduction

Rapid and practical methods are needed to detect the ecological impact of N deposition. Methods that rely on simply measuring changes in species composition are attractive since they amount to measuring actual biological responses among impacted organisms, but without the need for potentially costly chemical analyses of plant material. Species presence methods are used mainly to determine the impacts of atmospheric N deposition, but in some instances may also give some indication of the magnitude of enhanced N deposition fluxes. The most widely studied of these is the application of Ellenberg indicator values (Ellenberg 1988), but variant methods have also been devised that combine species characteristics with soils information, and these include the FNIS and N_{dev} methods developed by Diekmann and Falkengen-Grerup (1998, 2002).

6.2. Ellenberg indicator values

6.2.1. Introduction

Ellenberg devised a comprehensive indicator system for vascular plants of central Europe (Ellenberg, 1979; Ellenberg *et al.*, 1991) to describe the response of individual species to a range of ecological conditions (light, temperature, continentality, moisture, pH and nitrogen). In several European studies, calculation of the Ellenberg index of one or more variables for a site has been used to indicate vegetation change, due to land use changes and to increased atmospheric pollution (acidity and N deposition), by comparing plots or by monitoring change in a single plot over time (e.g. Ellenberg, 1988; Tyler, 1987; van Dobben, 1992).

The Ellenberg N Index consists of allocating a N-score to each plant species, so that the overall community has a score on a scale of nutrient poor (1) to nutrient rich (10). The index has been used at a local scale (e.g. Pitcairn *et al.* 2002; Falkengren-Grerup 1995; van Dobben, 1992) and also at a national scale (Haines-Young *et al.*, 2000). While most studies have focused on higher plants, the approach has been extended to cover bryophytes (van Dobben 1993; Pitcairn *et al.* 2002) using indicator values from Siebel (1993).

Assessment of the applicability of Ellenberg N values in measuring the impact of N deposition can be considered in terms of two analytical aims. These are, firstly, *signal detection*, referring to the actual detection of a floristic shift consistent with eutrophication, and, secondly, *signal attribution*, which focuses on attempts to isolate the fraction of the detected signal due to N deposition from the range of other potential causes. In general, published evidence indicates that Ellenberg N values have proved themselves as useful tools in detecting floristic shifts consistent with increased nutrient availability. This is because, as

response variables, N values integrate the effects of multiple drivers of change in nutrient availability. However, it is exactly this property that may render them blunt tools for quantifying the partial contribution of N deposition from place to place.

6.2.2. Past application of Ellenberg indicators for nitrogen

a. Examples of local-scale application

The impact of N additions on the plant indicator score was examined by Van Dobben (1993) in Swedish coniferous woodland experiments in which N or acidity had been added over a period of 15 years. Ellenberg indicator scales (Ellenberg *et al.*, 1991) for vascular plants and lichens were used together with values from Siebel (1993) for bryophytes. Calculated mean indicator values were either **unweighted using presence/ absence** for each species or **weighted by using cover/abundance** of each species. The nitrogen fertilisation treatment had the strongest effect, with an addition of around 60 kg N ha⁻¹ y⁻¹ causing a shift from ericaceous species with acrocarpous mosses and lichens to a dense carpet of *Deschampsia flexuosa*, pleurocarpous mosses and ruderal species such as *Chamaenerion angustifolium* and *Rubus idaeus* after 15 years of treatment. The Ellenberg indicator value for nitrogen was a fairly reliable indicator of soil nitrogen availability, and there was a good relationship between N addition and the indicator scores for N. Better relationships were found between measured environmental variables and indicator values based on presence/absence data, rather than on cover/abundance data. Similar conclusions were reached by Diekmann (1995) in a study of a Swedish deciduous forest.

In southern Scotland, the Ellenberg N index was applied to woodland ground-flora along a gradient of ammonia concentration and N deposition downwind of two poultry farms where changes in species composition had been measured (Pitcairn *et al.* 1998). Ammonia concentrations were large at woodland edges close to the poultry houses (annual means 30-60 µg m⁻³) and total nitrogen deposition at the woodland boundaries was estimated to range from 50 to 80 kg N ha⁻¹ year⁻¹ at the two sites. N indices were determined using the modified Ellenberg values for British vascular plants (Hill *et al.* 1999) and indicator values from Siebel (1993) for bryophytes. Unweighted and abundance (% cover) weighted mean indicator values for all species (vascular plants and key bryophytes) at different distances from the poultry farms were calculated.

The mean Ellenberg N indicator values for transects close to the poultry houses were not as high as might be expected for sites receiving large inputs of N. They were however, larger than those for the more distant transects, but the decline with distance from the buildings was small and the standard deviations were very large. An index of 5.8 was found 30 m downwind of Poultry Farm L declining to about 4.8 at 50 m and beyond. At Poultry Farm E, the index at 15 m downwind, where species change was obvious, was 4.9 declining to 3.9 at 276 m (Figure 6.1). The relatively poor relationship between Ellenberg N index and N deposition at Poultry Farm E may be partly due to the abundance of *Deschampsia flexuosa* and *Holcus lanatus* close to the poultry houses. These species are not typical of N rich ecosystems and have low and intermediate Ellenberg numbers, respectively. However, they are able to respond to an increase in available N and thus their presence at this distance from the poultry buildings does confound the index to some extent. The poor relationship may also be due to a higher disturbance factor close to the poultry houses favouring ruderal and early colonising species, and factors such as increased temperature and dust levels.

In this study changes were sought within a short transect in which many of the same species are present throughout, but in different amounts. Hence abundance weighted indices were thought to be useful. While a reasonable relationship was found between distance and

abundance weighted Ellenberg N Index at Poultry Farm L, a surprisingly poor relationship was found at Poultry Farm E (Pitcairn *et al.* 2002). The probable reason is that a large abundance of the low Index grass species close to the buildings was matched by high abundance of low Index herbs and mosses at 300 m from the buildings and provides an important reminder of the potential for confounding signals within the Ellenberg Index approach. A closer relationship was shown between Ellenberg N Index for two poultry farms and total estimated atmospheric N deposition at different distances from the poultry houses (Pitcairn *et al.* 2002), but error bars were large and the method is a relatively poor indicator of N deposition.

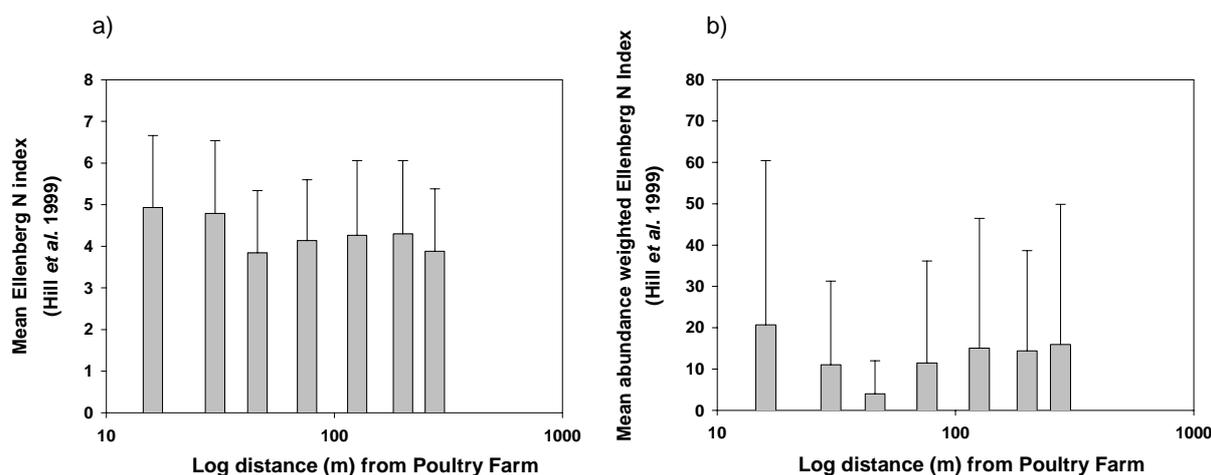


Figure 6.1: Ellenberg nitrogen indices of woodland ground flora in the vicinity of farm E reported by Pitcairn *et al.* (1998, 2002). Nitrogen deposition decreases with distance from the farm. a) Mean unweighted Ellenberg nitrogen index, b) Mean abundance weighted nitrogen index. Indicator scales from Siebel (1993) were used for bryophytes throughout. For higher plants and ferns, indicator scales are from Hill *et al.* (1999). Error bars are standard deviations.

b. Regional-scale application

Many published examples now exist of the successful application of Ellenberg N values in indirectly estimating spatial and temporal changes along productivity gradients. Examples carried out at regional scales include:

- Changes in regional floras (Preston 2000, McCollin *et al.* 2000);
- Changes across regional nature reserve series (Smart 2000)
- Regional woodland change (Diekmann & Dupre 1997, Hofmeister *et al.* 2002, Ejrnaes *et al.* 2003)
- National changes in plant species composition (Haines-Young *et al.* 2000, Smart *et al.* 2003, Smart *et al.*, in press, Preston *et al.* 2002)

Most applications of Ellenberg N values for quantifying floristic change have been primarily concerned with signal detection. Thus, it has often been enough to demonstrate changes in species composition along a productivity gradient. Ellenberg N values have been increasingly recommended for this purpose on the basis of published calibration studies. These have sought to validate both the N values attributed to each plant species and mean N values for mixed species assemblages by comparing them with independent measurements of biomass, tissue N and a range of soil variables. Examples include Ertsen *et al.* (1998), Meerts (1997), Melman *et al.* (1988), Thompson *et al.* (1993), Diekmann (1995), Schaffers & Sykora (2000), Smart *et al.* (2002) and Hill & Carey (1997). The majority of studies offer support for their

use as indirect indicators of eutrophication, although the strength of correlations varies considerably. For example, Hill & Carey (1997) reported an R-squared of 83% when weighted Ellenberg N values were used to predict annual hay yield. In contrast, Ertsen *et al.* (1998) reported an R-squared of only 54% in their Dutch calibration of N values against standing crop data.

6.2.3. Sensitivity of Ellenberg N values to other factors

In the absence of other nutrient limitations and given the presence of responsive species, increased N availability generally favours the establishment, persistence and increase in biomass of plant species inherently better able to exploit higher macro-nutrient availability. Since published studies generally support the positive correlation between Ellenberg N values and productivity, these values are clearly useful in detecting ecosystem eutrophication.

A range of other factors can potentially influence spatial and temporal change in mean Ellenberg values. Some are supported by published evidence, while others remain a theoretical possibility. These factors include: succession and disturbance, weather, light, bias and gaps in the original values, variable response curves and a range of other factors affecting species occurrence.

a. Correlations between Ellenberg values

Differences in Ellenberg N values can result from the positive correlation between plant traits associated with disturbance and low productivity. Schaffers & Sykora (2000) describe an example of an abandoned grassland part of which has been brought back into management as hay meadow and cut twice a year. Low indicator values for the mown section might not necessarily indicate lower nutrient availability relative to the undisturbed section, but simply a more favourable disturbance regime for the germination and persistence of shorter species which also tend to have lower N scores. On the other hand, biomass accumulation tends to favour taller species better able to compete for light, but biomass accumulation is also associated with increased accumulation of soil N, at least in the medium-term (e.g. Olf *et al.* 1993). Hence competitive, taller species tend to be those with high N values.

The confounding factor between nutrient availability and disturbance gradients is reflected by the negative association between the Ellenberg light (L) and nitrogen (N) values ($n=1267$, $F=50.58$, $p<0.0001$, $R^2\text{-adj } 3.7\%$ - using recalibrated GB values from Hill *et al.*, 1999). However, the low R-squared value indicates that the variation they jointly explain remains very low. This means that there should be scope for partitioning out change related to disturbance and shading from that related to change in nutrient availability. R (soil pH) and N scores are also intercorrelated but the relationship is curved rather than linear because extremes of pH coincide with low soil fertility. The use of analyses of covariance applied to R and N scores in German forests by Diekmann & Dupre (1997) is a good example of signal attribution where change correlated with acidification was isolated from that due to eutrophication. However, the intercorrelation between R, L and N values indicates that exploring the residuals of the one regressed onto the other will inevitably fail to partition the fraction of the variation exactly explained by both.

b. Weather

Very little published evidence exists on the effect of seasonal weather impacts on mean Ellenberg values. The problem of signal attribution, i.e. estimating the extent to which change in mean Ellenberg scores has been influenced by the weather independently of changes in nutrient levels, would be more feasible where tightly coupled climate and vegetation records were available from annual or more frequent recording schemes. In this respect the growing

time series available in the UK from the Environmental Change Network (ECN) sites may provide useful data for evaluating the extent to which local change in similar vegetation is correlated with weather effects. The problem is particularly acute for snapshot surveys such as the GB Countryside Survey, which cover large spatial scales, but at roughly decadal intervals. There is however, scope for searching for weather related impacts by testing hypotheses based on existing models of expected relationships (Dunnett & Grime 1999). For example, Dunnett *et al.* (1998) analysed a 38 year record of changes in plant species performance related to weather and hypothesised that more competitive plants (largely high N value) should be favoured by cooler, wetter summers, while warm and dry spring and summers should favour ruderal and stress tolerant species (low N value).

c. Light

The response of species to soil N levels will depend in part on other conditions including light and water content. Increased mineral nutrition may allow plants to grow in deeper shade on alkaline, neutral and weakly acidic soil than on strongly acidic soils (Peace and Grubb 1982, Ellenberg *et al.* 1991). Thus the presence of a species of low N requirements, but high light requirements in deep shade, may not be indicative of low N soil levels, but may be a response of that species to shade conditions. The possible dependence of one index on another emphasises the unreliability of this method for detecting changes in soil N, when other environmental variables are not constant.

d. Bias and gaps in original estimates

The original Ellenberg values reflected the behaviour of plant species in central Europe. Concerns that the values might not be appropriate in other parts of Europe have led to a number of recalibrations either against local environmental data or using local floristic data. In Britain, the original values have been recalibrated using floristic data from Countryside Survey and the National Vegetation Classification (Hill *et al.* 1999, 2000). The approach was to iteratively re-estimate Ellenberg values for each species based on the values of the neighbouring species in each plot across the datasets used. A number of species were originally omitted from the Ellenberg number system because they ranged widely along each environmental gradient and so lacked discriminatory power. Therefore, the other ostensible advantage of the Hill *et al.* (2000) recalibration for Britain was that these less specialist species were also given new Ellenberg values.

Although attributing values to previously wide-ranging species is useful from the point of view of completeness, the Hill *et al.* (2000) calibration thereby introduced species whose wide response curves offer the worst violation of the requirement for equal standard deviations of species along environmental gradients (Schaffers & Sykora 2000; ter Braak & Barendregt 1986). Clearly, it is ecologically unrealistic to expect equivalent tolerances. However, it does mean that a certain amount of lack-of-fit between Ellenberg N values and environmental measurements is built in when these wide-ranging species are included. A possible way round this is to restrict analyses to only extreme species (e.g. Thompson *et al.* 1993).

e. Random colonisation / extinction events and sampling error

Rare and transient species in a sample include those most atypical of prevailing environmental conditions (Grime 1998). Their turnover could exercise a major influence on mean Ellenberg N values by skewing the distribution of these values in a sample unit. These random events will result from sampling error - including mis-identifications and plot relocation error - as well as the random immigration and local extinction of species ultimately

unable to establish and persist. These effects are currently being investigated within the Countryside Survey data for GB.

When using unweighted means, all species contribute equally, including those present in small quantities. It is thus important to include all species in vegetation surveys, as infrequent species may have a high value as indicators.

f. Species richness and species frequency effects

It is possible that large spatial or temporal changes in species richness across sampled units could also influence changes in mean Ellenberg N values as a result of statistical sampling effects. For example, Huston (1997) discussed a so-called ‘selection probability effect’ and a ‘variance reduction effect’ in his critique of biodiversity and ecosystem function experiments. These effects refer to the increasing or decreasing floristic similarity between samples as each includes more or less species from the wider species pool, respectively. Consider the example of a highly disturbed plant community that consists of a very small number of species that is therefore predicted to be dissimilar to an average sample of the local species pool. This small set of species will also display a particular range of Ellenberg N values. Then, if the patch is released from disturbance and species richness increases, the resulting mean Ellenberg N value may approach the local modal N value for the species pool *but only because of passive sampling of the species pool rather than because of change in nutrient availability*.

This phenomenon assumes that low species richness will coincide with biased mean Ellenberg values. This is likely to be the case where individual species and hence, Ellenberg values differ from a uniform distribution across a landscape; a situation likely to be true in most cases biogeographic regions (Figure 6.2). The result is that the most common Ellenberg values in a species pool are more likely to be represented in a random draw.

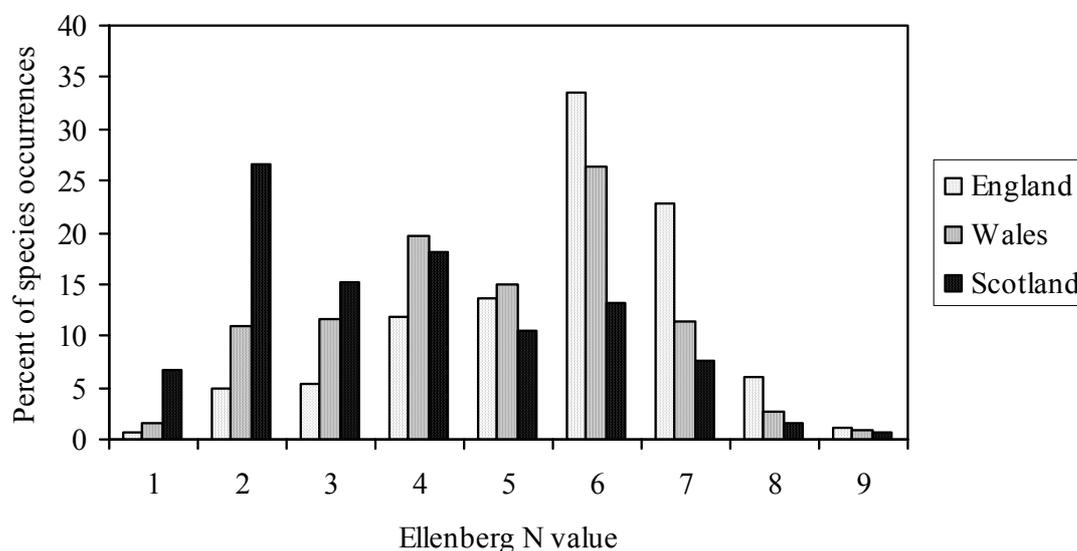


Figure 6.2: Percent frequency of Ellenberg N values in Countryside Survey vegetation plots recorded across GB in 1998 within England, Wales and Scotland (n=16,851).

The sensitivity of changes in mean Ellenberg scores to the uneven distribution of Ellenberg scores in local and regional species pools was also addressed by Schaffers & Sykora (2000). They proposed a solution where the contributions of species to each indicator value were downweighted according to their frequency in the species pool. The difficulty here is in defining a local species pool. An additional facet of the problem of uneven distributions of Ellenberg scores is the fact that Ellenberg values have an upper and lower limit. This means

that mean N scores sampled from vegetation representing extremes of the nutrient availability gradient could be based on extreme values and some intermediate values, but no values for even more extreme conditions. Hence, skewed distributions of N values in sample units would be more typical of these extremes than from intermediate situations (Schaffers & Sykora 2000).

g. Species pool variation and the availability of responsive species

Change in mean Ellenberg N scores is driven by changes in species composition. However, if sampled vegetation contains only stress-tolerant, low N value species, changes maybe modest or even undetectable even if nutrient availability increases. Consider the example of a blanket bog sampled such that the data consist entirely of stress-tolerant, slow-growing perennials with N values of 1 and 2. Nitrogen deposition may have increased, but these species merely respond by all increasing slightly in biomass, the net effect of which is that both unweighted and weighted mean Ellenberg scores remain the same. The artificially assembled calcareous grassland assemblage of Wilson *et al.* (1995) is perhaps an example of such resistance to perturbation. In their experiment, the expectation was that the invasive grass *Brachypodium pinnatum* would respond positively to added N and depress the abundance of neighbouring small calcicolous forbs. In this case, Ellenberg N values for forbs ranged between 1 and 3 while the value for *B. pinnatum* was 4. Their results however, showed a lack of response to increased N inputs. It is possible that the introduction of propagules of more responsive species would have resulted in more dramatic shifts in Ellenberg score, both as a result of increased cover of new dominants and reductions in competitively inferior species. In some situations, therefore, a lack of response maybe due to the absence of responsive species coexisting in sampled vegetation or absent from local species pools (Gough *et al.* 2000; Grace 2001).

The response of low N demanding species such as stress tolerant grasses to enhanced N deposition can confound the application of the Ellenberg index. These species are not typical of N rich ecosystems and have fairly low Ellenberg numbers. However they are able to respond to an increase in available N and can dominate total cover in high N deposition areas. Because some species typical of low N ecosystems are able to respond positively to increased N availability, the Ellenberg index may underestimate eutrophication and obscure species composition change in some cases. The increase in *Holcus lanatus* and *Deschampsia flexuosa* adjacent to a poultry farm, but with little overall increase in Ellenberg score (Figure 6.1) is a good example of this.

6.2.4. Suitability to indicate the effects of atmospheric N deposition

In themselves, Ellenberg N values cannot discriminate between different drivers of a detected floristic shift. For example, Lameire *et al.* (2000) analysed twenty years of vegetation change in a Belgian deciduous woodland. They hypothesised that floristic change had been driven by atmospheric N deposition and increased N mineralisation following lowering of the groundwater table. Whilst a marked change in the vegetation was detected, Ellenberg values only allowed them to quantify this signal. In the absence of independent measurements of water table depth and N deposition they concluded that “..it is impossible to separate the distinct effects of these two disturbance processes on the floristic composition of the forest ground vegetation.”

If changes in mean Ellenberg values are to be attributed to N deposition then the floristic response needs to be analysed along a gradient of N deposition (e.g. Pitcairn *et al.* 2002) and at varying levels of other suspected interacting factors. This amounts to applying the logic of a designed experiment, where the different contributory factors can be thought of as

treatments whose adequate crossing and replication across the sampling domain allows the partial contributions of each treatment and the interactions between them to be quantified. Large-scale monitoring and surveillance programs often lack such a controlled design because drivers may not be known before recording starts, while true replication of parts of the sampling domain maybe impossible (Stow *et al.* 1998). However, at the scale of a protected site, detailed recording of disturbance (i.e. management) and N deposition (biomonitored, directly measured or based on fine-scale model predictions) will at least provide data that can be used to attempt an analysis of the partial contributions of different drivers to changes in Ellenberg N scores. As the study by Lameire *et al.* (2000) illustrated, on their own Ellenberg values are likely to be of little use in signal attribution.

6.2.5. Links with other N variables

The Ellenberg N index has been correlated with a wide range of variables and has been shown to indicate soil and plant N status. For example, in Northern England in a survey of 45 species, good agreement was shown between plant tissue N content and N status of the habitat, while foliar N proved to be a good predictor of Ellenberg N index (Thompson *et al.*, 1993). Ellenberg indicator values for ground-flora have also been used to assess soil quality in British forests (Hawkes *et al.* 1997, Wilson *et al.* 2001). Hawkes *et al.* (1997) found soil parameters to be well correlated with abundance-weighted scores for nitrogen and pH (reaction). When the Ellenberg indices for N and pH were combined, the relationship was sufficiently robust to suggest the use of the combined index in separating sites in terms of nutrient status in the absence of soil analytical data, provided that species cover was adequate.

6.2.6. Ease of use

- Only botanical identification skills are necessary.
- The calculation of mean Ellenberg values is very simple while GB values are also readily available on the web at www.ceh.ac.uk/products_services/publications/, and means can be generated using the MAVIS software downloadable free at www.ceh.ac.uk/products_services/software/mavis.htm.
- A large published literature provides guidance on many aspects of the use of Ellenberg values.
- The simplicity of these indicators and their ready availability for the GB flora has also led to their recommendation as key community variables for future monitoring of agri-environment scheme land. Again though their strength is recognised as being signal detection rather than attribution (Critchley *et al.* 2002).

6.3. Other N bioindicators based on higher plant species composition

6.3.1. Functional Nitrogen Index for Species (FNIS)

While many studies have demonstrated the usefulness of Ellenberg's indicator system, there is uncertainty over the interpretation of Ellenberg's N values. The values, determined largely from phytosociological information and the somewhat subjective ecological behaviour of species in the field, have been shown to be related to a number of soil variables. As a response to these uncertainties, Diekmann and Falkengren-Grerup (1998) devised an index based on mineralization rates of various forms of soil N for forest vascular plants. Soil N mineralization rates for ammonium, nitrate and total N were determined by incubation

experiments for deciduous forest plots in southern Sweden. Species N indices for nitrate and ammonium were calculated as the weighted averages of mineralised N in those plots where the species were present. The species indices for ammonium and nitrate were then combined to produce a functional N index for each species (FNIS). When examined in relation to species composition data, the ammonium index alone and FNIS explained more floristic variability than Ellenberg N values. However, when transformed into classes, the values tended to be similar to Ellenberg's N values. While ammonium indices differed between regions due to differences in N deposition and mineralization and nitrification rates, FNIS (combining ammonium and nitrate indices) differed less and hence was more robust. The authors found FNIS to be a reliable expression of species response to N availability in deciduous forest soil of southern Sweden.

6.3.2. N_{dev} and Life History Traits

Diekmann and Falkengren-Grerup (2002) developed N_{dev} as a measure to reflect soil N status in respect to N and pH at which individual species occurred, and aimed to use it to predict response to enhanced N deposition. They calculated N_{dev} for all species in the study sites of southern Sweden, based on their observed versus expected nitrification rates at a given soil pH. They then used the measure to predict changes in species abundance and compared the results with life history traits of the species involved. The latter approach was recently developed by Lavorel *et al.* (1997, 1999), whereby species have been classified into groups sharing biological parameters that may be affected by disturbance. The life history traits used in the prediction exercise included life form, phenology, plant height, anatomy, growth rate, foliar N content and others. The exercise showed N_{dev} to be related to species frequency change in the Swedish study sites and other areas of central Europe and to also be able to predict species response to short-term fertilizer experiments. N_{dev} proved to be a better predictor than any life history traits measured, although plant height, leaf anatomy, foliar N and phenology were significantly correlated with species change. The authors concluded that species likely to increase in abundance in response to enhanced N deposition are those favoured by a high soil nitrification rate at a given soil pH, of tall stature with a hydro- or heliomorphic anatomy, large foliar N content and late phenological development.

Further studies from Sweden have focussed on the use of vascular plants as indicators of N enrichment in soils by calculating indices using Ellenberg's N values, FNIS and literature data on tissue N content of plants (Falkengren-Grerup and Schottelndreier 2003).

6.4. Higher plant and bryophyte N bioindicators: Conclusions and Application

6.4.1. Ellenberg Indices

- The Ellenberg nitrogen values of species may be used to provide mean Ellenberg scores or cover/abundance weighted scores of sites as a means of *detecting a signal* of change related to nitrogen availability.
- Ellenberg values by themselves, however, have no capacity for detecting the unique effects of N deposition, as compared with other sources of nitrogen, and *signal attribution* must rely on parallel recording of other potential drivers of the eutrophication signal. As a biomonitoring tool for assessing the impacts of atmospheric nitrogen deposition, the Ellenberg approach therefore needs to be applied jointly with local estimates of atmospheric N deposition, from other physical- or bio-monitoring methods.

- Analyses of vegetation change should take into account interactions with pH and disturbance/succession. This can be attempted using the analysis of covariance approach applied by Diekmann & Dupre (1997) to Ellenberg Light and Nitrogen scores, and more generally by analysing residuals after removing variation explained by change in the Ellenberg Light score. Interpretation will also depend on developing hypotheses about the inter-relationship between changes in pH, N and disturbance that are appropriate to the ecosystem. For example, disturbance may enhance responses to increased N in upland *Calluna* heath by allowing competitive grasses to invade (van der Wal *et al.* 2003), while in lowland mesotrophic grasslands release from disturbance can enhance the eutrophication signal because of biomass accumulation and the competitive displacement of smaller species with lower Ellenberg N values (Bakker 1987; Schaffers 2002).
- Where the variance explained by the Ellenberg N index in a study is low, attempts should be made to heighten the sensitivity of N scores by applying a cover/abundance weighting and testing the effect of a downweighting for the most common species pool members using the approaches suggested by Schaffers & Sykora (2000).

6.4.2. Other species based N indicators

- The Functional Nitrogen Index for Species (FNIS) has been developed for Swedish woodlands and shows a better relationship to estimated nitrogen deposition than the Ellenberg index. The limitation in the application of this approach to the UK is the requirement for intensive soil analysis and collation of published data.
- The N_{dev} index has been applied for Swedish woodlands and provides a measure to reflect soil N status in respect to N and pH at which individual ground-flora species occurred and to predict response to enhanced N deposition.
- There are currently insufficient data available to fully assess the suitability of these methods for monitoring purposes in the UK. Current evidence is not adequate to support a robust relationship between FNIS, N_{dev} or life history traits and N deposition.
- Bearing in mind the limitation of the Ellenberg N index in certain situations, initial pilot studies using sites where soil N data and species abundance data are available should be carried out to see if an index similar to FNIS or N_{dev} can be developed for the UK. Application of these methodologies would require an experienced botanist and/or soil scientist and suitable laboratory equipment.

7. BIOINDICATOR METHODS FOR NITROGEN BASED ON COMMUNITY SPECIES COMPOSITION: LICHENS

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7.1. Introduction

7.1.1. Background

Lichens have long been known to be highly sensitive to changes in atmospheric conditions and in particular to pollution caused by sulphur dioxide (SO₂) arising from industrial and domestic fuel burning. Early studies correlated lichen diversity with SO₂ concentrations, which led to the development of a number of methods based on epiphytic indicator species including both species sensitive to and those tolerant of pollution (de Sloover & le Blanc, 1968, 1970. Hawksworth and Rose, 1970; Kricke and Loppi, 2002). Areas lacking epiphytic lichen vegetation were described as ‘lichen deserts’. However, since peaking during the 1970’s SO₂ emissions and the resulting acid deposition have fallen dramatically both in industrial/urban areas and the wider countryside, so that today nitrogen deposition predominates over sulphur. In many urban areas, NO_x is often the major pollutant in the vicinity of roads and areas where traffic use is heavy, whilst intensification of agriculture in rural areas has resulted in enhanced deposition of ammonia (NH₃), ammonium (NH₄⁺) and organic N. On protected sites, lichen communities containing cyanobacterial photobionts such as the Lobarion were also shown to be affected by acid rain (Gilbert, 1986).

7.1.2. Nitrogen and lichen communities

Lichen communities associated with nitrogen-rich environments occur naturally (e.g. coastal rocks subject to bird excreta and/or locally high atmospheric NH₃ concentrations) and also accumulate the highest N contents (Rai 1988). Lichen communities in nitrogen- and phosphate-rich habitats provided by bird perches may include common widely distributed species such as *Xanthoria parietina* and also may contain a number of local or rare, including Priority BAP, species (James *et al.*, 1977, Church *et al.*, 1996). However, the majority of lichen-rich communities are found in conspicuously nutrient-poor habitats, so that enhanced nitrogen deposition may become a threat to the conservation of these communities.

Although there is plenty of evidence to demonstrate the toxic effects of nitrogen on vegetation and lichens, there is a poor understanding of how nitrogen acts to stimulate or depress growth in lichen thalli. Nitrogen is both an essential nutrient and/or a toxic substance according to the type and quantity of the compound deposited and the requirement of each species. Nitrogen is present in many forms e.g. inorganic / organic and as an acid or base. Ellenberg (1988) identified a range of environmental factors affecting the distribution of vegetation including nutrient status and toxiphoby. Subsequently, Wirth (1992) applied this method to lichens for Germany, based on expert judgement of the species and ecological

conditions. Wirth separated nutrient-N effects from toxic effects of sulphur dioxide or other compounds with a toxic effect, applying a scale of 1-9 for both categories.

Nimis and Martellos (2001) carried this further and tested the predictive value of indicator species in Italy for a range of conditions and have now applied this for 2,200 species of the Italian flora providing indicator values for pH, air humidity, solar irradiation and eutrophication which are available on the internet in ITALIC (<http://dbiodbs.univ.trieste.it/>) (Nimis and Martellos 2002).

7.2. Nitrogen and atmospheric pollution

7.2.1. Changes in atmospheric conditions

With falling SO₂ emissions in the 1990s, there has been a rapid increase in ‘nitrophytic’ vegetation, although there has been little change in annual atmospheric nitrogen deposition over the last 20 years (NEG-TAP 2001). This shift has been most conspicuous in areas where there was a large fall in SO₂ deposition. In urban areas the increase in NO_x associated with increasing volume of traffic is notable such as in the London area (Davies *et al.* 2002) and at Burnham Beeches where NO₂ levels have increased while SO₂ levels declined (Purvis *et al.* 2003).

7.2.2. Lichen diversity and substrate nutrient status

Lichen diversity has increased most markedly in urban areas and includes a conspicuous number of ‘nitrophytes’, as defined by van Herk (1999), while formerly frequent ‘acidophyte’ species are disappearing. The observations of van Herk (1999) in the Netherlands related particularly to enhanced concentrations of ammonia, which also leads to an increase in bark pH. The bark of many tree species is naturally acidic, and this provides a mechanism by which the acidophyte flora is replaced by nitrophytic lichen species. The extent to which SO₂ may favour natural acidophyte communities in the presence of ammonia is not clear, and it may be that the widely observed deleterious effects of SO₂ are a combination of strong acidic effects (Türk and Wirth 1975) (in contrast to the weak acids of bark) and toxic effects of sulphate.

7.2.3. Land management and atmospheric nitrogen

Over the same time period there have been dramatic changes in grassland management, in farming practices including stocking rates and in fertiliser and manure application, so that without also monitoring these changes it is difficult to predict how these have affected lichen communities. In the Netherlands ammonia concentrations have been widely recorded in the vicinity of intensive pig and cattle rearing units, allowing van Herk (2001) to define indicator species associated with high levels of ammonia using multivariate analysis. In the UK the monitoring of NH₃ concentrations levels is carried out at around 100 sites (Sutton *et al.* 1998, 2001b, Tang *et al.* 2001), and this data set is only now beginning to be used to investigate the relationship to lichen diversity. The limited number of available comparisons with atmospheric NH₃ and NO_x makes assessment of lichens as biological indicators more difficult.

7.2.4. Spatial models and local deposition

Although the rapid development of spatial models has meant that nitrogen deposition can be predicted over the UK (e.g. NEG-TAP 2001), the available monitoring data show a high degree of local variability, which coupled with topographical, microclimate and host substrate effects leads to a considerable variation in lichen species composition. In assessing the scale and purpose of biomonitoring methods, the pattern of distribution of nitrogen

compounds must also be considered in order to distinguish the very local effects of ammonia and nitrogen oxides, from the long-range dispersal and deposition of the resulting ammonium and nitrate in aerosols and precipitation.

7.2.5. The importance of bark pH

The effect of acidification on bark pH was demonstrated early on, with low bark pH associated with an increase in ‘acidophyte’ vegetation including the rapid increase of many species formerly associated with the naturally acidic bark of pine in Scottish woodlands (Hawksworth *et al.* 1974). Conversely, there has been a considerable body of evidence to show that an increase in bark pH is strongly correlated with a shift in lichen communities from naturally acidophytic to nitrophytic (de Bakker, 1989; Van Dobben and Wamelink, 1992; Van Dobben and ter Braak, 1993). De Bakker (1989) showed that bark pH was more important than ammonium concentration along a transect on oak from a nature reserve to an intensely farmed agricultural area. However, recent recording of bark pH of trunk and twigs of oak has shown little difference, despite a shift towards nitrophyte communities on the twigs (Wolseley and James 2002). Research by Loppi and De Dominicis (1996) in Italy showed no association of ‘nitrophyte’ species with bark pH or with nitrogen deposited on the bark. Further results of Loppi and Pirintsos (2000) in Italy and Greece suggest that dust may be an important factor in encouraging nitrophyte dominated lichen communities in southern Europe and that the typical Xanthorion lichen community develops with either acid or alkaline dust in areas without agricultural management. Hence, while clear relationships with ammonia emissions have been observed in northern Europe, there are clear limitations to the relationship in drier climates. However nitrogen is applied in many forms from gaseous and particulate forms and wet deposition from the atmosphere to solid forms in mineral fertilizers and dung. These authors also point out that most nitrophytes are also xerophytes. More research on the effects of nitrogen and dusts of varying chemical composition on individual lichen species is required to identify factors associated with these changes.

7.3. Lichens as pollution indicators

7.3.1. Lichen diversity indices

The use of lichen diversity as a method of assessing environmental changes has arisen from two different approaches: firstly, the identification of sensitive or tolerant species which are used to develop a qualitative scale using expert judgement to select appropriate species (Hawksworth and Rose, 1970; Deruelle, 1977; van Haluwyn & Lerond, 1988; Wirth, 1992) and the quantitative analysis of diversity and frequency of lichens determined by a strict sampling procedure (e.g. VDI 1995, Asta *et al.* 2002, Brunialti *et al.* 2002, Purvis *et al.* 2002). The results of both procedures have been used to map zones of lichen presence and frequency associated with levels of air pollution.

Since the fall in SO₂ concentrations, there has been an increase in lichen diversity especially in areas of former lichen “deserts”, but this did not follow expected patterns. Former ‘acidophytic species’ did not return, instead an increase in members of the Physcietum associated with high nitrogen was observed (Hawksworth and McManus 1989). However, this shift towards nitrogen tolerant species was also observed in rural areas, including areas that were formerly characterised by acidophyte communities in regions of high rainfall in the west of Britain. This change was recognised in other countries in Europe and the relationship between agriculture and lichen communities came to the fore in both Denmark and the Netherlands, where intensification of livestock farming was associated with a replacement of acidophyte communities by nitrophyte communities.

7.3.2. Indicator species and scales

Indicator scales have been developed using selected species with known sensitivity or tolerance to the factor identified (see van Haluwyn *et al.* 2000). Although scales have been applied to a range of factors such as ecological continuity of woodlands (Rose 1992, Coppins *et al.* 2002), to SO₂ concentrations (e.g. Hawksworth *et al.* 1970) and more recently to ammonia (van Herk 1999), the application is based on regional knowledge of the species distribution and ecological range. If a species varies in ecology across its distributional range, it may not be appropriate across the range or may be replaced by another in a different geographical region. In addition, experimental data for the selection of indicator species are often lacking. So although these have given useful results in regions of similar climatic character, the development of indicator scales appropriate to regions with a different bioclimatic character has to be assessed on an established dataset and, where possible, the physiological basis established for species that require high nutrient supply and those that are sensitive to excess nutrients.

7.4. Biomonitoring using lichens to detect atmospheric N in agricultural areas

The effect of agricultural intensification and increased ammonia emissions was investigated by Søchting (1995) in Denmark and in the Netherlands by de Bakker (1989) and van Dobben and de Bakker (1996). In these initial studies, there was still little evidence for mechanisms by which nitrogen affected lichens, and the concept of ‘nitrophytes’ was still a qualitative assessment. Concern in the Netherlands led to the establishment of widespread monitoring of atmospheric ammonia and when this was combined with detailed recording of lichens on c. 55,000 wayside trees, demonstrated a strong correlation of certain nitrophytic species with ammonia concentration (van Herk 1999, 2001). Further analysis of the data allowed van Herk (1999) to develop indices of nitrophyte (NIW) and acidophyte (AIW) lichens for the Netherlands (Figure 7.1). In France the association of nitrogen tolerant species with intensive land use and nitrogen sensitive communities with natural woodlands was used as a basis for selecting easy to identify indicator species in order to map areas affected by intensive farming (R. Lallement *et al.* 1999).

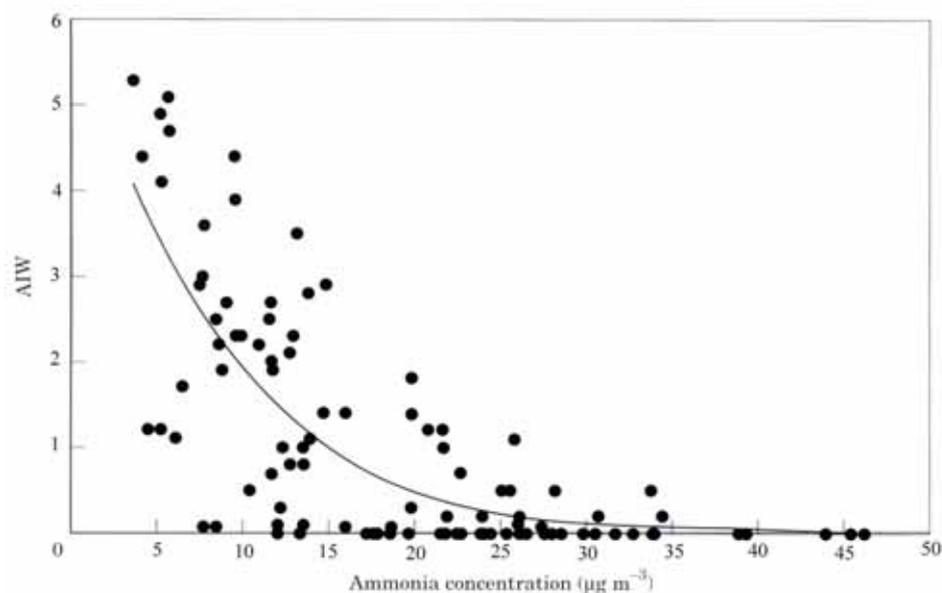


Figure 7.1: Relationship between the abundance of lichens on oak trunks that prefer naturally acidic bark conditions ('acidophytes', AIW) and annual mean atmospheric concentration of NH₃ from data across the Netherlands (van Herk 2001). At approximately 35 µg m⁻³ all acidophytic lichen species have disappeared from oak tree trunks.

7.4.1. Application of methods in UK in climatically different regions

Recent investigation of local sites in the east and west of Britain has demonstrated the need to define appropriate nitrophytes and acidophytes for UK climatic conditions (Wolseley and James 2002). For example, these authors found a good relationship between the occurrence of nitrophyte and acidophyte lichens (van Herk 1999) on oak and ammonia concentrations (Sutton *et al.* 2001) at the sampling sites. However many species known as acidophytes or nitrophytes in Holland were absent in the UK and conversely species were present in both communities in Britain that were excluded from the list of indicators for Holland (van Herk, 1999). Wolseley and James (2002) also extended the approach of van Herk (1999) to score for acidophytes and nitrophyte occurrence on oak twigs. Their results showed higher bark pH of twigs than trunks and this difference was consistent with increased occurrence of nitrophytes on twigs.

7.4.2. Comparison of methodology around a poultry farm in Scotland

A further test of the nitrophyte and acidophyte approach on trunks and twigs was made by Wolseley and James down wind of a poultry farm in southern Scotland (Pitcairn *et al.* 2004a, see Appendix I). In this case other acid bark tree species (spruce, pine, birch) were included in the analysis, which was conducted through a mixed species woodland stand. Although significant differences in shading occurred, leading to fewer species in the most shaded areas, the results showed a clear response to ammonia concentrations (Figure 7.2). As with the surveys in eastern and south west England, twigs acidophytes were found to be more sensitive than trunk acidophytes, while a combined score of NIW-AIW showed an approximate linear decrease with log of NH₃ concentration.

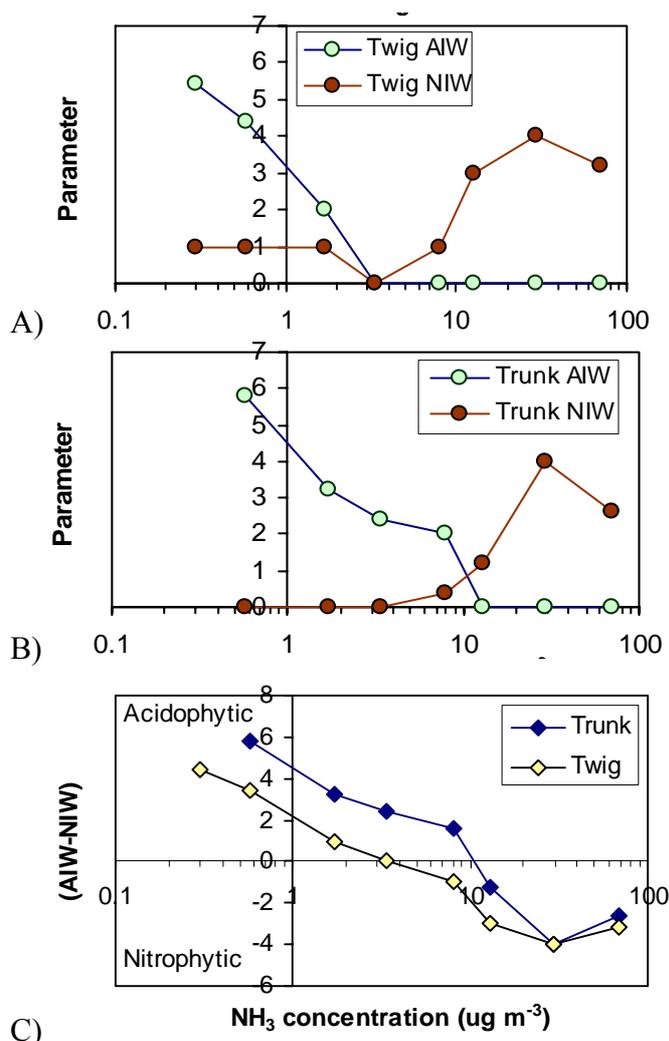


Figure 7.2: Occurrence of lichens on the trunks and twigs of a mixed woodland adjacent to a poultry farm in Scotland (Picairn *et al.* 2004a, see Appendix I). A) Lichens on trunks were scored using the van Herk (2002) approach, while B) lichens on twigs were scored using a modified sampling procedure of Wolseley (2002), with in both cases the AIW index representing ‘acidophytic’ species (which prefer naturally acidic bark) and the NIW index representing ‘nitrophytic’ species (which prefer nitrogen enriched more basic bark resulting from enhanced NH_3 concentrations). C) Subtraction of AIW-NIW provides an overall index of whether the flora is acidophyte or nitrophyte dominated.

What is particularly striking about the results from southern Scotland is the low NH_3 concentrations at which NH_3 is seen to be affecting lichens. The nitrophyte species only increase with NH_3 concentrations larger than $\sim 3\text{--}8 \mu\text{g m}^{-3}$, but the acidophyte species decrease at smaller concentrations: decreases occur below $2 \mu\text{g m}^{-3}$, with complete loss of trunk acidophytes at $\sim 12 \mu\text{g m}^{-3}$ and for twig acidophytes at $\sim 3 \mu\text{g m}^{-3}$. Comparison with the results of van Herk (1999) (Figure 7.1) shows that the trunk acidophytes disappear at much lower NH_3 concentrations in the study in Scotland than in the Netherlands. Further investigation is needed to explore these differences, but they may in part be due to higher acid gas concentrations (SO_2 , HNO_3 etc) in the Netherlands than in this relatively clean part of Scotland. Recent research on lichen distribution and wet deposition of pollutants including ammonium (NH_4^+) in 25 European atmospheric monitoring sites has shown a strong correlation of the loss of acidophytes in clean-air sites with low levels of ammonium, whereas the major increase in nitrophytes appears to be associated with an increase in bark pH due to NH_3 (van Herk *et al.* 2003).

The results of the Scottish study (Pitcairn *et al.* 2004a, see Appendix I), clearly show the NIW and AIW scores being consistent with increased bark pH on twigs and trunks near the farm NH₃ source. However, it was notable that the maximum bark pH occurred at the second sampling station from the poultry farm. At the closest point to the farm a small decrease in bark pH occurred, which was accompanied by a decrease in the NIW score for both twigs and trunks. This feature was also seen very close to a poultry farm in the study in eastern England (Wolseley and James 2002), and may be due to the parallel emission of nitrogen containing dusts from poultry housing. Further investigations are required to explore this effect.

The acidophyte/nitrophyte scoring system of van Herk (1999) provides an excellent description of lichen sensitivity to atmospheric NH₃ in Holland, but contains few of the same species in the UK, particularly in the more oceanic west. It is also rather labour intensive to carry out, often requiring the identification skills of a specialist lichenologist. There is therefore a complementary need for a practical approach that can provide a basic level of information and be conducted by non-experts. The use of a scoring system is applied to each species as a cover value (from 1-6) by van Herk and as an indicator value for each lichen species present for Ellenberg scores with a Nitrogen tolerance value from 1-9 (Wirth 1992). The Ellenberg approach takes less time since sampling can be conducted relatively quickly (based simply on species presence), and the method is also amenable to simplification where only a short list of the most easily identifiable species are included.

Figure 7.3 shows the application of the Ellenberg scoring system to the same lichen transect as indicated in Figure 7.2 (Pitcairn *et al.* 2004a, see Appendix I). In Figure 7.3A the average site Ellenberg scores were calculated from all the species identified. This shows a consistent response to elevated NH₃, particularly the twig lichens, but is not as sensitive to low NH₃ concentrations as the acidophyte's score calculated using the van Herk method (1999) (Figure 7.2). A simplification of the Ellenberg approach was tested whereby only well known and easily identifiable foliose and fruticose species were included (no crustose species were included).

The results of the simplified Ellenberg approach (Figure 7.3B) show that although the method is less sensitive to NH₃ (as would be expected), the effect of high NH₃ concentrations is still clearly seen. The further development of such simplified systems has the potential to involve non experts (including conservation agency field officers) and allow a screening of sites for which further more detailed monitoring by lichenologists is warranted.

7.5. Biomonitoring with lichens in urban environments

7.5.1 Urban environments and lichen diversity

Urban environments provide diverse habitats for lichen colonisation and in terms of biodiversity are now experiencing cleaner air than they have for 200 years. Owing to the historical effects of SO₂ air pollution, comparatively few rare species are found in the vicinity of major cities such as London, but are restricted to the urban fringes in relict habitats such as sub-urban ancient woodland e.g. *Pyrenula nitida* (Burnham Beeches). A survey on young oaks in Regent's Park in January 2002 recorded 38 lichens on young *Quercus* including some not seen prior to the impact of industrialisation in the 19th century (James *et al.* 2002). Parks, urban commons, graveyards, roadside verges and even derelict areas (urban wasteland and railway sidings) all create sanctuaries for lichens. The wide variety of tree species including exotics with varying bark properties, and buildings and monuments of different stone types (hardness and rock chemistry), help to create varied niches.

Approximately 11% of the species included in the UK Red List of Lichens are closely associated with the cultural landscape (Church *et al.*, 1996). In many urban environments there is a lack of information on lichen communities present prior to urbanisation. Exceptions include areas of ancient woodland (e.g. Epping Forest James and Davies 2004) and heathland (e.g. Hampstead Heath), which represent traditional hunting grounds of early lichenologists who deposited samples in national herbaria. The loss of lichen diversity is due not only to pollution, but also to many other factors to do with land management and loss of ecological continuity.

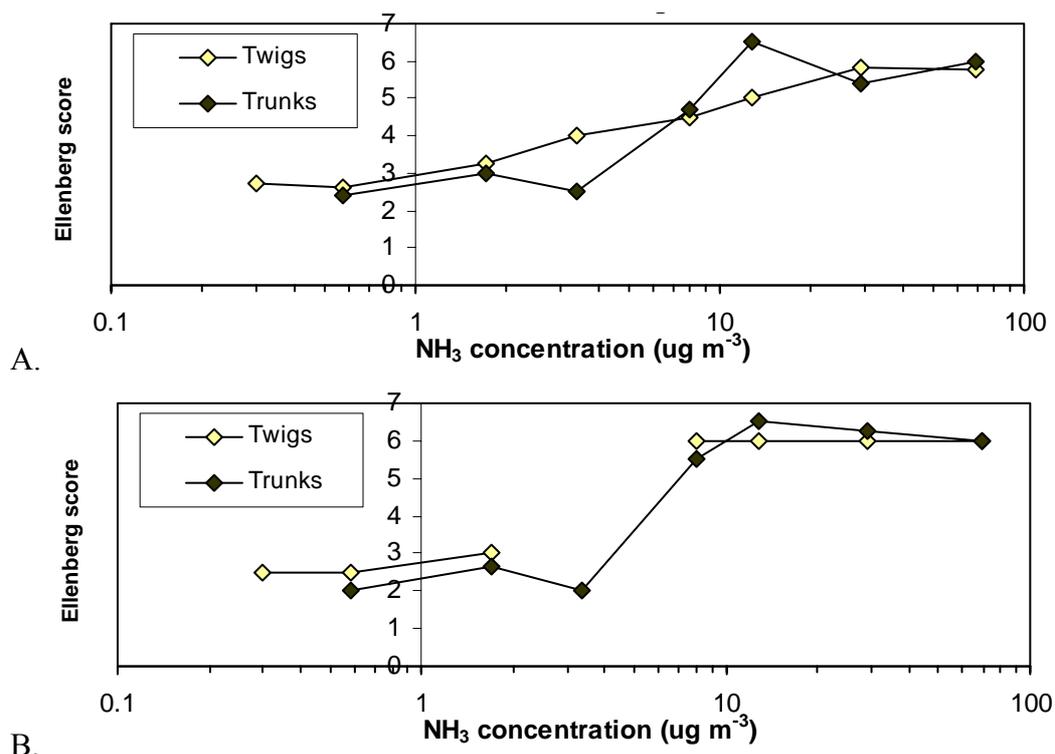


Figure 7.3: Application of the ‘Ellenberg’ scores for lichen flora (Wirth 1992) to a transect through a deciduous woodland adjacent to a poultry farm in southern Scotland (Figure 7.2). A) overall site Ellenberg index based on presence of all species identified; B) simplified Ellenberg index based on only easily identifiable foliose and fruticose lichen species.

7.5.2. Biomonitors of environmental health

Many of the methods developed to monitor changes are derived from a concern for environmental health in densely populated areas where the environment has been substantially altered over a long period of time. Various standards have been set for NO₂ concentration to protect human health, vegetation, and ecosystems, but current EU standards to protect sensitive species from negative effects of NO_x were determined in a very different pollution climate than today. Unlike SO₂ and NH₃ in the Netherlands where lichen distribution studies correlated with physicochemical data lead to the development of indicator scales, the impact of NO_x remains poorly quantified. Both the IAP and the VDI (1995) methods are dependent on a standardised procedure on selected trees distributed over a wide geographical area, but this has yet to be fully tested in UK cities. The EU recording method (Asta *et al.* 2002) has been tested in London and showed differences in lichen diversity between inner and outer London (Davies *et al.* 2002). Higher numbers of nitrophyte species were recorded in central London compared with agricultural areas.

There is an urgent need to identify new methods to monitor the effects of N pollution on lichens in relation to protective legislation and to determine if semi-quantitative scales using indicator species relevant to the current pollution climate of cities can be established.

7.6. Biomonitoring effects of atmospheric N deposition on statutory nature conservation sites in the UK

7.6.1. Location of lichen species-rich sites

Many of the species-rich communities of the UK are acidophytic and associated with oceanic conditions of high rainfall in the south and west of Britain, or of acidic vegetation on nutrient-poor uplands. In the past the highly oceanic distribution of rainfall meant that many of these lichen communities in rural areas survived historical changes in air pollution. It is therefore now of great concern that there is a marked increase in nitrophytes across the country in a great range of habitats from epiphytic to terricolous and saxicolous. Many of the lichen-rich woodlands are ancient sites with a high number of indicators of ecological continuity (Rose 1992, Coppins and Coppins 2002), which are highly sensitive to changes in environmental conditions.

The research on lichens as indicators of environmental change has concentrated on assessing changes in epiphytic communities at the species and community level and where possible testing this against atmospheric conditions. As there has been little data available until recently on atmospheric nitrogen, little work has been done to correlate the results.

7.6.2. Assessing changes in lichen species sensitive to increased N

The use of fixed quadrats on sensitive lichen communities to monitor changes over time was undertaken in epiphytic, terricolous and saxicolous habitats. A UK wide project undertaken by the NHM between 1986-1990, to assess changes in species of *Lobaria* and the Lobarion community in protected sites, used fixed quadrats to monitor changes. Sites were selected in close proximity to atmospheric monitoring stations, but in the conditions of falling SO₂ values the evidence for thallus loss being associated with acidification was only strong in regions where acid deposition remained high (e.g. Pennines). In other areas, deterioration of *Lobaria* species continued despite the amelioration of acid deposition. Many of these sites are in open wood pasture and parkland adjacent to agricultural improvement, where atmospheric nitrogen would have an effect, although nitrogen may be only one of a number of factors affecting these species (Looney & James 1989; Wolseley & James 2000).

Where changes in thallus area are combined with parallel information on atmospheric conditions, it is possible to assess changes in relation to nitrogen deposition and also accumulation (Purvis *et al.* 2003). Research on lichen communities on oak twigs on internal and boundary margins of a National Nature Reserve (NNR) in Wales showed that there were significant differences in the twig flora of internal margins of long established glades within the woodland site and margins adjacent to a range of land use from intensive grassland management, to old pasture and heather moorland (Wolseley and Pryor 1999).

Recent physiological research by Hyvärinen & Crittenden (1998a,b) has shown the damaging effects of nitrogen on species of *Cladonia* in heathland, but the development of methods for quantifying accompanying loss of diversity per unit area has only been applied to a few species in the UK (e. g. species of *Lobaria* (Wolseley and James 2000)).

7.6.3. Assessing effects of N on rare / local BAP species

Changes in the distribution of rare and local BAP species may be more difficult to interpret as many of these species are associated with local microhabitats and have highly specialised requirements. There are many species that have shown a decline that may be related to a change in nitrogen levels resulting from agricultural intensification. Species showing a decline in inland and agricultural areas include BAP species *Teloschistes flavicans* and *Fulgensia fulgens*. *Teloschistes* was formerly found inland on trees and in unimproved terricolous communities but is now lost from inland sites and restricted to a few maritime sites (Gilbert and Purvis 1996; Purvis and James 1995; Wolseley and James 1997). However, whilst there is no measurement of nitrogen deposition at these sites, the presence of ubiquitous 'nitrophytes' in areas where the species formerly was abundant suggests that N rather than climatic factors, such as humidity, may be responsible for this decline. The monitoring of the distribution and health of a population was undertaken in island and mainland habitats (Wolseley & James 1997) and the rapid deterioration of the mainland population was associated with adjacent land improvement, which again suggests a link to increased NH₃ emissions.

7.6.4. Terricolous species

Fulgensia fulgens is a terricolous lichen of short-cropped calcareous grassland that has declined in many areas. A conspicuous loss of this species and other rare species in the Brecklands including *Buellia asterella* and *Squamarina lentigera* has been monitored by Gilbert (2001). These species are associated with close-cropped turf and low nutrient status and their loss is associated here and in other places with an increase in coarse grasses, bramble and bracken. In Wales there was a rapid decline in a population of *Fulgensia fulgens* at the Stackpole NNR, where the site was adjacent to intensively stocked grassland with a strong smell of livestock during a winter survey of the site (Wolseley & James 2001). Ammonia concentrations were monitored at Stackpole as part of the National Ammonia Monitoring Network (Sutton *et al.* 1998, 2001e) during October 1996-November 1997. The mean monthly concentration was 2.6 (range 0.42 – 7.2) µg m⁻³, with the peak concentrations coinciding with autumn and spring when manure spreading is most common. Although these concentrations are clean by comparison with Dutch conditions, they are larger than the concentrations shown to affect twig acidophytes in Scotland (Figure 7.2), and background concentrations in the UK are around 0.05-0.3 µg m⁻³. This suggests that the loss of *Fulgensia* at Stackpole is entirely consistent with increased NH₃ emissions.

7.6.5. Saxicolous species

Rock surface (saxicolous) habitats are also lichen-rich and considerably affected by nitrogen deposition where surface nutrient may encourage algal growth of *Desmococcus* and other species on the surface of the lichen which in effect shades out and kills the photobiont of the lichen. A survey of Sarsen stones on Fyfield Down in south west England identified considerable damage to lichen communities. It was observed that these changes followed a change in surrounding livestock grazing, which increased the number of stock four fold for shorter periods of time (O'Dare & Coppins 1994).

Recent work comparing saxicolous, terricolous and epiphytic habitats in Scotland using a method that has been applied over 7 European countries has provided data on distribution of lichen species and their association with farming and forestry conditions (Scheidegger *et al.* 2002). This work also suggests that using multivariate analysis of both lichen and deposition data would allow us to detect association's characteristic of increased nitrogen in terricolous and saxicolous sites. However, in situations where ecological continuity has allowed stable

communities to develop on rocks, it may take some time for crustose communities to respond to changing conditions. This was apparent in Pembrokeshire where lichens on slate gravestones and ash twigs were sampled on a transect from a point source of oil refineries. The result showed high correlation of distance from source with the twig lichen flora but no correlation for the gravestone lichen flora (Purvis et al. 1998).

Nitrogen also affects many of our fresh water systems where excess nitrogen is often available at high levels in streams and rivers during periods of fertiliser application on surrounding arable or grass leys. There is little research on the effects of this on lichen communities in the UK, but recent monitoring of populations of the rare *Collema dichotomum* shows that this species is associated with salmon or trout rivers where surrounding landuse is unimproved and water quality high (O'Dare & Coppins 1995).

7.7. Lichens as N bioindicators: Conclusions and Application

- Eutrophication through deposition of nitrogen is a major environmental problem in both urban and rural areas. As many lichen-rich sites of high conservation interest are associated with acidic nutrient-poor habitats and substrata, changes accompanied by an increase in available nitrogen may represent a major threat to sites of conservation interest. The effects of nitrogen on base-rich substrata require further investigation.
- In the available datasets it is often difficult to identify causality where there are multiple factors as well as pollution gradients. A quantitative approach to lichen recording is therefore essential in combination with physical monitoring of atmospheric N concentrations in order to improve the characterization of lichen species N sensitivities.
- Further investigation is needed to identify and examine the effects of lichens of different components containing nitrogen in for instance; organic and inorganic fertilisers, car exhausts and industrial effluents.
- The vicinity of point sources provides a particularly valuable context to interpret the impacts of N deposition in different regions of the UK. The most useful results are obtained where complementary interpretive measurements are made, for example, comparison of estimated N deposition, lichen species occurrence, lichen N chemistry and pH of the substrate.
- The use of lichen communities on twigs in areas where trees are a component of the landscape in hedgerows, parkland and woodland edges complements the traditional approach of recording lichens on tree trunks. Lichens on twigs respond more rapidly to changing environmental conditions and provide an early warning system where changes have not yet affected the lichen communities of trunks. The data from both substrata can be used to establish calibrated indicator values for species in the UK.
- While progress has been made in analysing national relationships to N deposition in the Netherlands, it is a remaining challenge to accumulate data for the UK over wider areas where lichen diversity can be compared with nitrogen deposition and other factors.
- Much of Scotland and Wales has very little tree cover, although there are extensive saxicolous and terricolous lichen communities over large areas of land. Recent work in Scotland suggests that these communities of acid rocks and heathland are highly susceptible to increased N deposition and that further sampling in combination with

accumulation of data on N deposition would enable the development of appropriate indicators.

- It is envisaged that overall detection of deterioration in lichen communities due to nitrogen could be established across protected sites. However, the effects on individual BAP lichen species may be more difficult to assess. As their distribution may be associated with a number of factors it is imperative to have monitoring systems in place which include all species in the community to allow assessment of shifts in associated species that may be indicative of nitrogen deposition or other environmental changes. As the distribution of individuals may change with time it is important to devise a sampling system that can pick up changes in the cover and distribution of a rare species (Wolseley *et al.* 2002).
- In parallel, there is a need to further develop and test simpler systems for biomonitoring of N using macro-lichens that are easily identifiable by non-experts. In this respect the use of lichen Ellenberg scores appropriate to the UK may provide a basis for generalized assessment, with reasonable results being possible through identification of the most common and easily identifiable species. Further development is required in this area, particularly in characterizing N preferences of characteristic lichen communities.

8. BIOINDICATOR METHODS FOR NITROGEN BASED ON COMMUNITY SPECIES COMPOSITION: SOIL ORGANISMS AND INVERTEBRATES

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8.1. Introduction

Other organisms that have been shown to respond to atmospheric nitrogen deposition include soil flora and fauna and other invertebrates. In particular mycorrhizal fungi play an important role in the nutrient acquisition of many higher plants and there is substantial evidence that the populations of these fungi are reduced as a consequence of enhanced nitrogen deposition. Aside from their functional importance, many of these species (particularly basidiomycetes) are of interest for their fruiting bodies – mushrooms and toadstools. Invertebrate populations in soils have also been shown to be affected by enhanced nitrogen supply, as have insect pest populations. Each of these responses could potentially provide a basis for bioindication and biomonitoring of the impacts of atmospheric nitrogen deposition.

8.2. Changes in ectomycorrhizal fruiting bodies (mushrooms/toadstools)

Many plant species benefit from symbioses with mycorrhizal fungi. These enhance nutrient exploration and uptake and protect roots from pathogens and drought. The autotrophic plant fixes carbon from the atmosphere and provides carbohydrates to the heterotrophic fungi. In ectomycorrhizal (ECM) plants, the fungal hyphae enclose the plant root as a fungal mantle and penetrate between the epidermal and cortical cells of the root axis to form the ‘Hartig net’. The ECM fungi that produce large epigeous fruit bodies are usually associated with trees. Nutrient foraging occurs mostly via extraradical mycelium, which also provide the basis for fruit body formation and it is these structures that have been shown to be sensitive to N deposition and thus could provide an indicator of N deposition.

The ectomycorrhizal symbiosis, like most mycorrhizal symbioses (others include the ericoids with *Hymenoscyphus ericae* and vesicular arbuscular with *Glommus* spp.), is regarded as an adaptation to nutrient limited conditions. N deposition can affect fruit body formation, the production and distribution of the extra radical mycelium in the soil and the formation of ECM (Wallenda & Kottke 1998). Data from long-term N deposition studies have shown loss of species diversity both above and below-ground. ‘Generalist’ species, forming a symbiosis with a wide range of tree species are less affected than ‘specialist’ species. Negative effects below-ground reflect high N concentrations. Observations of the effects of an N manipulation experiment at CEH Edinburgh have shown varying effects depending on whether the N supplied as NH_4NO_3 is also supplied with sulphuric acid. In addition, the responses have changed as the stand has aged so that the initial restriction of *Lactarius rufus* and domination by *Tylospora fibrillose* has become less obvious as the stand has aged.

Ritter (1990) observed that fruit bodies associated with *Pinus sylvestris* were not present within a certain distance of a pig farm, only occurring at lower levels of N deposition. Lilleskov & Fahey (1996) measured an N gradient around a fertilizer factory in Alaska and found that the ECM fungi *Laccaria laccata*, *L. bicolour*, *Lactarius theiogalus* and *Paxillus involutris* occurred all along this gradient, whereas *Cortinarius spp* and *Russulas* declined drastically in both abundance and diversity with increasing N deposition. Thus the production of fruit bodies species show different sensitivities to N. Work at CEH has also shown that even such sensitivities may not be consistent in all situations. *Russula* and *Cortinarius* species have shown declines in both fruit body yield and species diversity (Wallenda & Kottke 1998). A critical load of 20 kg N ha⁻¹ yr⁻¹ was proposed by Bobbink *et al.* (1992) for change in fruit body numbers, which is supported by the more recent evidence (Achermann and Bobbink 2003).

High inputs of inorganic N reduce the level of C-assimilates in the roots (Wallenda *et al.* 1996), which in turn restricts fruit body formation. However, change in fruit body occurrence can occur for many reasons, and fruit body production can vary frequently both spatially and temporally. Assessments must be made on a sustained basis (several years of visits, fungal fores), as an isolated count is unlikely to provide meaningful data. Numbers are significantly reduced in cold dry conditions. Identifying the different above ground fruit bodies requires training. The age of the trees in woodland also exerts a strong influence on the ECM; species that are successional respond to the quality of the litter and wood substrate. Probably the biggest drawback to assessing fruit body numbers is the requirement for visits at several times of the year to take account of the different requirements of the different species, and multi year observations required to account for large annual differences reflecting the weather. Finally, the absence of some species may also reflect the lack of a suitable host.

8.3. Changes in soil fauna

The effect of enhanced nitrogen input on soil fauna has received little attention. Application of high doses of nitrogen has had mixed results on soil fauna. For example, the abundance of *Nematoda*, *Oligochaeta* and microarthropodes, especially *Collembola*, had increased in some studies, but decreased in others after fertilizer addition (>150 kg N ha⁻¹ yr⁻¹) (Abrahamsen and Thompson, 1979; Huhta *et al.*, 1983; Vilkamaa and Huhta, 1986). A one-time application of 100 kg N ha⁻¹ yr⁻¹ (as NH₄NO₃) in the Fontères state forest in Eastern Brittany, France, produced an effect on soil micro-organisms which was still significant after 23 years. There were decreases in *Oribatida*, *Camarida*, *Collembola*, *Symphyta* (small *Myriapoda*) and *Pseudoscorpionida*; the atmospheric deposition at this site was estimated as 10-20 kg N ha⁻¹ yr⁻¹ (Deleporte and Tillier, 1999). A reduction in the nitrogen deposition in a *Pinus sylvestris* stand (NITREX site Ysselsteyn) to preindustrial levels increased the species diversity of microarthropods due to a decreased dominance of some species (Boxman *et al.*, 1995). A significant decrease by 66% of the abundance of earthworms was observed after seven years of fertilization in a young beech stand with 20 kg N ha⁻¹ yr⁻¹ (atmospheric deposition 12 kg N ha⁻¹ yr⁻¹) (Flückiger and Braun 1998). Simultaneously, the pH of the upper soil layer (30 cm) decreased from 3.7 to 3.5. In Sweden, a significant decrease of snails over 14-46 years in areas with N deposition of 15-25 kg N ha⁻¹ yr⁻¹ was observed, while in areas with N deposition of 3-6 kg N ha⁻¹ yr⁻¹ no significant changes were found (Gårdenfors *et al.*, 1995). In N manipulations in an acid peat, numbers of enchytraeid worms declined in the 48 kg N treatment, though the effect was modified by the presence of sulphuric acid (Standed, per comm.). However, numbers of worms show a large temporal and moisture dependence.

There is scope for the development of standard soil survey methods to monitor the impact of N deposition on soil invertebrates. However, the existence of confounding factors such as soil type, moisture and temperature levels requires further research to characterise the interactions and species preferences. Despite this, the over-riding limitation for biomonitoring nitrogen impacts on soil fauna is likely to be the substantial time required for surveying. Because invertebrate identification requires expertise and is very time-consuming, invertebrate responses are unlikely to replace other more established bioindicator methods.

8.4. Changes in insect pest populations

It is generally thought that the increased infestations of insect pests particularly sucking insects, observed following N addition from the atmosphere or as fertiliser, is a response to increased N content of the plants. For example, the infestation of beech aphid *Phyllaphis fagi* in a N fertilization experiment, increased significantly with increasing N concentration in leaves and N:P ratios respectively (Flückiger and Braun, 1998). The attack on beech nuts in Swiss permanent observation plots by the tortricid *Cydia amplana* was also significantly increased with increasing N:P ratio in leaves (N-deposition 15-60 kg N ha⁻¹ yr⁻¹) (Braun and Flückiger, 2002).

The occurrence of insect damage to pine needles in permanent observation plots in the UK was found to be positively correlated with modelled N deposition (range 7-22 kg N ha⁻¹ yr⁻¹), but only within Scotland. A negative relationship was found between the duration of needles retention and modelled N deposition (NEGTAP, 2001).

Whereas the better performance of the insects listed above may be explained by a better nutrition with soluble nitrogen compounds, the situation is more complicated in the case of the red-black pine bug *Haematoloma dorsatum*, originating from the Mediterranean region, which causes severe needle damage to pine stands in the Netherlands. The nymphs of the bugs suck on the roots or basal stem parts of *Deschampsia flexuosa* exclusively. Only the adults cause damage in the trees. *D. flexuosa* seems to be crucial for nymphal development, because it is a wintergreen grass and because it increases significantly when N deposition exceeds 10-15 kg N ha⁻¹ yr⁻¹.

While the presence of any of the above pests may indicate enhanced N deposition, the absence does not indicate the opposite. Hence the method cannot be actively used to biomonitor change. Pests cannot be introduced to a site and the response monitored, because of potential damage to the site and surrounding vegetation. It may be possible to develop a lab-based study whereby plant material (i.e. tree branch) is brought into the laboratory and manually infected with a pest with a recognised life history and previously calibrated growth rate. However such a method may only follow the results of other bioindicators (e.g. tissue N concentration).

8.5. Invertebrates and soil organisms as N bioindicators: Conclusions and Application

Mycorrhizal fruiting bodies

- Mycorrhizal fungi enhance nutrient exploration and uptake and protect plant roots from pathogens and drought. The ectomycorrhizal (ECM) fungi that produce large epigeous fruit bodies are usually associated with trees. It is these structures that have been shown to be sensitive to N deposition and thus could provide an indicator of enhanced N deposition.

- Data from long-term N deposition studies have shown loss of species diversity both above and below-ground. ‘Generalist’ species, forming a symbiosis with a wide range of tree species are less affected than ‘specialist’ species.
- Many factors affect the presence of different mycorrhizal fruiting bodies, including suitable soil conditions and the presence of an appropriate host plant species. As a consequence, mycorrhizal fungi are not suitable for bioindication of atmospheric nitrogen deposition or impacts by sampling at one time and comparing results between locations. They may, however, provide a valuable tool for long term biomonitoring of the impacts of N deposition.
- Fungal fruit body production can vary frequently both spatially and temporally. Biomonitoring for N impacts according to fruiting bodies must be made on a sustained basis (several years of visits, fungal foreses).
- Numbers of mycorrhizal fruiting bodies are significantly reduced in cold dry conditions, necessitating several visits. Also different species exhibit different timing for fruit body production, so that again several visits to a site may be necessary per year to monitor the different species of interest.
- Since changes in other factors may affect the occurrence of mycorrhizal fungi over time, biomonitoring of fruiting bodies should be accompanied by other methods indicating N availability more directly (e.g. physical monitoring, N accumulation based biomonitoring).

Soil fauna

- Available evidence suggests substantial effects of N on soil invertebrate species occurrence and abundance. Observed effects of increased N deposition include reductions in abundance of snails, earthworm, enchytraeid worm species and microarthropod species diversity.
- Because of the multiple causes of change on soil invertebrates, use of these species for nitrogen impacts assessment is limited to long-term biomonitoring at fixed locations.
- Lack of knowledge of characterizing species responses is an important limitation at present, but the over-riding limitation is resource requirement for surveying. Invertebrate identification requires specialist skills and is very time-consuming, so surveying soil fauna is unlikely to replace other established biomonitoring methods.

Insect pests

- Increased infestations of insect pests, particularly sucking insects, observed following N addition from the atmosphere or as fertiliser is thought to be a response to increased N content of the plants. Effects of N on pests have been shown for both beech and pine woodlands.
- While the presence of insect pests may indicate enhanced N deposition, the absence does not indicate the reverse. Hence the method cannot be easily used to biomonitor change, particularly since infestations occur on an occasional basis.

9. BIOINDICATOR METHODS FOR NITROGEN BASED ON TRANSPLANTATION: NATIVE RECIPROCAL TRANSPLANTS

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9.1. Introduction

Transplantation of species between locations provides a means to indicate both the amounts and impacts of atmospheric nitrogen deposition. There are two basic types of transplantation: firstly, native species may be moved from one site of interest to another. This is particularly relevant when the species concerned is of conservation interest and considered to be under threat at a protected site. Most information on this kind of transplantation is available for lower plant species, both because of their frequent sensitivity to nitrogen and because of the ease with which they may be transplanted. The second type of transplantation consists of exposing standardised 'model' cultures in conditions of differing nitrogen deposition. This approach has mostly been applied for cultivated grass species (e.g. *Lolium*), specimens of which are germinated and grown under controlled conditions, and then exposed for a standard period (Section 10). For both approaches, it is most common to measure growth rates and total tissue N concentrations, although other nitrogen accumulation or response parameters may also be applied (Sections 3-5).

This section considers, first, examples where native transplants have been used to assess N pollution. This is followed by a review of the methods and problems with native transplantation experiments, based on experience for a variety of air pollutants, including nitrogen. Given the availability of information, most attention is given to the use of lower plants.

9.2. Transplanting of native plant species: lower plants

In the absence of a root system for nutrient uptake, terrestrial bryophytes are largely dependent on the atmosphere for the acquisition of major nutrients such as nitrogen (Brown, 1982; Brown & Bates, 1990; Bates, 1992, 1994). Lichen species without a nitrogen-fixing algae component are similarly dependent on the atmosphere for nitrogen, although it is believed that some species (e.g. *Cladonia*) can recycle N from the necromass. Thus lower plants may be especially sensitive to changes in atmospheric N pollution levels and able to respond to changes in both concentration and deposition of N. High concentrations of N can be phytotoxic to bryophytes, disrupting metabolic processes such as pH regulation and N assimilation by nitrate reductase (Woodin *et al.*, 1985; Woodin & Lee, 1987; Morgan *et al.*, 1992; Woolgrove & Woodin, 1996; Soares & Pearson, 1997). These effects may reduce the growth rates of bryophytes. In addition to growth effects, there can be a good correlation between atmospheric N deposition and N concentration in bryophyte tissues (Pitcairn *et al.*, 1995, 1998; Section 3).

In the past, lichen community composition was used as a direct measure of air SO₂ pollution (Hawksworth & Rose, 1970) and now similar schemes are being developed for N pollution (Section 7). However, N transplant experiments have so far been used as methods to assess the impact of N pollution on a given species, rather than to provide a direct measure of N pollution levels. Transplant experiments involve moving a group of individuals from relatively unpolluted sites to highly polluted sites or *vice versa*. Ideally all environmental and physical variables except the pollution levels, should be the same at the two sites, allowing an assessment of the pollutant effect to be made.

9.2.1. Examples of transplants and N pollution

Bakken (1995b) transplanted *Dicranum majus* between two areas in Norway with different nitrogen deposition rates, (13.3 and 2.1 kg N ha⁻¹ year⁻¹) to test whether differences in bryophyte tissue nitrogen, protein and chlorophyll concentrations were of genetic origin or were environmentally induced. This reciprocal transplant experiment showed that although transplantation between the study sites resulted in some changes in nitrogen, protein and chlorophyll concentrations, the environment cannot be assumed to be the key factor determining the concentrations of total nitrogen, protein and chlorophyll concentrations. By contrast, transplantation studies both between regions and between sites within a mountain system in the UK demonstrated the importance of atmospheric N deposition in determining the tissue nitrogen concentration of *Racomitrium lanuginosum* (Baddeley *et al.*, 1994).

In a further study in the UK, four epiphytic species of bryophytes (*Isothecium myosuroides*, *Dicranum scoparium*, *Frullania tamarisci* and *Ulota crispa*) were transplanted reciprocally between two Atlantic (west coast) oak woodlands receiving an estimated deposition of 12 and 54 kg N ha⁻¹ y⁻¹ (Mitchell *et al.* 2004). The study indicated a detrimental impact of increased N deposition on the bryophyte species studied and a possibility for recovery following a decrease in atmospheric N, after a period of only 12 months (for which the plants were exposed). The results showed that the extent of recovery after this period was less than the reductions in growth and increase in foliar nitrogen that occurred in transplanting from the clean to the polluted site, providing evidence that recovery takes place more slowly than damage. Of the species studied most striking results were seen for the leafy liverwort *Frullania tamarisci*, as shown in Figure 9.1.

The effect of acidity and wet nitrogen deposition on the biomass increments in the cushion-forming heathland lichen *Cladonia portentosa* was assessed at five heathland sites in the UK from pre-weighed thalli (Hyvarinen & Crittenden, 1998). Nitrogen concentration in both apices and bases of transplanted lichens increased significantly when specimens were transplanted to high N deposition sites. By contrast, owing to retarded growth at these locations, estimated total N uptake by the lichen (N concentration in the thallus base x mass increment) was broadly similar at all sites. This indicates that while the method proved suitable for assessing the impacts of N deposition, it was less suited in this instance to estimating rates of N deposition.

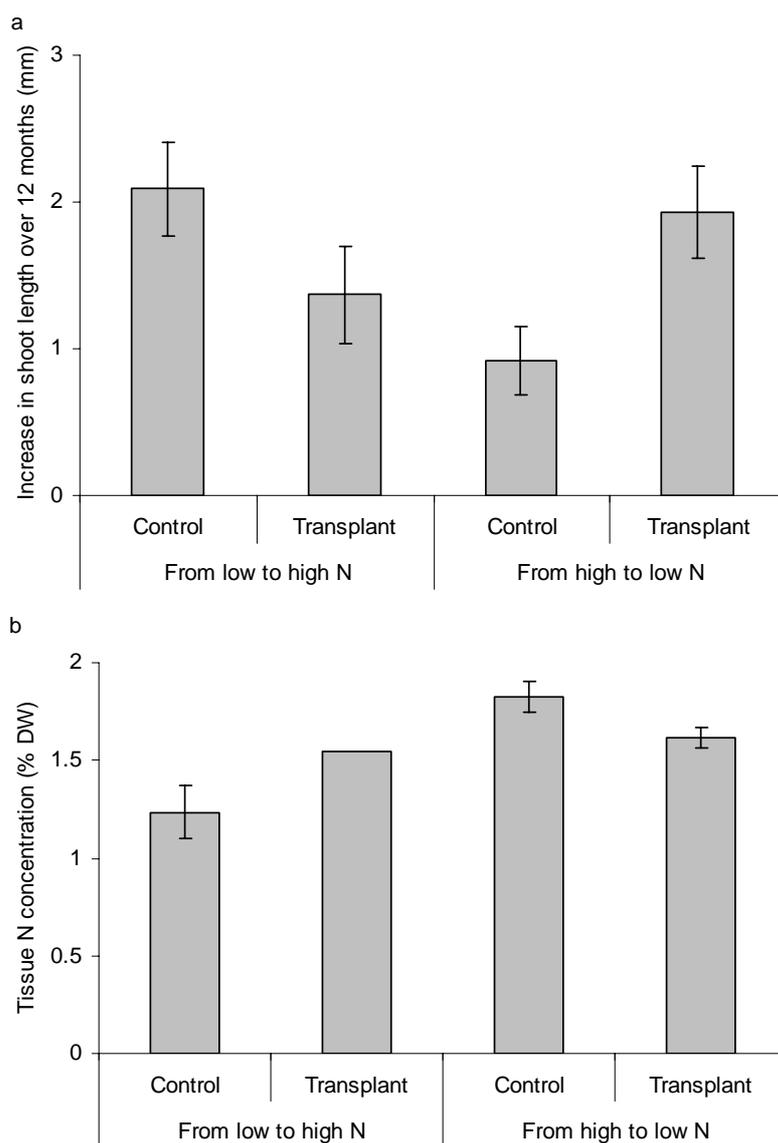


Figure 9.1. Changes in (a) growth and (b) tissue N concentration following transplantation of the leafy liverwort *Frullania tamarisci* between two Atlantic oak woodlands in western Britain, with low ($12 \text{ kg N ha}^{-1} \text{ y}^{-1}$) and high ($54 \text{ kg N ha}^{-1} \text{ y}^{-1}$) estimated N deposition (Mitchell *et al.* 2004). The results show that growth declined and tissue N concentrations increased when plants were transplanted to the high N deposition site and that growth increased and tissue N concentration decreased plants from the high N deposition site were transplanted to the low N deposition site.

By contrast, the lichen *Hypogymnia physodes*, has been shown to be a useful tool for monitoring total N deposition (Søchting, 1995). Thalli, with a tissue N concentration of 1.4 %, were transplanted from a clean environment in North Zealand, Denmark to the vicinity of a pig farm in southern Jutland, Denmark. Samples were placed at varying distances from the farm and four months after transplantation thalli had increased levels of nitrogen. Values of up to 2.9% tissue N were found in thalli closest to the farm where some thalli had died. The effect of the nitrogen pollution could be traced in samples of *Hypogymnia* up to 1000 m away.

9.2.2. Methods of transplantation

Pearson (1993) lists general principles of best practice when transplanting lichens for air pollution monitoring purposes and these principles also apply to bryophytes:

- The material to be transplanted must consist of healthy specimens of the biomonitor species.
- It is recommended that the number of collection sources is limited in order to minimize variability.
- The biomonitor species should be easy to collect, transplant and observe.
- If physiological studies are to be made the species used should be well within their normal range of adaptation, both at the source site and at the site to which they are to be transplanted.
- In order to make comparisons between sites meaningful, it is important that the site environmental conditions are as similar as possible to each other and ideally only differ in the level of the pollutant received.

It is important to cause as little disturbance to the plants as possible during transplanting, and this is best achieved by moving the plants with the substrate to which they are attached. For terricolous species this is relatively easy as turfs or cores may be cut and transplanted to the new site (Baddeley *et al.*, 1994; Bakken, 1995a&b; Hyvarinen & Crittenden, 1998). Saxicolous lichens and bryophytes may be transplanted by moving the rock on which they are growing, assuming the rock is small enough to be moved. The best known method of transplanting epiphytic lichens is that developed by Brodo (1961) in which lichens are transplanted to a new environment on a disc of the bark on which they are growing. These discs can then be re-attached to trees, boards or posts using epoxy resin (e.g. Ferry & Coppins, 1979). The species most commonly transplanted in this way is *Hypogymnia physodes*, but other species such as *Cladonia stellaris* (Kauppi, 1976), *Lobaria pulmonaria* (Hallingback, 1990), *Parmelia perlata*, *P. caperata*, *Ramalina farinacea*, *R. fastigiata* and *Xanthoria parietina* (Ferry & Coppins, 1979) have all been transplanted in this way. Lichens and bryophytes growing on twigs e.g. *Hypogymnia physodes* and *Ulota crispa*, may be transplanted by cutting the twig from the tree and tying the twig onto the host tree or post (Søchting, 1995; Mitchell *et al.*, 2004).

It is not always practical or possible to transplant species with their substrate attached. In such instances the plants should be transplanted to the same kind of substrate from which species were taken (Pearson, 1993). Methods of attaching the lower plants to the substrate must not be harmful. Epiphytic lichens may be put into bags of polyethylene netting and tied on to the tree with a nylon rope (Sloof, 1995). Epiphytic bryophytes may be removed from trees and reattached by either placing the bryophyte in a nylon net bag or a fine nylon hair-net (Gordon *et al.*, 1995; Mitchell *et al.*, 2004). The bags or nets can then be either tied or stapled to the trunk of the tree ensuring that the bryophytes are held tightly against the trunk of the tree. It is important to ensure that the bags, nets and string release no toxic substances. By contrast, nitrate is known to adsorb to nylon, and this effect may be minimized by using the widely spaced fine gauge netting. Sometimes the lichen thallus or bryophyte may be glued directly to the tree trunk, rock or stone wall in order to re-attach it (Richardson, 1967). Any glue used must hold well even in rain and must not give off toxic fumes (Pearson, 1993). As the transplanting process may affect the bryophyte or lichen, it is important to ensure that transplants are established within sites as well as between sites; this allows the effects of transplanting to be separated from the effects of increased or decreased pollution (Bakken, 1995b; Mitchell *et al.*, 2004).

9.2.3. Measuring the effect of N pollution on transplants

The responses of the biomonitor that may be measured are either physiological or chemical accumulation of pollutants in the tissues (Pearson, 1993; see also Sections 3-5). High concentrations of N can be phytotoxic to bryophytes, disrupting metabolic processes such as pH regulation and N assimilation by nitrate reductase (Woodin *et al.*, 1985; Woodin & Lee, 1987; Morgan *et al.*, 1992; Woolgrove & Woodin, 1996; Soares & Pearson, 1997) and these effects may reduce the growth rates of bryophytes. Growth may be measured by tagging the shoot at a set distance from the shoot apex, (Mitchell *et al.*, 2004) or using a cranked wire or fluorescent spray to mark the tip of the shoot from which new growth is measured (Jonsdottir *et al.*, 1996). Terricolous lichens and bryophytes may be cut to certain length (2 or 3 cm) from the tip and replaced in the turf and growth measured (Pearce & van der Wal, 2002; Karenlampi, 1972). Growth of lichens is slower than that of bryophytes and, as such, growth measurements are harder to make. One of the best ways to measure growth is therefore by weighing the thalli (Jonsdottir *et al.*, 1996), although precautions must be taken to harvest the lichen under wet conditions, as dry lichens can be fragile.

There can be a good correlation between atmospheric N deposition and concentration of N in bryophyte tissues (see Section 3). Changes in tissue N concentration are therefore commonly measure in bryophyte transplant experiments involving N pollution (Baddeley *et al.*, 1994; Bakken, 1995a,b; Mitchell *et al.*, 2004). Tissue N of lichens is less commonly measured, although this was done by Hyvarinen & Crittenden (1998) for *Cladonia portentosa*.

Different micro-habitats may affect the deposition rates received by lichens and bryophytes. For example, Søchtin (1995) suggested that total nitrogen in *Cladonia portentosa* and in *Hypogymnia physodes* growing close to the ground and not receiving canopy throughfall reflected the wet nitrogen deposition levels, while the nitrogen content of *Hypogymnia physodes* growing epiphytically on *Picea abies* reflected total (dry and wet) nitrogen deposition.

Chlorophyll and protein levels in mosses have also been related to N deposition in transplant studies (Bakken 1995b). The effects of a variety of pollutants, including N, on *Cladonia stellaris*. were determined by changes in morphology, net assimilation rate, chlorophyll content, pH and electrical conductivity (Kauppi, 1976) (Figure 9.2). Photography of lichen transplants has been used to assess growth rates and discolouration, both of which may be a measure of the effect of the pollutant. Use of Munsell colour charts to describe changes in lichen colours has been recommended by Pearson (1993).

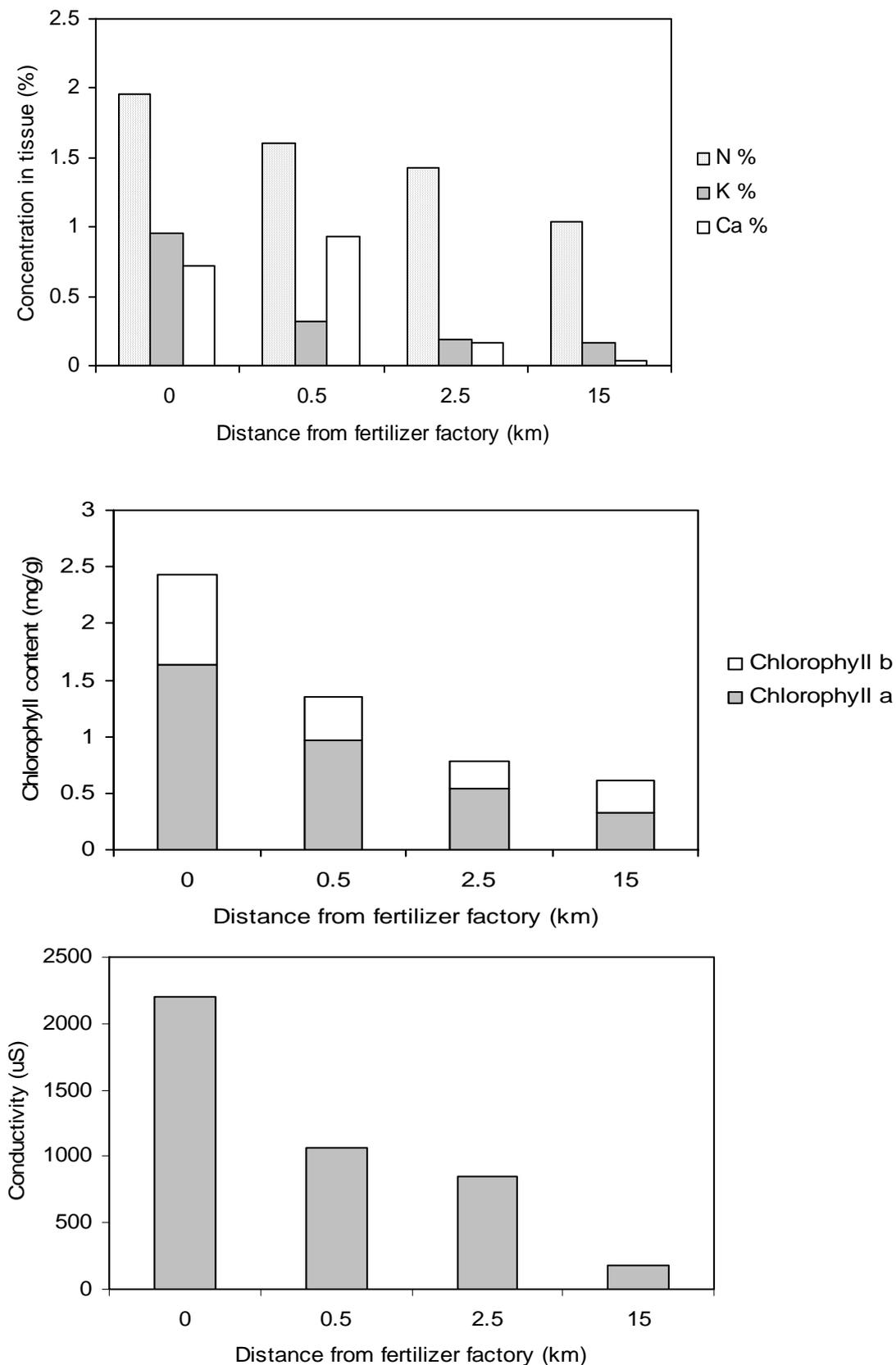


Figure 9.2. The effects of a variety of atmospheric pollutants, including N, on *Cladonia stellaris* transplanted at different distances downwind of a fertilizer factory (Kauppi 1976). Other responses were found for net assimilation rate and total tissue pH.

9.2.4. Lag and faint memory effects

Although bryophyte tissue N concentrations generally reflect atmospheric N loads there can be a lag time before the bryophyte tissue N concentration equilibrates with the atmosphere N concentration. In some cases this lag effect may exceed 16 months (Bakken, 1995b). As noted above, the lag effect for transfer from high to low N loads may exceed that for low to high N loads (Mitchell *et al.*, 2004). It is possible that bryophytes (and lichens) from high N sites are able to recycle N either within the shoot or within the bryophyte clump from senescent or dead cells in other parts of the plant (Brown, 1982; Bakken, 1995a,b; Aldous, 2002, Hyvarinen & Crittenden, 1998). This would enable them to retain high tissue N concentrations for a considerable time following transplantation to a site receiving lower inputs of atmospheric N. By contrast, the tissue N of bryophytes appears to respond more rapidly than vascular plants to changes in atmospheric N deposition, since the pool of N available to bryophytes (particularly epiphytes) is smaller than that available to plants obtaining nutrients from the soil.

Reis *et al.* (2002) developed calibration models that take account of the fact that lichens present a memory for their exposure history, which fades out in time. Such models give better performance than simple linear regression models, as they take into account this lag effect. Figueira *et al.* (2002) developed a multiple-regression model to predict the dry deposition of saline elements from the concentration of salt tracers in lichens. While neither of these models deal directly with N pollution, a similar approach could be used to model faint memory effects on tissue N levels in lichens and bryophytes.

9.2.5. Stepped effects v gradual changes in pollution levels

Transplant experiments are done to either monitor pollution levels or to assess the effects of increased/decreased pollution levels on a species. In the latter case it is important to realize that transplantation provides a sudden change in pollution levels and does not mimic the gradual increases/decreases more commonly found in the environment. The assumption is that the response of the plant to a sudden change in pollution levels is the same as that to more gradual changes, but this has not been tested. This issue indicates the need for caution when applying native transplants to assess the impact of long-term historical air pollution at sites. By contrast, in the regulatory context, where it is of interest to consider the expected effect of new air pollution source, the stepped change in conditions matches closely to that which would be experienced.

9.2.6. Timing of transplant experiments

The value of transplant experiments increases with length of exposure, since time counteracts the influence of the lag effect. Most transplant experiments on cryptogams run for at least a year, as this includes all seasons and therefore minimizes any seasonal effects on the results. However, if the transplants are left out for too long, patterns of response may become less clear as other factors may start to obscure the pollutant effects. Sõtching (1995) found that while transplanted samples of *Hypogymnia physodes* upwind of a N source showed a clear gradient of tissue N corresponding to distance from the source, samples down wind showed a less clear pattern with even remote transplants having high tissue N concentrations. It is thought that a shorter exposure time, than the 4 months of this experiment, would have been more suitable to identify the main footprint of ammonia from this farm. Transplant experiments studying reduced pollution levels may need to run for longer than those studying the effects of increased N pollution levels. A programme of sequential sampling would provide additional information on the rate of change, but would require additional transplants to be set up, which is often too costly or time consuming.

9.2.7. Other factors affecting transplant response to pollution levels

Transplant experiments are not conducted under controlled conditions and are thus likely to be influenced by a variety of other factors. Responses of epiphytic bryophytes to changes in atmospheric N may vary according to how they obtain their nutrients, the chemistry of the bark and the roughness of the bark and also how it traps dust particles (Brown, 1982). Species that grow on acid and basic barked tree species respond differently to SO₂ pollution levels (Ferry & Coppins, 1979) and the same is true for lichen responses to NH₃ (Section 7), therefore it is most appropriate to use a single host-tree species in epiphyte transplant studies. Climatic differences between sites may also confound results. Where comparisons are made between widely spaced locations, meteorological data on temperature, precipitation and windiness should if possible be obtained for each site to help interpretation of results. In this respect, transplant studies using lichens appear to be more sensitive to microclimate than those using bryophytes, which can affect the quality of the results (Cirimele *et al.* 2002).

9.3. Transplanting of native plant species: vascular plants

Very little has been reported in the literature for transplant experiments using native vascular plants. Spinks and Parsons (1995) transferred turfs of upland ombrotrophic mire from an unpolluted site in north Wales to a polluted site (Holme Moss) in the southern Pennines. Increases in root and shoot N concentrations were found at the polluted site compared with the control site after 2 years exposure in *Narthecium ossifragum* (L.) (Bog Asphodel) (Figure 9.3). By contrast, no increases were found for the more competitive species *Eriophorum vaginatum* L. (Cotton grass), which may have used the additional N for growth.

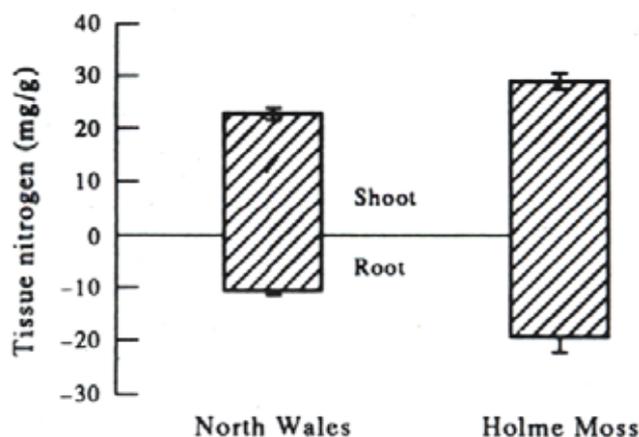


Figure 9.3: Changes in tissue N concentration in *Narthecium ossifragum* (bog asphodel), following increasing and decreasing N deposition from Spinks and Parsons (1995).

Hicks (1996) also carried out a reciprocal transplant experiment for one year, using small turfs of semi-natural upland vegetation (0.3 m x 0.3 m) containing *Calluna vulgaris* L. and *Nardus stricta* L. to assess the effects of N deposition and altitude on foliar N. Turfs were taken from Beinn an Fhurain, in N. W. Scotland (low N deposition) and transplanted to Great Dun Fell (high N deposition) in the northern Pennines, while turfs from Great Dun Fell were transplanted to Beinn an Fhurain. *C. vulgaris* transplanted to Great Dun Fell did not reflect the increased available N deposition at this site. By contrast to the results for *Calluna*, *Nardus stricta* transplanted to Great Dun Fell did show increases in tissue N concentration, but this relationship was modified by temperature differences between the sites.

9.4. Native transplant methods: Conclusions and application

- Transplant experiments involve moving a group of individuals from relatively unpolluted sites to highly polluted sites or *vice versa*. Ideally all environmental and physical variables except the pollution levels, should be the same at the two sites, allowing an assessment of the pollutant effect to be made.
- Lower plants are particularly well suited for transplant based bioindication of atmospheric nitrogen pollution since they are both sensitive to atmospheric N supply and are easily transplanted. Both lichens and bryophytes may be transplanted in this way, although in some cases a change of microclimate in transplantation can have a negative effect on the performance of lichens in transplants.
- Native reciprocal transplants have the advantage that both damage (due to transplant to polluted locations) and recovery (transplant to clean locations) can be demonstrated in relation to atmospheric N levels.
- Available data have shown significant recovery within one year for bryophytes on transplanting to clean conditions, but that the rate of recovery is slower than the reductions in growth and increase in foliar nitrogen that occur in transplanting to polluted conditions.
- Native transplants are less suited to estimating N deposition, since high N deposition can reduce growth rates. For this purpose, plants which are not sensitive to high N levels are more suited (e.g. standardized *Lolium* plants).
- Lower plants can be transplanted with their substrates (e.g. bark, rocks) or re-attached to a similar substrate at the new location, e.g. using inert glue or fine netting.
- Where growth rates are difficult to measure in slow growing species (e.g. lichens), specimens may be weighed or photographed.
- Transplanting provides a sudden step change in nitrogen conditions. While this may be of interest to simulate the effect of a new polluting development, it may not accurately simulate the effect of gradual historical changes in nitrogen deposition.
- The use of a range of plant material from different locations may help to clarify the existence of genetic adaptation.
- There have been few studies on the application of native higher plants for reciprocal transplantation. Because associated soil also needs to be transplanted, such studies need to be conducted over longer periods (>1-2 years) since the time constant of change will be slower than for lower plants.
- Native transplants have the advantage that uniform material from a common site can be applied at multiple locations of specific interest. By contrast, a limitation in regional transplant studies is to ensure comparable meteorology and microclimate.

10. BIOINDICATOR METHODS FOR NITROGEN BASED ON TRANSPLANTATION: STANDARDISED MODEL PLANTS

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10.1. Background

Standardised plants can be used to biomonitor nitrogen deposition over short periods and provide a graphic demonstration of nitrogen deposition impacts for stakeholders. There is a long history of such approaches in air pollution monitoring, such as the application of the sensitive tobacco (*Nicotiana tabacum*) BelW3 for biomonitoring ozone and the application of moss bags as biomonitors for heavy metal deposition. There has, however, been much less study on the application of standardized plants to biomonitor enhanced nitrogen concentrations and deposition.

Recent interest in standardized plants as biomonitors for air pollution has been stimulated through the European Network for the Assessment of Air Quality by the use of Bio-indicator Plants (EuroBioNet, 2003). EuroBioNet defined bioindicators as “organisms or communities of organisms which react to environmental effects by changing their vital functions and / or their chemical composition thus making it possible to draw conclusions on the state of their environment”. EuroBioNet assessed and evaluated the effects of air pollution by using bio-indicator plants within a network of 11 European cities. The suite of standardized bioindicator plants applied by EuroBioNet included: BelW3 tobacco and poplar (*Populus nigra*: clone “Brandaris”) as bioindicators of ozone pollution, Italian rye grass (*Lolium multiflorum* Lam) as a bio-indicator of sulphur compounds and heavy metals/trace elements, curly kale (*Brassica oleracea acephala*) to indicate polycyclic aromatic hydrocarbons (PAH) and *Tradescantia* (clone # 4430) to indicate mutagenic substances. Although EuroBioNet did not include nitrogen, it has helped develop standardized methods of cultivation, exposure and assessment for the application of model plants as biomonitors that are also relevant for nitrogen.

10.2. Examples of standardized plant transplants to assess N deposition

Sommer (1988) applied barley (*Hordeum vulgare* var. Harry) as a bio-indicator of ammonia deposition around a dairy farm. He grew barley plants in pots with an inert medium of rockwool and these were exposed for 1 month along a transect 0 – 300 m from the farm. The N content of the green biomass was found to increase closer to the farm, reflecting an increase in N deposition from NH₃. In a subsequent study, Sommer and Jensen (1991) assessed foliar absorption of NH₃ using *Lolium multiflorum* Lam along a transect of 0 – 130 m from a dairy farm. In this study, plants labelled with ¹⁵N were exposed for 47 days. Atmospheric ammonia concentrations (measured as NH_x, representing total NH₃ plus aerosol NH₄⁺) were also measured and ranged from 6-89 µg NH₃ m⁻³ along the transect. Dry matter production (g/pot) was greatest close to the source, but did not decrease substantially with

distance from the point source. Conversely, total foliar N concentration decreased significantly with distance from the point source, although the foliar N concentrations were generally low. A low nutrient sand was used to grow the plants and this may have contributed to the low N concentrations in the tissue and to low biomass production.

Sommer and Jensen (1991) applied their results to demonstrate the enhancement in total NH_3 deposition adjacent to the livestock farm sources. However, further analysis of the results of that study by Sutton *et al.* (1993) showed that it was possible to use the bioindicator information to estimate deposition velocities of NH_3 . Sutton *et al.* showed that although the deposition inputs were strictly in terms of total N from the atmosphere (including wet and oxidized N deposition) and that the air concentrations were measured as total NH_x , it could be assumed that the local spatial gradient would have been almost completely due to NH_3 dry deposition. As a consequence differentiation of the total N deposition to the atmospheric NH_x concentration yielded estimates of the NH_3 deposition velocity to the biomonitors (Figure 10.1). The analysis showed that the deposition velocity decreased closer to the farm, indicating a tendency for saturation of deposition at very large concentrations (cf. Section 4). The standard approach for estimating pollutant dry deposition to apply micrometeorological techniques, but these are not readily applicable in situations with substantial local horizontal concentrations (such occur as near point sources). Therefore the standardized grass plant approach provides a complementary tool to investigate the dynamics of nitrogen dry deposition close to sources.

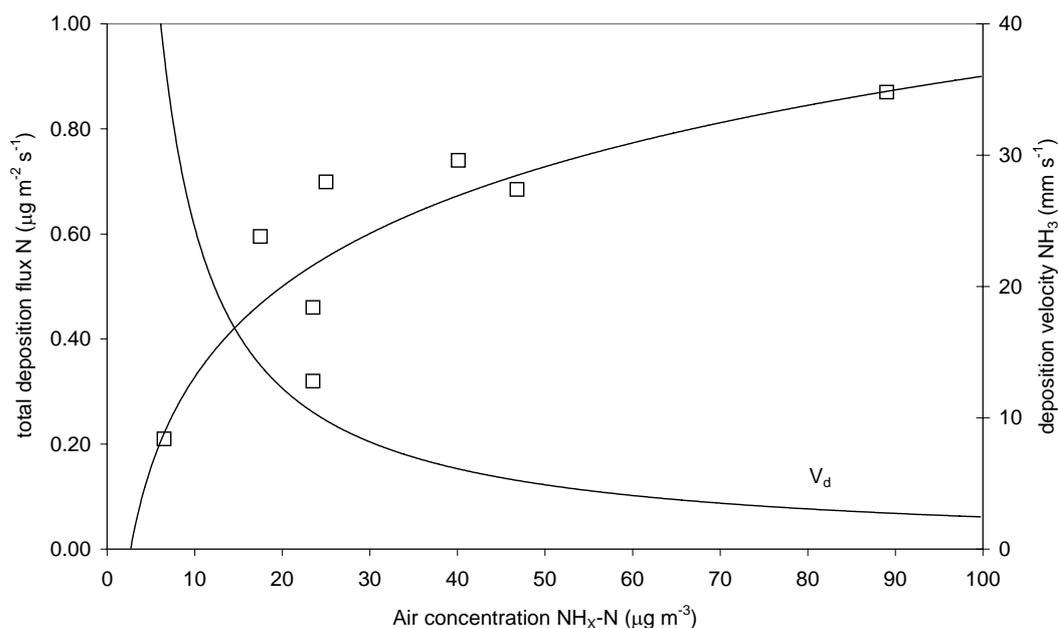


Figure 10.1. Analysis of NH_3 dry deposition rates adjacent to a dairy farm in Denmark. Sommer and Jensen (1991) set pre-cultured plants of Italian ryegrass at different distances from the farm to determine N deposition. Sutton *et al.* (1993) showed how the data can be applied to investigate the NH_3 concentration dependence of the deposition velocity.

The use of grass plants as biomonitors for nitrogen deposition has recently been tested downwind of a poultry farm in southern Scotland in a comparison of several nitrogen bioindicator methods (Picairn *et al.* 2004a, see Appendix I). This showed strong relationships between foliar N concentration, biomass and ambient NH_3 along a transect 10 – 270 m downwind of the farm in perennial ryegrass (*Lolium perenne*) plants exposed for 37 days. The results showed a graphic demonstration of the effects of NH_3 deposition, with the

biomass of the plants nearest the farm almost double those at 270 m (Figure 9.6). At the same time, total foliar N content was also highest in the plants nearest the farm.

In this study, the transect passed through a mixed woodland with very variable light levels, while meteorological conditions were often not favourable to growth (October-November). Therefore it is not surprising that there is significant scatter in the biomass and foliar N results. However, it was interesting that the shading effect on biomass was largely cancelled-out by growth dilution effects on the tissue N. Hence, when the total N inventory of the plant was considered (biomass * foliar N content), there was a substantially improved correlation with NH₃ exposure (Figure 10.2).

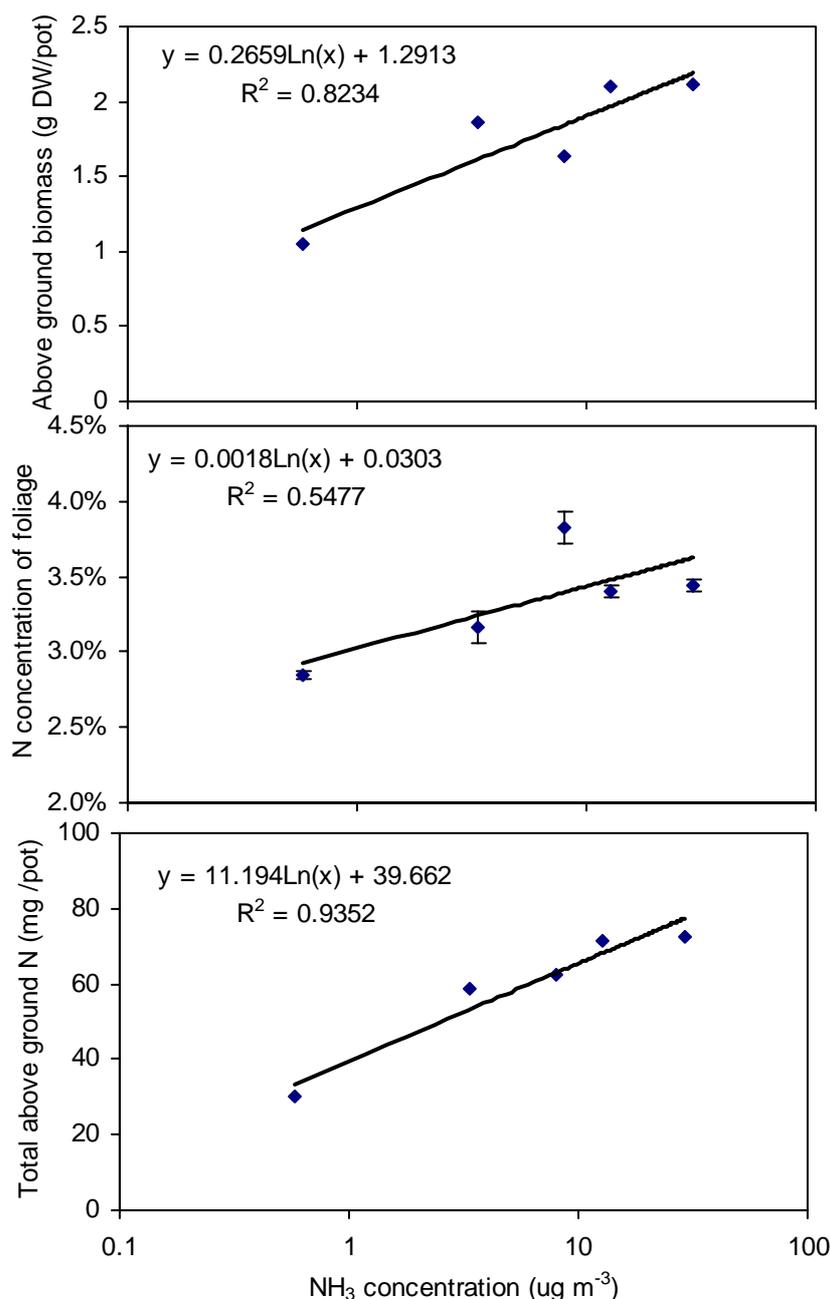


Figure 10.2: Application of standard *Lolium perenne* plants to assess N deposition and its effects downwind of a poultry farm in southern Scotland (Picairn *et al.* 2004a, see Appendix I). The increase in total above ground N inventory of the plants correlates better with NH₃ exposure since the effects of variable shading (under a woodland canopy) on biomass and foliar N concentration are largely cancelled out.

10.3. Methodological aspects

More recent field testing by Leith *et al.* (unpublished results) has applied *Lolium multiflorum* plants downwind of a controlled field NH₃ release system over a raised bog transect 60 m long (Leith *et al.*, 2004). Whereas the previous studies using standardized grass plants had considered only above-ground biomass, Leith *et al.* grew the grass plants in a root medium (Agsorb) was chosen to reduce the time taken to extract the roots, permitting the analysis of both above- and below-ground biomass. They found that over 85% of the increase in biomass under conditions of enhanced NH₃ occurred in the above-ground biomass. This is a significant practical result, since the analysis of root biomass is very labour intensive even in the Agsorb medium, and it justifies the much quicker application where only above-ground biomass is harvested and analysed.

Eurobionet (1999) produced extensive and detailed criteria and protocols for cultivation, exposure injury assessments and sampling procedures. One of the most important factors is ensuring correct irrigation in potted studies. Too much water is as detrimental to the plants as too little. To deal with this, Eurobionet introduced a simple system where plant pots include wicks connected to a water reservoir, which allow the plants to be left unattended for several weeks.

A further practical issue is the requirement to avoid grazing of the grass plants. This was avoided in the study by Pitcairn *et al.* (2004a, see Appendix I) by the use of a rabbit-proof fence around the replicate grass plants at each location (Figure 9.7).



Figure 10.3: Photographs of the *Lolium perenne* biomonitoring system used by Pitcairn *et al.* (2004a, see Appendix I). Plants were grown from seed in 20 cm diameter pots incorporating three 4 mm wicks, and cut to 5 cm height immediately before setting out in the field. Six replicate pots were applied at each location, protected from grazing by a rabbit-proof fence.

10.4. Limitations and requirements for further development

The advantage of *Lolium* as a bioindicator for nitrogen is that it grows under a wide range of nitrogen conditions and is fast growing, allowing field tests to be applied over just a few weeks. This suits it well to testing in the vicinity of point sources, such as farm where a rapid

demonstration of impacts may be of interest, by comparing plant responses along a transect away from the source. By contrast, it is noted that in many upland areas total nitrogen deposition is dominated by wet deposition in precipitation. A key feature of wet deposition is that it is highly episodic, so that much of the annual wet deposition may fall in a small fraction of time. This represents a drawback for fast growing species such as *Lolium*, since many sample runs and frequent site visits would be necessary, making the method unreasonably expensive. Development is therefore required using slow growing species better adapted to these environments, and further investigation is currently testing the application of *Deschampsia spp* for these situations.

The other main source of nitrogen deposition in the atmosphere is nitrogen oxides, and these may occur in high concentrations near roads, together with increased concentrations of ammonia. At present, however, such grass biomonitors have yet to be tested in this context, although there is no reason why they should not perform well.

10.5. Standardized model plants: Conclusions and Application

- Standardised plants can be used to biomonitor nitrogen deposition over short periods and provide a graphic demonstration of nitrogen deposition impacts for stakeholders. Recent experience has helped to standardized methods of cultivation, exposure and assessment for the application of model plants as bioindicators.
- Most information is available concerning the use of *Lolium spp* to assess deposition and impacts from ammonia near farms. Plants are grown from seed in pots using a standard treatment and cut prior to exposure in the field. Exposure periods are typically 20-50 days. This method has the advantage that it is able to provide information on deposition rates in situations with substantial horizontal concentration gradients.
- Varying light levels can affect growth rates leading to scatter in biomass and foliar N results. By contrast, total above ground biomass in *Lolium* appears to be relatively unaffected by light levels.
- In situations with locally varying N deposition, most of the variation is apparent in *Lolium* shoots rather than roots. This has the advantage that substantial cost savings can be made by restricting the analysis to above ground biomass and nitrogen concentrations.
- Practical aspects in the application of grass biomonitors are the need to irrigate plants and avoid damage by grazing. Simple approaches can overcome both these potential problems.
- The high episodicity of wet N deposition in upland areas means that long exposure-periods of, at minimum, several months are necessary, making fast growing species such as *Lolium* less suitable. Further testing with slow growing species, such as *Deschampsia* is necessary to allow application in upland areas.
- Standardized model plants have yet to be applied to assess N deposition impacts from nitrogen oxides adjacent to roads, but there is no reason why they should not be suitable.

11. EVALUATION OF BIOINDICATOR METHODS FOR APPLICATION TO STATUTORY NATURE CONSERVATION SITES

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11.1. Introduction

From the previous sections it can be seen that there is a wide range of bioindicator methods suitable for assessing the exposure and impacts of reactive atmospheric nitrogen. Since the intended application of bioindicators is to provide a sensitive signal to exposure and ecosystem effects, it is not surprising that the bioindicator methods reviewed reflect many of the clearest demonstrable biological and ecological responses to nitrogen. These responses include accumulation of nitrogen, changes in biochemical processes and changes in community species composition. Many of the methods involve observation of existing field conditions, either through comparison between sites or by long term repeated monitoring at fixed locations. In addition, transplant methods have been applied where either native plant material is moved to a new environment or standardized plants are set out, with the methods monitoring the chemical and growth responses of the plants.

The approaches vary in their robustness, ease of use and suitability for different purposes. Given the large number of different approaches, it is therefore necessary to select and recommend the most practical methods for further application. This is done in this section using two approaches.

- a. Firstly, a general rating of the different methods is made regarding their suitability for general purpose N bioindication by the conservation agencies. Here criteria are set to consider i) robustness, ii) ease of use and iii) extent of method development/ establishment. Based on these criteria, replicated expert assessments are made to identify the most promising methods.
- b. Secondly, the suitability of the methods is considered for different interests and contexts. This is largely based on the most successful general methods, but also includes specialist methods that may be suitable in certain contexts.

In relation to the second of these approaches, it must be recognized that knowledge on many bioindicators is restricted to particular habitats, while methods differ in their suitability to indicate N concentrations, deposition and ecological impacts. Some are suited to application at one time in spatial comparisons, while others require long-term biomonitoring over many years to obtain meaningful results.

11.2. General assessment of practical bioindicator methods

The different bioindicator methods were compared by allocating performance scores for different criteria to each method. Performance was rated on a Likerts scale of 1 to 5, where 1

represents very poor performance and 5 excellent performance. A standard set of criteria were established, that match the method datasheets described in Appendix II. Since such an assessment is necessarily subjective, it was carried out here using replicated expert judgement. A basic scoring-table was drafted (Table 11.1) and this supplied to each of three experts familiar with the different methods. The experts were asked to score each of the methods according to the different criteria, but were free to modify the system if they wished.

In the first evaluation (Expert A, Table 11.1) the table was used as supplied, with allocation of rating scores based on the intrinsic suitability of a method to fulfil selected criteria and the level of evidence to support that suitability. Scores for each criterion were summed. Based on 10 criteria, the minimum score is 10 and the maximum 50. Out of the 16 methods tested, 6 scored more than half marks:

Tissue N – 33; Ellenberg N index – 30; Transplants/standard plants – 29; Transplants/native – 28; Dutch (van Herk) Lichen survey – 27; NHM lichen/twig survey – 25.

A similar ranking of methods scoring more than 25 was identified in the second evaluation (Expert B):

Tissue N – 34; Ellenberg N index – 31; Transplants/standard plants – 30; Dutch (van Herk) Lichen survey – 29; NHM lichen/twig survey – 27; Other N accumulation bioassays – 27; Transplants/native – 25; Soil trace gas emission – 25;

In the third evaluation (Expert C, Table 10.3) the form was modified to make the distinction between different criteria types. Criteria were divided into Group A, showing how good the method is and Group B, showing how easy the method is to apply. The allocation of the score was based solely on the intrinsic suitability of a method to fulfil selected criteria. The scores were summed for each group and a score based on the product of the two subscores was produced (Total A x Total B). This score was seen as representing the potential suitability of the method. A further criterion, C, was included to assess how well tested the method was. By multiplying the potential score by C, the suitability of the method for current application was evaluated (A x B x C). The ranking of the scores for Method Potential (values >100) was:

Transplants/standard plants – 200; Tissue N – 192; Other N accumulation bioassays – 192; NHM Lichen/twig survey – 180; N:P ratio of foliage – 140; Transplants/native – 135; Ellenberg N index – 130; Dutch(van Herk) Lichen survey – 119; Amino acid analysis – 119.

The ranking of the scores for practical application (values >400) was:

Tissue N – 960; Transplants/standard plants – 800; Other N accumulation bioassays – 576; N:P ratio of foliage – 560; NHM Lichen/twig survey – 540; Transplants/native – 540; Ellenberg N index – 520; Dutch(van Herk) Lichen survey – 476; Amino acid analysis – 476.

Interestingly, although the scores were changed slightly for the practical application, the ranking of methods turned out to be similar. The three sets of scores provided by the different experts are seen to be rather similar in their composition, highlighting a reasonable consensus on the most suitable general methods.

To further refine these lists of methods into a short list of best approaches, a trade off was considered between how robust the method is and how easy it is; a more difficult method to apply would only be considered useful where it delivered proportionately more robust results.

Bioindicator and biomonitoring methods for assessing the effects of atmospheric nitrogen on statutory nature conservation sites

Criteria	Total tissue N	Amino acids	Enzyme activity	N:P ratio	Ellenberg N index	Other N indicators	Lichen biodiversity				Transplants		N isotopes	Frost hardiness	Chloro-fluoresc	Soil gas fluxes	Soil inverts
							LDV	Dutch	Fr	Twig	Model	Native					
Suitability to indicate N concentrations	4	1	1	1	1	1	1	4	1	1	3	2	2	1	1	1	1
Suitability to indicate N deposition	5	4	2	1	3	2	3	4	1	4	4	3	3	3	1	5	2
Sensitivity to depos. levels	3	3	2	3	2	2	1	1	1	1	2	3	3	2	2	2	1
Suitability to indicate N impacts	2	2	1	2	4	3	1	3	1	4	2	4	1	1	1	1	2
Sensitivity to other factors	4	3	2	2	2	2	1	3	1	3	3	1	3	1	2	3	1
Time constant	4	2	1	4	3	2	3	4	4	4	3	3	4	3	3	3	1
Limitations to applicability	4	3	1	4	5	2	3	3	2	3	3	2	3	2	3	3	1
Simplicity – field	4	2	1	4	3	1	1	1	4	1	4	3	3	2	4	2	2
Simplicity – analysis	1	1	1	1	4	1	1	1	5	1	3	2	1	1	1	1	1
Cost	2	2	1	1	3	1	3	3	2	3	2	2	1	1	2	1	1
TOTAL SCORE	33	23	13	23	30	17	18	27	22	25	29	25	24	17	21	22	13

Scores have been allocated for selected criteria on a 1 to 5 scale. 1 – least advantageous; 3 – intermediate; 5 – most advantageous

Table 11.1: First empirical evaluation (Expert A) of the suitability of different N bioindicator methods for application by conservation agencies

Bioindicator and biomonitoring methods for assessing the effects of atmospheric nitrogen on statutory nature conservation sites

Criteria	Total tissue N	Amino acids	Enzyme activity	Other N bioassay (subst N, foliar NH ₄ ⁺)	N:P ratio	Ellenberg N index	Other N indicators	Lichen biodiversity				Transplants		N isotopes	Frost hardness	Chloro fluores	Soil gas fluxes	Soil inverts	Ecto Mycorrhizae	
								LDV	Dut	Fr	Twig	Model	Native						Nos	Div
Suitability to indicate N concentrations	4	1	1	1	1	1	1	1	4	1	1	3	2	2	1	1	1	1	1	1
Suitability to indicate N deposition	5	4	2	3	1	3	2	3	4	1	4	4	3	1	3	1	5	2	3	2
Sensitivity to depos. levels	3	3	2	4	3	2	2	3	3	3	3	2	3	3	2	2	2	1	2	3
Suitability to indicate N impacts	2	2	1	2	2	4	3	1	3	1	4	2	4	1	1	1	4	2	4	4
Sensitivity to other factors	2	3	4	3	2	2	2	1	3	1	3	3	1	3	1	2	3	1	1	1
Time constant	4	3	2	2	4	3	2	3	4	4	4	3	3	4	3	3	3	1	1	1
Limitations to applicability	4	3	1	3	4	5	2	3	3	2	3	3	2	3	2	3	3	1	2	2
Simplicity – field	4	2	1	3	4	3	1	1	1	4	1	4	3	3	2	4	2	2	2	1
Simplicity – analysis	3	1	1	3	1	4	1	1	1	5	1	3	2	1	1	3	1	1	3	2
Cost	3	2	1	3	1	4	1	3	3	2	3	3	2	1	1	3	1	1	1	1
TOTAL SCORE	34	24	16	27	23	31	17	20	29	24	27	30	25	22	17	23	25	13	20	18

Scores have been allocated for selected criteria on a 1 to 5 scale. 1 – least advantageous; 3 – intermediate; 5 – most advantageous

Table 11.2: Second empirical evaluation (Expert B) of the suitability of different N bioindicator methods for application by conservation agencies.

Bioindicator and biomonitoring methods for assessing the effects of atmospheric nitrogen on statutory nature conservation sites

	Criteria	Total tissue N	Amino acids	Enzyme activity	Other N bioassay (subst N, foliar NH ₄ ⁺)	N:P ratio	Ellenberg N index	Lichen biodiversity				Transplants		N isotopes	Frost hardiness	Chloro Fluor	Soil Gas fluxes	Soil inverts	
								LDV	Dut	Fr	Twig	Model	Native						
A How good	Suitability to indicate N concentrations	1	1	1	1	1	1	1	4	2	4	3	1	1	1	1	1	1	
	Suitability to indicate N deposition	4	4	2	4	4	2	1	3	1	3	4	2	1	1	1	3	1	
	Sensitivity to depos. levels	4	4	4	4	4	3	1	3	2	4	5	5	2	2	1	3	2	
	Suitability to indicate N impacts	3	4	3	3	3	5	1	4	3	4	4	5	1	2	2	4	2	
	Sensitivity to other factors	4	4	2	4	2	2	1	3	2	3	4	2	2	2	1	3	1	
	Time constant Long or short	L	L	S	S	L	L	L	L	L	L	M	S	S	S	L	S	L	L
	Total A	16	17	12	16	14	13	5	17	10	18	20	15	7	8	6	14	7	
B How Easy	Simplicity – field	5	5	3	5	5	3	2	2	3	3	3	3	4	3	4	3	2	
	Simplicity – analysis	3	1	1	3	2	4	3	3	4	4	3	3	1	2	4	2	2	
	Cost	4	1	2	4	3	3	3	2	3	3	4	3	1	3	3	2	2	
	Total B	12	7	6	12	10	10	8	7	10	10	10	9	6	8	11	7	6	
Potential Method: Total AxB		192	119	72	192	140	130	40	119	100	180	200	135	42	64	66	98	42	
C	How well tested	5	4	3	3	4	4	3	4	2	3	4	4	4	4	3	4	3	
Practical Method: Total A x B x C		960	595	216	576	560	520	120	476	200	540	800	540	168	256	198	392	126	

Scores have been allocated for selected criteria on a 1 to 5 scale. 1 – least advantageous; 3 – intermediate; 5 – most advantageous

Table 11.3: Third empirical evaluation (Expert C) of the suitability of different N bioindicator methods for application by conservation agencies. In this approach the scoring system is modified to reflect A. How good, B. how easy and C. how well tested is the method.

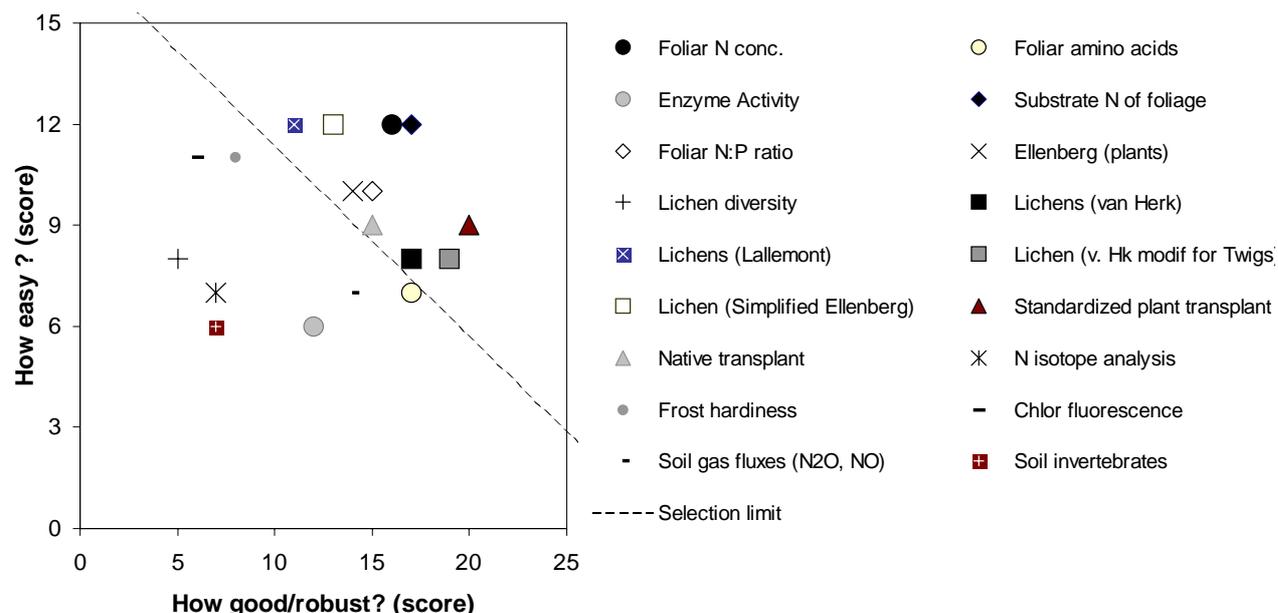


Figure 11.1: Empirical assessment for the general application of different N bioindicator methods. The highest scoring (best) methods are shown to the top-right of the figure. The diagonal line indicates a limit of method selection.

At this point the expert results were amalgamated to provide agreed scores for methods according to the relevant headings (groups A and B of Table 11.3). The results are summarized in Figure 11.1 and a diagonal line drawn to indicate a limit of selection of the methods for general purpose application by conservation agencies. Finally, it was considered that where two similar methods both scored higher than the selection limit, that only the highest scoring of the two methods was included. This applied, for example, to Foliar N content and to Foliar N:P ratio, with the latter being excluded by this filter.

The limits of such a qualitative assessment mean that it should not be over interpreted. However, it provides a means to help identify a short list of recommended methods. The scoring also highlights the limitation of biochemical or species response based bioindicators, which are not specific to nitrogen (e.g. frost hardiness, chlorophyll fluorescence and lichen diversity). Similarly, biochemical responses broadly specific to nitrogen (e.g. soil emissions of N₂O, enzyme responses), tended to be less suited to indicating N deposition than N accumulation based approaches (foliar N, foliar soluble N, amino acids). One reason for this is that the biochemical response is usually a function of both the N accumulation (more directly measured by accumulation-based methods) and other factors, such as moisture, light, soil type etc.

11.3. Leading general methods for practical biomonitoring of nitrogen loads and effects on statutory nature conservation sites.

As a result of the empirical selection process, seven methods were identified as being the most suitable for general application:

Chemical methods:

- 1) Total foliar N concentration,
- 2) soluble foliar nitrogen or ammonium,

Diversity methods:

- 3) Ellenberg Index (higher plants, bryophytes),
- 4) Lichen diversity (van Herk approach),
- 5) Lichen diversity (Wirth approach),

Transplant methods:

- 6) Standardized plants,
- 7) Native reciprocal transplants.

The benefits and limitations of each of these methods are reviewed in turn below.

11.3.1. Chemical methods: Total foliar N concentration

The measurement of total foliar nitrogen is undoubtedly the most robust in terms of evidence and published work relating to both N deposition and tissue N concentration under natural conditions. The range of studies and measurements has enabled the development of simple models of the response of tissue N in bryophytes and *Calluna vulgaris* to atmospheric N deposition. Most examples of the use of this bioindicator in field studies illustrate its suitability to indicate enhanced N deposition with little distinction between reduced and oxidised N or wet and dry deposition. Recent simulation experiments in open-top chambers have shown a greater effect of NH₃ rather than NH₄ on tissue N and a large scale field simulation experiment is currently in progress to determine the effects of wet and dry deposition and oxidised and reduced N on mire vegetation, including effects on tissue N in mosses, lichens and higher plants. Work under the GANE Thematic Programme has also shown that tissue N responds more positively to deposited N in the form of NH₃ rather than wet deposited NH₄⁺ + NO₃⁻ and should provide further information in the future. Response-times for tissue N may be shorter than previously thought depending on the species. For example, some *Sphagnum* species have a very short time scale of response (days) but longer scale for recovery (weeks). For *Calluna* (annual increment) and trees (current year's leaves), response time is longer, probably integrating over the growing season and possibly over several growing seasons if recycling processes are considered.

Measuring foliar N, once a sampling protocol has been designed for collection and sample preparation, is relatively straightforward. Analysis bears a cost, but, compared with many of the biochemical techniques, the method is well described with far ranging application and costs are modest. Equally there are many examples of good agreement between foliar N and N deposition and a reasonable understanding of why agreement may be less good.

There is however, a need for development work in order to interpret the data with a view to describing a conservation site as, already impacted by N, at risk from N etc. It is recommended to establish a database for N values for species – with information on the collection protocol, how and when measured etc. soil type, climate, geology. This could be used to help assess the significance of the N value.

11.3.2. Chemical methods: soluble foliar nitrogen

Soluble foliar nitrogen and ammonium are more recently developed parameters that appear to show larger relative responses than total N, as they reflect smaller plant pools that are more directly perturbed by atmospheric N deposition. In particular, the analysis appears to be well suited to pleurocarpous moss species, with the species tested so far having a similar response to total N deposition. Both total soluble N and foliar ammonium have been measured, but the available results to date indicate that the clearest responses are seen for foliar ammonium.

This is also relatively straightforward to analyze based on grinding a frozen sample of leaf material, although further work is warranted to compare the effects of different extraction approaches and identify simpler procedures, further reducing costs. A key advantage of the method over the analysis of foliar amino acids is that the accumulation of the amino acids is species dependent, with different plant species accumulating different amino acids. Therefore analysis of total soluble N or foliar ammonium should provide more generalizable results.

11.3.3. Diversity methods: Ellenberg Index for higher plants and bryophytes

The Ellenberg index for higher plants and bryophytes is widely used to show the impacts of N eutrophication on species composition in Europe. While the Ellenberg index does not distinguish causes of eutrophication, it has been strongly linked to changes in the N status of ecosystems resulting from enhanced N inputs (from fertiliser and atmospheric deposition) and can provide a quick status report on the condition of a site. For a meaningful comparison, vegetation should be assessed along a gradient of anthropogenic influence (edges of the site inwards). Surveys should be carried out regularly - every 3-5 years. Another approach is to compare a site index with that of a known pristine site of the same habitat, bearing in mind that some habitats such as woodlands tend to support more N-loving species than others (e.g. calcareous grasslands). It may be possible and useful to develop a system of key sites for specific habitats, which could be used to calibrate scores for sites under investigation.

Because of the buffering effects of soil and competitive changes in species composition, the Ellenberg index is not a rapid response to concentration changes or to short term changes in deposition, but is more suited to longer term deposition changes occurring over a period of > 3 years and has been shown to be related to N deposition in some studies. As with most methods, the index is most effective in indicating impacts at the higher end of the deposition range and is not sensitive enough to pick up small deposition changes.

With suitable training, the method can be applied by agency staff. The method is time-consuming but could be part of survey and monitoring of protected sites. It is clearly important to avoid or at least be aware of confounding factors and to have a good knowledge of past management practices at the site. Informal application of the method could also provide a very useful first assessment of a protected site.

11.3.4. Diversity methods: Lichen diversity using the van Herk approach

The recent evidence from the UK reported in Section 7 has shown the van Herk (1999) method to be highly sensitive to atmospheric NH₃ concentrations, providing an indication of both the loss of acidophyte lichen species and the consequent increase in nitrophyte species. Importantly, the results also suggest that the most sensitive acidophytes are lost at lower NH₃ concentrations than the increase in occurrence of nitrophyte species. This means that lichens at a conservation site may be affected by NH₃ even in the absence of clear nitrophytic species. By contrast, there is less evidence of an effect of wet N deposition or of NO_x concentrations according to this method.

Differences in bark pH caused by atmospheric NH₃ concentrations are critical to the interpretation of the lichen diversity results. Because of this, the van Herk (1990) approach should be applied only for acid bark trees, the best studied of which is oak. Consistent results have also been obtained for pine, spruce and birch. Because of the interaction with bark pH it is highly desirable to accompany the application of this method with direct measurements of

bark pH. This can help interpret the results in relation to physical monitoring of atmospheric concentrations of nitrogen in the atmosphere.

The van Herk (1999) method consists of a highly detailed system for scoring lichens on tree trunks. However the recent work by Wolseley et al. (Section 7) has shown that the method can also be applied in a simpler fashion for analysis on the bark of twigs. Because of the naturally higher pH of twigs than tree trunks, these tend to be more sensitive to NH_3 than trunks, and may provide one of the most sensitive bioindicators of atmospheric NH_3 .

The approach is more suitable for temperate climates such as the UK, since in warmer, dusty climates, nitrophytic species are a natural constituent of the flora. The main disadvantages of the method are the requirement for a specialist botanist to identify species and the need to fulfil method requirements and to find sufficient trees with acid bark.

11.3.5. Diversity methods: Lichen diversity using the Wirth (Ellenberg) approach

While the van Herk (1999) approach represents a detailed scheme for lichen assessment that is very sensitive to low concentrations of ammonia, there is a need for simpler methods that can be conducted more quickly and also applied by non-experts. The Ellenberg approach for lichens developed by Wirth (1993) provides a tool that is useful in this respect. Firstly, a location can be scored relatively quickly, noting the presence of different species, and assigning an average Ellenberg score. Secondly, there is potential to simplify the method by incorporating only key easily identifiable species. In the present study (Section 7), a short list was made by considering only well-known foliose and fruticose species, and ignoring the more difficult to identify crustose species entirely. While the results are obviously of a lower quality than detailed assessment of all species, it showed a clear response to elevated NH_3 concentrations, indicating potential as a screening approach. This approach is also of interest as it provides a means to engage non-specialists in the biomonitoring of atmospheric N compounds.

As with the van Herk (1999) approach, both the detailed Ellenberg and simplified Ellenberg approaches are applicable on twigs and trunks. Analysis of both components may provide a useful way to improve the quality of the assessment and give an indication of any temporal changes, since twig lichens respond more to present conditions, while trunk lichens may in part respond to conditions over previous years to decades.

11.3.6. Transplant methods: Standardized plants

Plants grown under standard conditions may be placed out in the field and the total N (tissue N x biomass) assessed following subsequent harvest. The approach has been tested with *Lolium* plants and may be implemented to indicate both deposition and biological impacts over a short timescale (several weeks). The method provides very visible results, which are useful for stakeholder demonstration. A key advantage of transplant methods using standard plants is relative simplicity in the field and laboratory and reasonable costs. The approach delivers clear evidence that atmospheric deposition may alter biological processes. Conversely, the method does not formally guarantee that the observed effects are also occurring on designated species in a protected habitat. The method therefore relies on the supposition that a clear biological response in the standardized plants implies a high likelihood of responses by the native species present.

The published use of standard plants as bioindicators of N deposition is limited. However, during the recent field study (Appendix I), this method appeared to be suitable for indicating both ammonia concentrations and N deposition. Despite unfavourable meteorological

conditions, growth and tissue N in standardised *Lolium perenne* plants responded to a gradient of N deposition over an exposure period of 1 month. There is some potential for calibration of the method using exposure facilities in open-top chambers and field facilities, which might reduce number of sampling locations that need to be monitored at each site.

Plants could be grown up commercially to an agreed standard and set out and monitored by agency staff. Care must be taken in selecting sampling positions with regard to wind exposure, temperature and light. Growth may be stimulated by a range of conditions, however, expression of the response as total N per plant overcomes problems associated with growth dilution. While standard plant species typical of agricultural usage have been used to date, these tend to grow too fast for application to longer-term monitoring of wet deposition inputs (which are more temporally variable, requiring longer monitoring periods). However, if native species (e.g. *Deschampsia* spp) were selected and propagated, longer-term application may become more practicable.

11.3.7. Transplant methods: Native reciprocal transplants

Reciprocal transplanting of plants already occurring in sites of interest is a valuable way to demonstrate local damage to specific plant components. The method has been tested for epiphytic bryophytes, which are easily transplantable, but is less reliable for lichens, due to microclimatic sensitivity, which can reduce the success following transplantation. While the approach is rather high cost (because of the need for detailed growth measurements as well as chemical determinations), a key advantage is that it can be used to demonstrate recovery of species from a particular site following transplanting to conditions with lower N levels.

The method of transplanting native species to monitor pollution has been well used and reported on in Europe and has the major advantage of directly indicating impacts of N deposition on vulnerable species. Conversely, major disadvantage of the method is difficulty in selecting sites with comparable meteorological conditions. For this reason, it the method is best applied using several species that represent a range of climatic preferences. The requirement for similar climatic conditions and careful growth measurements, indicates that specialist staff need to be involved in site selection and transplanting when applying this approach.

11.4. Decision tree approach for selecting nitrogen bioindicator methods

Each of the above methods has benefits for the general bioindication and biomonitoring of atmospheric N deposition and its effects. It is noted, however, that the different methods have contrasting benefits and limitations, which make them variously suited for application depending on the specific questions being asked. Based on these differences, an alternative selection procedure consists of a 'decision tree' approach, where users are guided to the most appropriate methods based on the likely questions of interest.

Box 11.1 presents a preliminary decision tree for selecting bioindicator methods for atmospheric nitrogen deposition and impacts. By following the decision tree a user is guided to think through the exact questions they are asking, considering the context of the habitat of interest. In doing this, they may realize that they have several purposes in conducting biomonitoring (e.g. the assessment of both atmospheric deposition loads and impacts), and may therefore need to work through the decision tree several times to different end points.

An important point in the use of bioindicators for nitrogen is that no one method is perfect and that application of suite of methods is likely to give the most robust results. The decision

tree approach therefore helps users to identify a short selection of methods that are best suited to each context. Similarly, consideration should be given to the joint application of bioindicators and physical measurement of reactive N concentrations in the atmosphere (e.g. using passive samplers). Where costs permit, this provides a much more robust assessment, which links the biological responses measured directly to atmospheric pollution levels. Physical monitoring may also be the only approach available, where reliable biomonitoring methods do not exist (e.g. NO_x concentrations).

Box 11.1: Preliminary decision tree for selection of nitrogen bioindicator methods

Q1: Is the nature of the task to:

- a. **Assess the impacts** of enhanced N concentrations or deposition? *Go to Q2.*
- b. **Attribute the source** of enhanced N concentrations or deposition? *Go to Q3.*
- c. **Assess the magnitude** of atmospheric N concentrations or deposition? *Go to Q9.*

Q2: What is the timescale to which the N bioindicator should refer?

- a. Monitoring **short term** responses (e.g. <2 months). *Method: Standardized grass plants (*Lolium*).* (Note not suited to high altitude sites or testing impacts of high wet N deposition due to intermittency of rain events)
- b. Monitoring of **medium term** responses (e.g. 2-12 months).
 - i) *Method: Standardized grass plants (*Deschampsia*)* (Slow growing plant also suited to exposed UK high altitude sites with large wet deposition).
 - ii) *Method: Substrate N or foliar ammonium concentration of native grass or pleurocarpous mosses.* (A newly identified parameter expected to show faster responses to N deposition than total tissue N).

- c. Monitoring **long-term** responses (e.g. minimum of 1 year, but typically several years to decades). *Go to Q4.*

Q3: What is the nature of the site in relation to obvious local sources?

- a. **Obvious local source present:** Visual assessment of the site and odours (e.g. vehicle fumes or livestock smell) may be sufficient. In the case of point or line sources, measurements using bioindicators should focus on differences with distance from the source.
- b. **Significant uncertainty as to the main N source:** *Method: Natural abundance of ¹⁵N isotope.* (The method is suited to distinguishing volatilised agricultural ammonia from N emissions due to combustion processes, but is less well suited to attribution of N sources in areas dominated by wet deposition).

Q4: What kind of ecosystem response is of most concern?

- a. **Nitrogen accumulation in plants** as an early indicator of change. *Go to Q5.*
- b. **Growth responses of plants** to total N deposition especially for stakeholder demonstration. *Go to Q6.*
- c. **Chemical responses of soils** as an indicator of soil N saturation. *Method: Trace gas emissions from soils.* (Note: measurements can include nitrous oxide (N₂O), nitric oxide (NO) and methane (CH₄). N₂O and NO are relevant for both well drained and wet soils, while CH₄ emissions from bogs increase with N deposition).
- d. **Species composition of plant communities** as the ecological consequence of enhanced N concentrations or deposition over several years. *Go to Q7.*

Q5: Is the need to monitor plant response to several forms of additional N or specifically identify the impact of large gaseous ammonia concentrations?

a. **Foliar responses** to different forms of additional nitrogen. (Note that the dose response relationships probably differ for different N forms).

- i) *Method: Total tissue N of foliage.* (Note: Suited to pleurocarpous mosses and certain well studied higher plants, e.g. *Calluna*). A well-studied parameter.
- ii) *Method: Substrate N or ammonium content of foliage.* (Note: A newly identified parameter, which appears to show a very large response to enhanced N). Particularly large responses for pleurocarpous mosses.
- iii) *Method: Foliar Amino Acids.* (Note: Amino acids present differ between species. The method may be useful when focusing on key species. Particularly large responses for pleurocarpous mosses.)

b. **Bark response** to large gaseous ammonia concentrations. *Method: Measurement of bark pH:* (Note: Ammonia increases the pH of bark, which is detrimental to certain lichen species.)

Q6: For plant growth responses, what kind of response is of most interest?

- a. To demonstrate a **standardized response** to nitrogen deposition. *Method: Standardized grass plants (*Lolium* or *Deschampsia*).* (Note: the method is good for visual demonstration and directly records additional nitrogen absorption by the plants. For long-term application, a series of plants must be exposed over time.)
- b. To demonstrate responses for a **key native species** of concern. *Method: Reciprocal transplants of native species.* (Note the method is particularly relevant for bryophytes, which can be easily transplanted. Transplanting specimens from polluted to clean sites can also be used to demonstrate recovery).

Q7: For changes in species composition, what habitat is of interest?

a. Is the site wooded or are **trees present**? If, yes:

- i) *Method: Ellenberg analysis of higher plants and bryophyte species.* (Note: this is usually conducted for the ground-flora of woodland, but may also consider epiphytic bryophytes.)
- ii) Lichen diversity methods: Go to Q8.

b. Is the site **treeless**? If yes:

- i) *Method: Ellenberg analysis of higher plants and bryophyte species.* (Note: Interpretation of the method may be complicated by local differences of habitat management and soil types, and is therefore best suited to application at sites with well defined site history.)

Q8: For the lichen diversity methods, what is the level of detail required?:

- a. A **detailed survey** conducted by expert lichenologists? *Method: Full lichen survey identifying all species, and calculation of Acidophyte / Nitrophyte Indices following van Herk.* (Notes: Responds primarily to NH₃. Sampling is normally conducted on tree trunks, but sampling on twigs can give an indication of present rather than historical conditions and is more sensitive. See also bark pH).
- b. A **simplified survey** conducted by trained non-experts? *Method: Survey of key lichen species and calculation of Ellenberg or Acidophyte / Nitrophyte scores.* (Notes: The method gives a broad indication of NH₃ exposure. Sampling is normally conducted on tree trunks, but sampling on twigs can give an indication of present rather than historical conditions and is more sensitive to low NH₃ concentrations. See also bark pH).

Q9: What component of atmospheric N concentrations or deposition is of prime interest?

- a. **Atmospheric NH₃ concentrations:** *Method: Full lichen survey identifying all species, and calculation of Acidophyte / Nitrophyte Indices following van Herk.* (Notes: Sampling is normally conducted on tree trunks, but sampling on twigs can give an indication of present rather than historical conditions and is more sensitive. See also bark pH. The method provides a qualitative indication of NH₃ concentrations, and further work is not sufficiently developed to provide quantitative estimates of NH₃ concentrations. Consideration should also be given to physical monitoring of NH₃ concentrations proven sampling methods with adequate QA/QC).
- b. **Atmospheric NO_x concentrations:** There is no bioindicator method currently available to indicate NO_x concentrations. Methods may yet be developed using lichens, but apart from near NO_x point or line sources responses appear to be stronger to NH₃. Apply low cost methods for physical measurement of NO_x concentrations (e.g. diffusion samplers).
- c. **Atmospheric N deposition.** *Go to G10.*

Q10: What is the timescale to which the N deposition bioindicator should refer?

- a. Monitoring present **short term** N deposition near point or line sources only (e.g. <2 months). *Method: Standardized grass plants (Lolium).*
- b. Monitoring of **medium term** N deposition (e.g. 2-12 months). *Method: Substrate N or foliar ammonium concentration of pleurocarpous mosses.* (A newly identified parameter that is very sensitive, although further work is required to improve the quantitative relationships).
- c. Monitoring **long-term** N deposition. *Method: Total tissue N of foliage.* (Note: Suited to pleurocarpous mosses and certain well studied higher plants, e.g. Calluna). A well-studied parameter.

11.5. Staged site assessment using nitrogen bioindicators

In assessing the impacts of atmospheric reactive nitrogen at a protected site it is of great interest to be able to screen priority sites of interest. It is therefore useful to be able to conduct simplified preliminary assessments at sites that indicate the requirement for further assessment. A two-stage approach can therefore be identified with the initial screening approach taken depending on the expertise of local staff.

11.5.1. Initial screening of sites using bioindicators

An initial site screening can be conducted based on an informal assessment of the plant species occurring at the site, including any changes across the site in relation to nearness to any point or line sources. Informal recording of species with known extreme Ellenberg scores, either for higher plants, bryophytes or lichens can help build up a picture of whether a site is affected by enhanced N levels. In particular, recording of the main foliose and fruticose lichen species on tree trunks and twigs provides a sensitive initial assessment of exposure to atmospheric ammonia. Such a site visit should also assess any odours (which may be indicative of nitrogen emitting sources in the vicinity), as well as the occurrence of visible injury to particular species known to be sensitive to excess nitrogen.

Such an initial assessment could may include the sampling of plant material for simple analysis of a few specimens, for example for total foliar N levels or foliar ammonium concentrations, particularly in pleurocarpous mosses. Although only limited weight can be given to analysis from a few samples, any extreme values would be clearly indicative of the need for further assessment.

11.5.2. Detailed site based assessment using bioindicators

Based on initial observations and findings a more detailed site assessment may need to be carried out. The appropriate level of assessment will depend on the exact issues applicable for the site, and therefore the level of resource given to each situation may vary. However, the application of a selection of complementary methods is recommended for the most robust assessment. Where a nearby source is under investigation, use should be made of local transects with distance from the source, while low-cost monitoring of NH₃ concentrations, NO_x concentrations and or N wet deposition is recommended, depending on the local N form of most concern.

Bio-indicators may be applied by measurements at one time, with results compared across sites for local gradients, or with other national data, for sites more distant from sources. However, the most robust results are to be obtained by biomonitoring over time. A detailed assessment may therefore extend the application to use either short term exposure of transplants (standardized or native species), or the long term repeated assessment of the methods (e.g. after six months operation of a process or every few years).

11.6. Discussion and recommendations

11.6.1. Suitability and limitations of bioindicators for atmospheric N deposition

Available bioindicator methods for atmospheric nitrogen offer a range of advantages and limitations specific to each method in relation to the questions being addressed. For the purpose of estimating atmospheric nitrogen deposition, methods based on measuring an accumulation of nitrogen are the most robust, and these can be used to help refine local estimates of critical loads exceedance by the definition of critical foliar nitrogen concentrations. By contrast, methods measuring a biochemical response to nitrogen tend to be more uncertain indicators of atmospheric deposition. This is because the biochemical responses tend to be both a function of nitrogen accumulation and other changes or limitations in the system. Such biochemical response methods may, however, be useful for assessing processes associated with adverse biological effects.

Species composition changes represent the end-point of N deposition effects that are most of interest to the conservation agencies. Protected sites are designated on the basis of their habitats and species, so any losses of designated features constitutes an unambiguous negative impact on site condition. As a consequence, bioindicators based on these approaches are of particular interest to conservation agencies. The weakness of such methods is that there may be many other causes of change other than nitrogen, making attribution of the observations difficult. Such issues apply particularly for Ellenberg indices for higher plants and ground dwelling lower plants, with land management and disturbance being major parallel drivers of change. By contrast, the analysis of epiphytic lichens has the advantage that species composition is less influenced by land management, apart from where there are interactions through emissions to the atmosphere. The major challenge concerning lichen-based approaches is to classify responses in relation to host tree species and in relation to twig versus trunk bark conditions. In this respect, the supporting analysis of bark pH provides a valuable tool to help interpret results.

In considering the diversity methods, it is notable that there are two contrasting approaches using lichens. In the Wirth approach, each species is allocated a characteristic score, so that an average Ellenberg value for a site may be derived from the full list of species present. By

contrast, in the van Herk method, certain species are assigned as either favouring or avoiding a high nitrogen supply, and the species score in each group used to rate the site. The experience from the lichen assessment is that the second approach is more sensitive than the first. This may provide a useful pointer to further development of species based approaches for higher plants, where such a nitrophyte-nitrophobe approach has been little explored. It may be that identifying a series of characteristic nitrophytes and nitrophobes appropriate for different habitats would improve the sensitivity of assessments based on higher plants and bryophytes.

Reciprocal transplanting has the benefit of being suitable to show both damage due to high pollution loads and recovery on transplanting to clean conditions. This may be particularly useful for stakeholders to demonstrate the benefits of pollution abatement. In the case of a test with Atlantic bryophytes in the UK, the extent of recovery on transfer to a clean site after one year was found to be less than the extent of damage on transfer to a polluted site and this supports the expectation that rates of recovery are slower than damage. Conversely, the limitations of such approaches are the need to ensure that the physical microclimate is similar between the exchange sites, the effective restriction to lower plants and the labour involved in making growth measurements.

The use of standardized model plants provides a means to estimate both nitrogen deposition in complex terrain and to demonstrate visible impacts of nitrogen deposition for stakeholders. A key advantage of the method is the ability to provide a demonstrated biological impact of atmospheric nitrogen deposition in a rather short period (e.g. one month). By contrast, such changes do not strictly imply that parallel changes occur on the designated species of a protected site, although in many cases a connection could reasonably be assumed.

11.6.2. Relationship between bioindicator responses and the condition of protected sites

The uncertainty of the relationship between the response of standardized plants to nitrogen and actual adverse effects on a protected site reflects a general issue with many bioindicator methods. With the exception of the Ellenberg approach for higher plants, most of the bioindicator methods do not actually measure the condition of designated species. In some cases sites may be designated for their lichen interest, in which case the lichen methods also provide a direct measure. However, to what extent do, for example, changes in lichen flora indicate adverse effects on other elements of a habitat? Similarly, at what bryophyte foliar nitrogen level should wider impacts on the habitat be expected?

In the case of methods that can be used to estimate atmospheric nitrogen deposition, this question can partly be answered by identifying the value that matches to the critical load for that habitat. Estimates are possible in this way for both foliar nitrogen and foliar ammonium. However, to answer the question more fully, the extent of functional linkage between the bioindicator and possible adverse effects needs to be examined, and this can only be done on a case-by-case basis. For example, if a site is designated for a rare lichen species, which is not included in any lichen index, would the use of lichen bioindicators be sufficient to infer an adverse effect? In this case, a sound approach is to consider the species community with which the rare species is normally associated. If this community is broadly acidophytic, then the species may be reasonably assumed also to be sensitive to nitrogen. Conversely, a rare species characteristic of nutrient enriched locations may only suffer at extremely high levels of nitrogen deposition.

Another example of a functional linkage between a bioindicator and species response would be when using foliar nitrogen in pleurocarpous mosses to question whether a nitrogen-sensitive species of woodland ground-flora might be adversely affected. In this case the foliar nitrogen level is indicative of a shortage or oversupply of nitrogen in the ground flora, and related plant competition effects may be expected to determine an effect on the sensitive species, allowing the connection to be made. Conversely, if a site is designated for its bird populations, the functional linkage with biomonitored nitrogen responses may in many cases be considered too distant to make a link. If the bird species has a specific requirement for an element directly threatened by nitrogen deposition, then a link may be established. However, in most cases the bird may be more general in its requirements, and able to meet its requirements in an environment with significant vegetation change.

Following on from the question of a functional link between bioindicator and a designated feature is the question of relative sensitivity. For obvious practical reasons, bioindicators are selected to represent some of the most sensitive responses to atmospheric nitrogen. Hence the dose at which some change in the bioindicator is observed may be less than the dose required to adversely affect the designated feature. An example of this could be the comparison of changes in lichen species composition against the health of deciduous trees, with the evidence pointing to the latter as being much less sensitive. Such differentials can be dealt with in two ways. Firstly, bioindicators may be considered as 'early warning' tools for later long term effects, for which actual observation of damage would be too late to allow easy recovery. Secondly, the bioindicator responses can provide a 'currency' by which other effects are scaled. For example, (with appropriate refinement of the information), it becomes possible to say, what lichen community or foliar nitrogen concentration corresponds to changes in ground flora composition or loss of vitality in deciduous trees. The development of such exchangeability between indicators and ecosystem responses highlights the need for studies that simultaneously consider a wide range of indicator methods and ecological responses.

In applying bioindicator methods to assess site condition, it is also important to relate this to the legal context. The onus and burden of proof vary in different contexts and this has consequences for the application of bioindicator results. For example, in assessment of potential effects under the EU Habitats Directive, any proposed development (referred to as a plan or project) has to demonstrate that there will be no adverse effects on the integrity of the protected site before it can be approved. In this case, reasonable scientific evidence from bioindicator measurements indicating a likely, but not conclusive, adverse effect should be sufficient to prevent approval of the plan or project, since if an adverse effect is likely, it cannot be concluded to be demonstrated that there would be no effect. By contrast, it is expected that stronger evidence would be necessary in the context of litigation following suspected damage to a nationally protected site from an existing process.

One of the strengths of the bioindicator approach is that it is extremely diverse and flexible. It is diverse in the sense that a broad range of techniques is available, and flexible in that an assessment might use one or many of the techniques simultaneously, with or without associated physicochemical monitoring of atmospheric concentrations. The flexibility allows methods to be applied for quick initial screening or for a comprehensive assessment. The diversity of methods means that, where several methods are applied, the resulting picture obtained is very robust. Hence in the context of litigation if all the different bioindicator methods applied point in the same direction, this should be sufficient to demonstrate conclusively an impact of atmospheric nitrogen deposition.

A further link between bioindicators and site condition arises in the condition monitoring of protected sites. One of the major challenges faced by conservation agencies is to allocate sufficient resources for condition monitoring of all sites, and this places a requirement for quick and cheap methods. It may be that for many sites, only the initial screening (Section 11.4.1) is possible, with this repeated at e.g. 5 yearly intervals. However, where key concerns are identified it is obviously preferable if that a detailed assessment be made. In this case, each of the approaches (biochemical, species diversity, transplant) have merits and could be applied. In particular, however, studies may focus on the repeated application of a) foliar N accumulation methods and b) species diversity-based assessment.

11.6.3. Research challenges to improve bioindicators for atmospheric nitrogen

Although a number of the nitrogen bioindicator methods have been available for many years, from an operational perspective, it is clear that bioindication and biomonitoring for atmospheric nitrogen is still in the stage of scientific development rather than exploitation. A key purpose of this review has been to clarify what is known and what is uncertain so as to encourage the operational use of bioindicators. While many scientific questions remain, the key challenge is therefore how to standardize, quantify and compare the performance of different bioindicator and biomonitoring methods for nitrogen.

To meet these challenges, further research is required that:

- 1) *applies bioindicator methods in a wider range of circumstances* than have previously been tested (e.g. new habitats, effects of different nitrogen forms)
- 2) *improves the quantification of species responses* to nitrogen for each of higher plants, bryophytes and lichens, thereby improving the classification of nitrogen sensitivities between species and their interactions with other parameters (e.g. light levels, substrate pH etc).
- 3) *investigates new ways of scoring* the nitrogen preference of species assemblages, in particular exploring the wider use of nitrophyte-nitrophobe systems for higher plants according to different habitats (e.g. heathland, bogs, woodland ground flora, calcareous grassland etc).
- 4) *compares different bioindicator methods* simultaneously to improve the ability to quantitatively inter-relate bioindicator responses and ecological changes.
- 5) *refines the bioindicator methods* for ease of applicability and improved standardization, including the development of easy to apply protocols.
- 6) *extends the application of bioindicator methods* to biomonitoring on a realistic number of protected sites that can be repeated in the future.
- 7) *applies the bioindicator methods to help revise critical loads and levels*. In particular, the lichen data already provide evidence that the critical level for NH₃ is too high and further application of this approach is required to allow a revised critical level to be set internationally.
- 8) *considers bioindication and biomonitoring in the context of existing physico-chemical monitoring* of air pollution and seeks to utilize the existing air monitoring data as a means to improved the bioindicator methods.

Although it will take several years effort by many researchers to make progress with all of these challenges, a follow up program funded by JNCC, CCW, English Nature and SNH is

now underway which addresses several of the key points. Based on the leading methods recommended here, multiple bioindicator methods are being applied in parallel at several sites that will extend the information available for different habitats and pollutants. The following pollutant, habitat combinations are being investigated in detail:

1. Road emissions (NO_x and NH_3), effects on woodland, as assessed using a) Foliar N, b) Foliar ammonium, c) bark pH, d) Ellenberg index for higher plants, e) Lichen biodiversity (van Herk and simplified approaches) for twigs and trunks and f) standardized grass transplants.
2. Ammonia emissions from agriculture, effects on lowland oak woodland using each of the above approaches.
3. Enhanced wet deposition of ammonium and nitrate on an upland moorland, using methods, 1, 2, 3, 4, 5 and 6 listed in Section 11.3.
4. Effects of different nitrogen forms (NH_3 , NH_4^+ , NO_3^-) on peat bog vegetation from an experimental manipulation study, using methods 1, 2, 3, 4, 5 and 6.

In parallel with these detailed studies, total foliar N, foliar NH_4^+ , bark pH and simplified lichen diversity assessment are being tested at around 30 sites across the UK at sites where air monitoring data are available, with a particular focus on protected sites. While the detailed assessments will yield improved understanding on the responses and relationships between the different methods, the national scale assessment will engage with conservation agency staff seeking to engage them in the application of nitrogen bioindicators as a practical tool to aid the assessment air pollution impacts in relation to site condition.

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APPENDIX I:

Field inter-comparison of different bio-indicator methods to assess the impacts of atmospheric nitrogen deposition

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1. Introduction

In order to compare the different bioindicator methods, and to minimize costs, the experimental work was focussed on a single local gradient in N deposition that has already been well studied (Pitcairn *et al.* 2002, 2003). This has the advantage that information on several additional bio-indicators is already available for comparison, so that these methods do not need to be repeated. This annex describes the field site and summarizes the results from previous studies at the site. The subsequent sections then describe the approaches and results of the additional methods tested for the present project. The additional methods tested were a) standard grass transplants, b) novel biochemical methods in pleurocarpous mosses (foliar ammonium and soluble nitrogen, compared against total tissue nitrogen) and c) the use of lichen biodiversity to indicate atmospheric nitrogen levels.

2. Site description

The site selected for study is a poultry farm situated in southern Scotland at an altitude of 230 m in an agricultural area with background concentrations of ammonia and nitrogen oxides $<1 \mu\text{g m}^{-3}$ and $<3 \mu\text{g m}^{-3}$, respectively. Impacts of N deposition resulting from ammonia emissions on the surrounding woodland have been studied extensively since 1995 and results have been published in peer-reviewed journals (Pitcairn *et al.* 1998, 2002, 2003; Skiba *et al.* 1998; Fowler *et al.* 1998).



Figure A1. Poultry unit at Earlston, southern Scotland

The poultry farm has been operating for 21 years and contains approximately 120,000 birds. The birds are farmed on a roughly 54-day cycle, 60 % of the birds being removed after around 40 days. There are several other units in the area and some litter spreading occurs on surrounding arable land. The east of the site was bounded by dense coniferous plantation of mainly *Pinus sylvestris* with some *Picea sitchensis* and little or no ground flora. However this area has recently been felled leaving a thin fringe of trees. The most extensive stretch of woodland which can be said to be downwind of the unit is to the north. This area of fairly open, largely coniferous woodland is composed of 30-40 year-old *Pinus sylvestris*, with some *Betula pubescens* and *Sorbus aucuparia*. The soil is a poorly drained non-calcareous gley and the ground flora is a mosaic of shade tolerant species of fern, herbs and moss. Past and present studies have been concentrated in this area.

3. Summary of previous work at this site

3.1. Ammonia monitoring

Ammonia was measured continuously for 12 months from February 1995, using open-ended passive diffusion tube samplers at 5 sites around the farm and 5 sites along a transect through woodland north of the farm, at 16, 46, 76, 126 and 276 m from the poultry buildings.

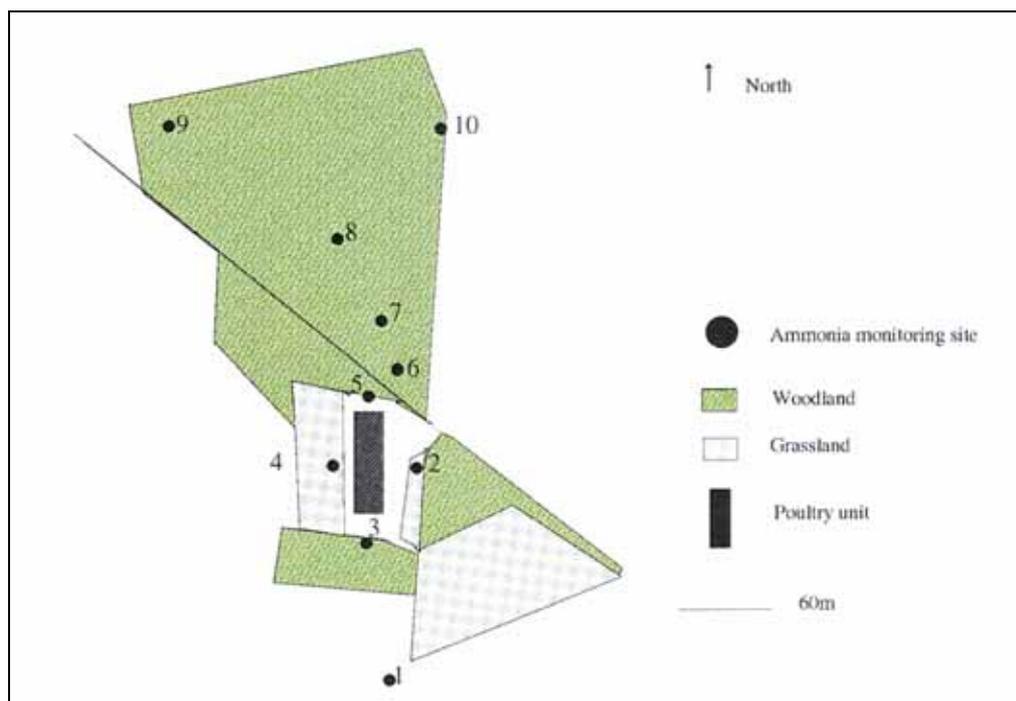


Figure A2. Site plan of Earlston Poultry Farm Field Site 1995/9

Annual mean ammonia concentrations ranged from $29 \mu\text{g m}^{-3}$ close to the livestock buildings to $<1.0 \mu\text{g m}^{-3}$, 300 m downwind of the buildings and exceeded critical levels for NH_3 ($8 \mu\text{g m}^{-3}$ annual mean). Concentrations for some individual sampling periods covering the latter half of the poultry cycles reached values in excess of $300 \mu\text{g m}^{-3}$. This ammonia concentration gradient also provides a major gradient in nitrogen deposition, which has been estimated by Fowler *et al.* (1998) to be approximately $50 \text{ kg N ha}^{-1} \text{ year}^{-1}$, at the woodland boundary exceeding critical loads for acidic coniferous forest (i.e. $15\text{-}20 \text{ kg N ha}^{-1} \text{ year}^{-1}$ to protect ground flora).

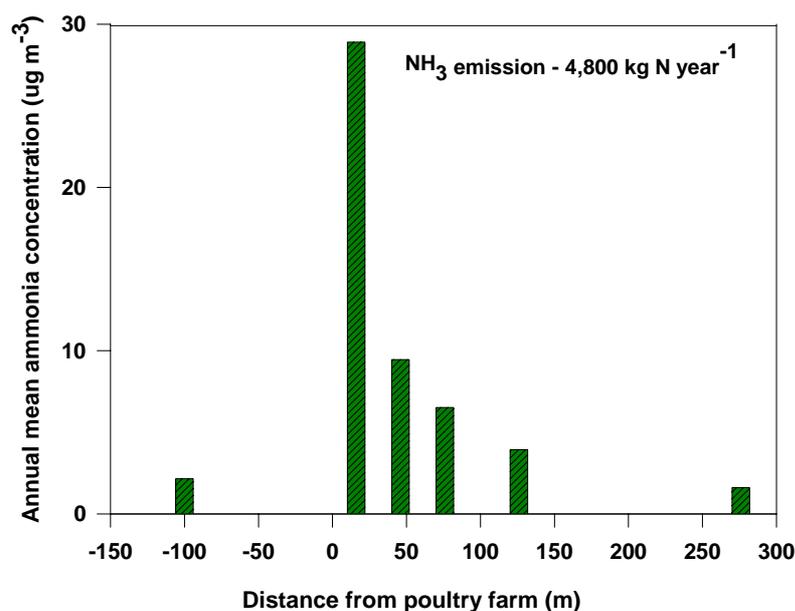


Figure A3: Annual mean ammonia concentrations ($\mu\text{g m}^{-3}$) downwind of the poultry farm (1995-96)

3.2. Vegetation surveys

The aim of the surveys was to quantify the frequency and cover of the higher plant species present and the major bryophyte species along a transect from livestock buildings out to a distance of ~ 0.5 km. Results indicated an abundance of nitrogen loving species such as *Holcus lanatus* and *Chamaenerion angustifolium*, close to the houses. ‘More sensitive species’ such as *Oxalis acetosella*, *Galium odoratum*, *Potentilla erecta* and the mosses such as *Polytrichum commune*, *Plagiothecium undulatum* and *Pseudoscleropodium purum* increased in abundance with distance from the houses.

3.3. Ellenberg Index

Ellenberg nitrogen indicator values modified for British conditions by Hill *et al.* (1999), were determined for each transect. Indicator values from Siebel (1992) were used for bryophytes. Unweighted and abundance (% cover) weighted mean indicator values for all species (vascular plants and bryophytes) at different distances from the farm were calculated.

The N index was able to distinguish differences in composition along the transect downwind of the farm (Figure A4a). Mean Ellenberg N indicator values for transects close to the livestock buildings were larger than those for the more distant transects, but the decline with distance from the buildings was small and the standard deviations were very large. A mean Ellenberg Index of >4.5 may indicate a change in species composition of woodland ground flora but error bars are large. The mean abundance-weighted Ellenberg indicator values for transects close to Poultry Farm E also showed a trend with distance from the livestock buildings (Figure A4b).

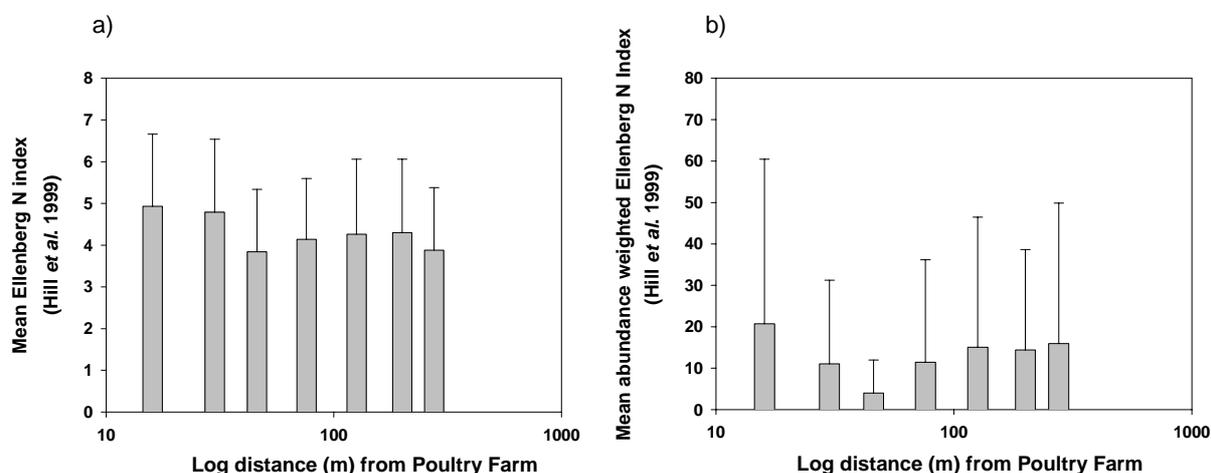


Figure A4: Ellenberg Nitrogen indices of woodland ground flora in the vicinity of the poultry farm. a) Mean unweighted nitrogen index, b) Mean abundance weighted nitrogen index. Indicator scales from Siebel (1992) were used for bryophytes throughout. For higher plants and ferns, indicator scales are from Hill *et al.*, (1999). Error bars are standard deviations. (Pitcairn *et al.* 2002)

3.4. Soil trace gas emissions

The gases NO and N₂O are produced in soils by nitrifying and denitrifying bacteria with the magnitude of the emissions controlled by the availability of N as NH₄ or NO₃ and also by certain climatic and soil properties which promote nitrification or denitrification, e.g. temperature, rainfall, organic matter content. Studies of a range of semi-natural ecosystems which have received various forms of N deposition suggest that measurements of soil NO and N₂O emissions may be useful indicators of soils where N supply exceeds demand of vegetation (Skiba *et al.* 1998). N₂O emissions were measured at 4 distances downwind of the farm on 5 occasions during summer 1997 and N₂O and NO emissions were measured on 2 occasions during autumn 1997. Nitrous oxide fluxes were measured using static chambers (3 per location), which remained *in situ* throughout the measurement period (May to November). Chambers were sealed for 1 hour when gas samples were withdrawn, stored in PTFE bags and analysed by ECD gas chromatography. The measurement of nitric oxide fluxes requires more complex equipment and must be measured on site. Methods are described in Skiba *et al.*, (1993). Soil emissions of N₂O and NO were large close to the poultry houses and decreased with increasing distance from the poultry houses (Figure A5).

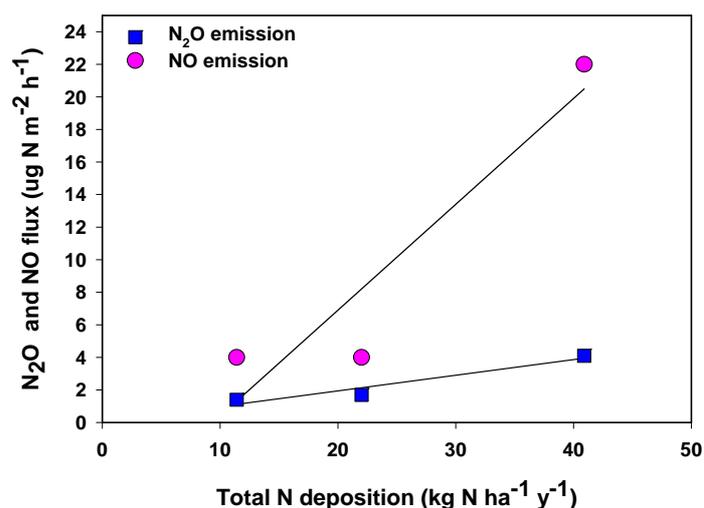


Figure A5: Nitrous oxide and nitric oxide fluxes downwind of the poultry farm.

3.5. Stable isotope $\delta^{15}\text{N}/^{14}\text{N}$ content of leaves

Stable isotope studies can help to identify the source and fate of N added to the environment by anthropogenic activities. Isotopic composition is expressed in terms of δ values – parts per thousand (per mil ‰) differences from a standard:

$$\delta^{15}\text{N} = [(R_{\text{sample}}/R_{\text{standard}}) - 1] * 10^3$$

δ values are measures of the amounts of heavy and light isotopes in a sample (¹⁵N/¹⁴N), $\delta+$ means an enhancement of ¹⁵N and *vice versa*.

Ammonia produced by livestock farms has very negative $\delta^{15}\text{N}$ values as the ammonia volatilised is preferentially enriched with the lighter ¹⁴N.

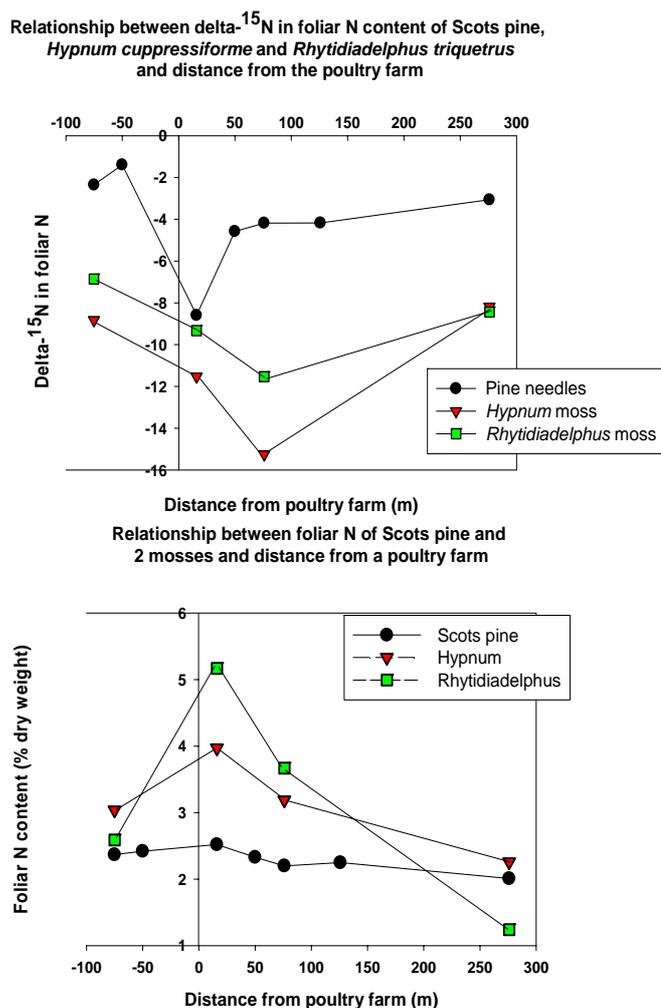


Figure A6: Comparison of $\delta^{15}\text{N}$ and total N measurements, February 1999 (Harrison *et al.* 1999).

$\delta^{15}\text{N}$ values of vegetation sampled upwind and downwind of the poultry farm were shown to reflect the deposition of ammonia and its uptake into plant tissue (Harrison *et al.* 1999). $\delta^{15}\text{N}$ values decreased from -6.8‰ and -8.8‰ for *Rhytidiadelphus triquetrus* and *Hypnum cupressiforme* respectively, upwind of the poultry buildings to -9.3‰ and -11.5‰ respectively, downwind of the poultry buildings. Values increased to the upwind values at a distance of 276 m downwind (Figure A6).

3.6. Foliar nutrient concentrations

Plant samples were collected for foliar N analysis in the vicinity of each NH_3 monitoring site in July 1995. Collections were made (where present) of *Pinus sylvestris*, *Picea sitchensis*, *Betula pubescens*, *Fagus sylvatica*, *Dryopteris dilatata*, *Oxalis acetosella*, and mixed ectohydric moss species. Further samples have been collected on numerous occasions between 1995 and 2002.

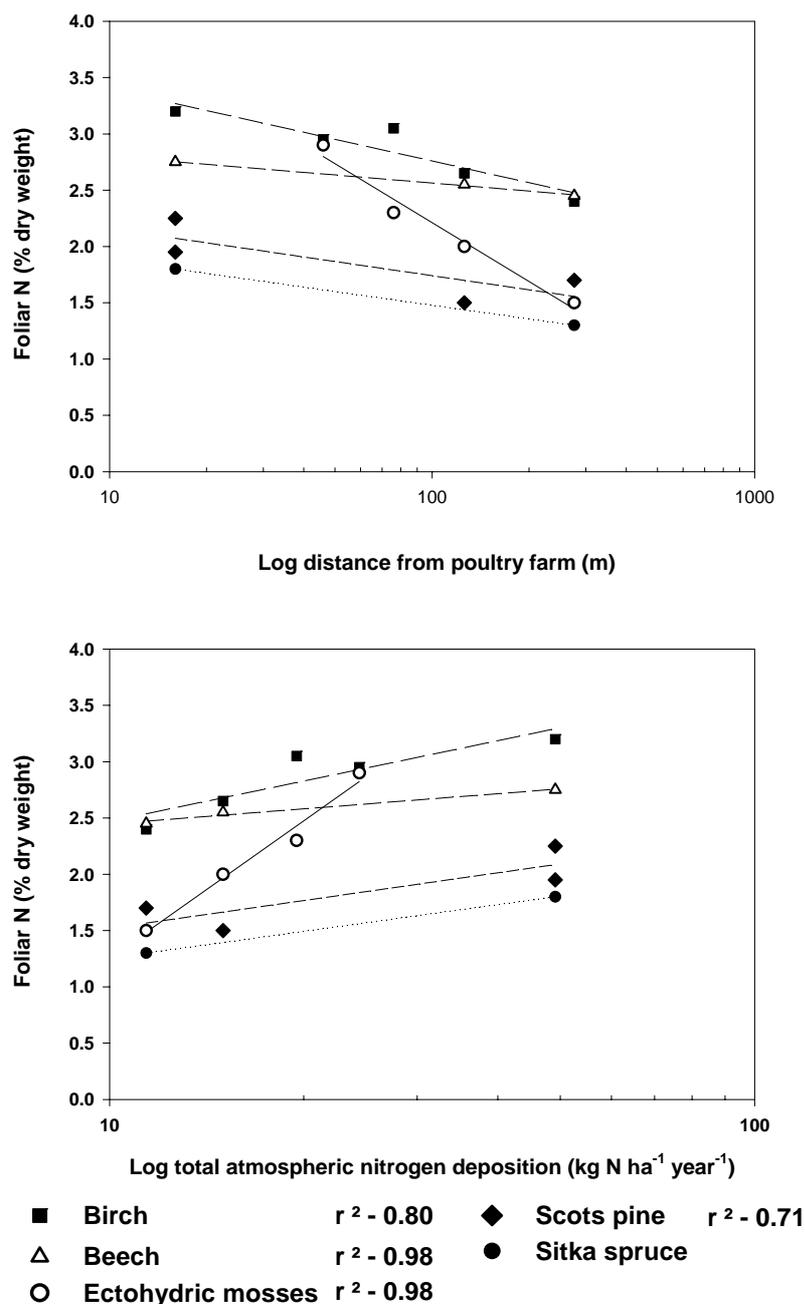


Figure A7: Relationship between foliar N and a) log distance from the poultry farm and b) log total atmospheric N deposition at those distances, for 4 tree species and ectohydric mosses (Pitcairn *et al.* 1998).

Foliar N concentration of sampled vegetation declined with distance from the poultry buildings. Results for 4 tree species and ectohydric mosses are illustrated in Figure A7. The slope of the regression lines for tree species especially beech is very shallow compared with that for ectohydric mosses and demonstrates the buffering effect of stored N on changes in foliar N in large trees relative to the ectohydric mosses, which rely primarily on the atmosphere for the N supply. Conversely, the very steep slope of the regression line for ectohydric mosses emphasises the close link between foliar N concentrations in ectohydric mosses and atmospheric N inputs providing further support for the use of ectohydric mosses as indicators of NH_3 concentrations and N deposition. When foliar N content is plotted

against estimated total N deposition at each distance, the relationships are very similar, suggesting that N deposition is responsible for the changes in foliar N with distance from the livestock building

3.7. Amino Acid analysis

Results from the a summer campaign in 2001 and published results from the literature confirmed that amino storage is minimal in the summer and occurs mainly in late autumn. Further sampling took place in November 2001 and included a range of moss species: *Rhytidiadelphus triquetrus*, *Rhytidiadelphus squarrosus*, *Hypnum cupressiforme*, *Pseudoscleropodium purum* and *Brachythecium rutabulum*. Although only *R. triquetrus* was present at all sites, data from the other species provided additional information about species specific amino acids and the composition of the amino acid pool.

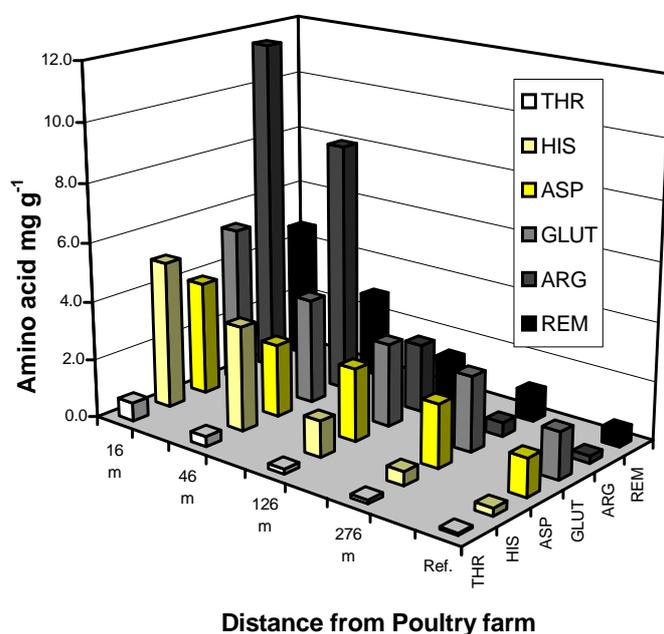


Figure A8: Free amino acid concentrations in *Rhytidiadelphus triquetrus* at 4 distances downwind of the poultry farm and a 'clean' reference site (Ref.), November 2001. (THR-threonine, HIS-histidine, ASP-aspartic acid, GLUT-glutamic acid, ARG-arginine, REM- remaining amino acids) (from Pitcairn *et al.* 2003).

Free amino acid concentration in all three moss species showed a strong relationship with N deposition Pitcairn *et al.* (2003). Arginine was the dominant amino acid at high N deposition close to the buildings in the three species, but was especially dominant in *R. triquetrus* representing 30% of total free amino acids. Concentrations close to the poultry buildings (except in *R. triquetrus*) were similar to those reported by Nasholm *et al.* (1994) and Nordin *et al.* (1998) following N fertilizer additions to mosses of under storey vegetation in boreal coniferous forest. Note also that histidine concentrations decrease with distance from the poultry houses. Histidine concentrations are very small under clean conditions, which may make it a good candidate for bioindication for these species.

Changes in the relative composition of the amino acid pool in response to changes in ammonia concentrations downwind of the poultry farm are shown in Table A1. The composition of the amino acid pool at 300 m downwind of the farm, where NH₃

concentrations are around background level, showed a co-dominance of aspartic acid and glutamic acid in the 3 species, with arginine comprising < 8%. Closer to the farm buildings (46 m), the composition of the amino acid pool changed from aspartic acid and glutamic acid dominance to arginine dominance in *R. triquetrus* and *P. purum*, whereas in *B. rutabulum*, aspartic acid was still dominant. At a distance of 16 m from the poultry buildings, where measured NH₃ concentrations were largest, the amino acid pool was dominated by arginine, especially in *R. triquetrus*. Clearly a change in dominance from aspartic acid and glutamic acid to arginine would denote a change from low to high N deposition. The different uptake strategy of *B. rutabulum*, compared with those of *P. purum* and *R. triquetrus*, and the arginine dominance of the amino acid pool only close to the poultry buildings, may partly explain its frequency close to sources of N and its ability to accumulate tissue N.

Table A1: Relative percentage of 3 key amino acids in the amino acid pool of 3 mosses at 3 distances downwind of a poultry farm. (Hist – histidine; Asp-aspartic acid; Glu – glutamic acid; Arg – arginine) From Pitcairn *et al.* (2003).

Moss species	Relative % of amino acids downwind of the poultry farm											
	276 m				46 m				16 m			
	Asp	Glu	Arg	Hist	Asp	Glu	Arg	Hist	Asp	Glu	Arg	Hist
<i>Rhytidiadelphus triquetrus</i>	31.6	37.7	7.1	7.0	11.6	16.8	40.0	16.0	12.5	17.3	37.1	16.1
<i>Pseudoscleropodium purum</i>	36.9	34.7	4.5	5.9	14.8	15.3	29.6	9.0	14.5	17.3	23.7	12.2
<i>Brachythecium rutabulum</i>	34.5	23.5	7.3	11.2	23.0	14.6	16.0	15.9	13.8	13.5	23.6	16.9

The histidine component is small at 300 m in *R. triquetrus* and *P. purum* but larger in *B. rutabulum*. It increases to around 15% close to the houses. Arginine + histidine comprise 10-18% of the amino acid pool at 300 m, but 30->50% at 46 m from the houses. A change in the relative composition of the amino acid pool may therefore prove to be early indicator of enhanced N deposition.

4. Field Survey 2002

Procedures for testing *Lolium perenne* as a standard plant bioindicator and soluble N as a biochemical indicator were coordinated with the poultry farm bird cycle to maximise exposure to ammonia. One-day old chicks were placed into the houses on 30 September 2002 and 60% of the birds were removed on 7 November 2002. Plants were thus exposed from 1 October 2002 until 7 November 2002, and NH₃ concentrations were continuously monitored throughout this period. In addition to these measurements of CEH, staff of NHM surveyed epiphytic lichens through the transect, together with measurements of bark pH on trunks and twigs.

The site map for the 2002 field survey, with revised numbering to cover the 6 monitoring sites used, is shown below in Figure A9.

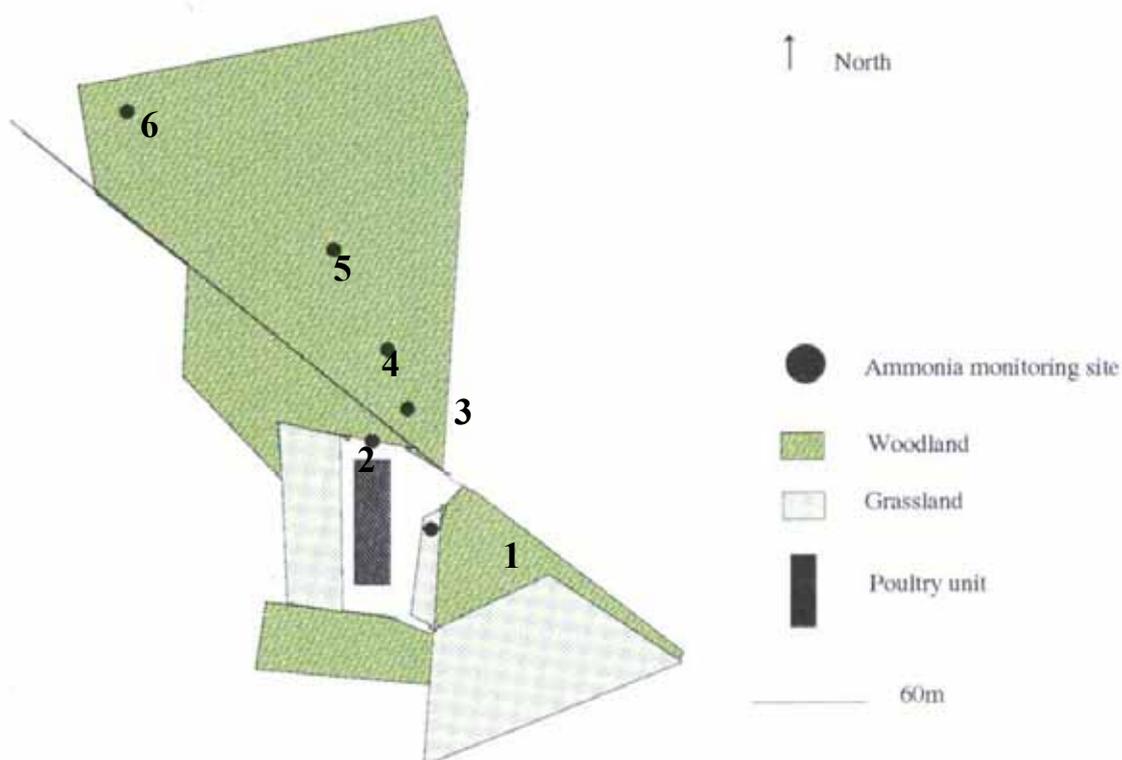


Figure A9: Site plan of Earlston poultry farm field site, showing the location of monitoring points for sampling in 2002.

5. Meteorological data

The meteorological data used in this study are from the CEH experimental site at Whim Moss in the Scottish Borders, a site at a similar altitude ~20 km west of Earlston. The data from Whim show that air temperatures ranged from a maximum of 21⁰C to a minimum of -3⁰C during the experimental period.

The woodland transect broadly downwind of the poultry farm was exposed to ammonia from the farm when the wind direction was in the sector 130⁰-180⁰. The data from Whim show that the wind was in this sector for 43% of the 37 day exposure period.

The total rainfall for the experimental exposure period was 200 mm. During the study there was a dry period near the start but also some very wet days with prolonged rainfall (i.e. days 21 and 22 with 22 and 37 mm, respectively).

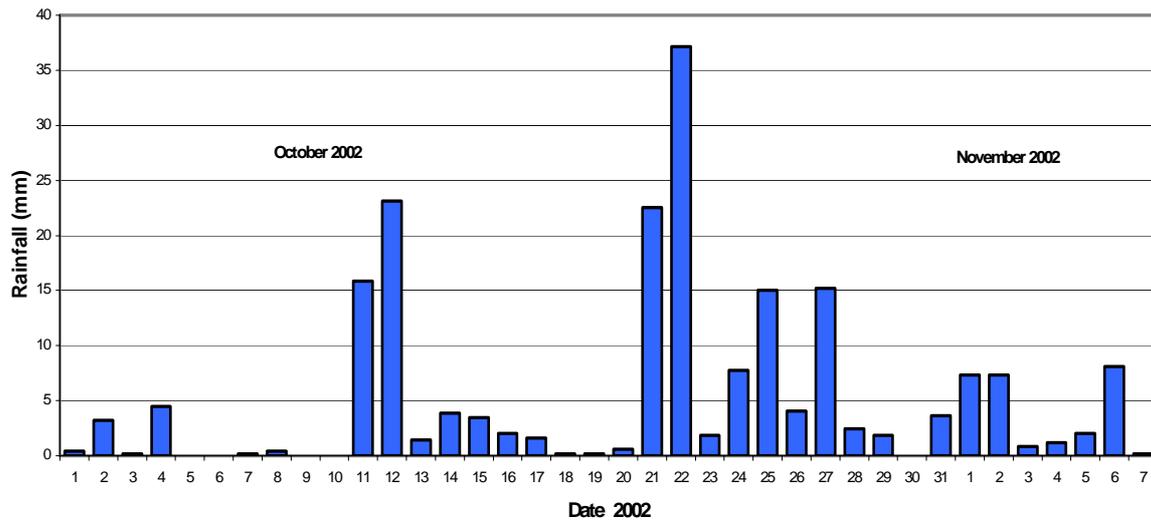


Figure A10: Daily total rainfall data (mm) measured at Whim Moss during the course of the present bioindicator exposure period.

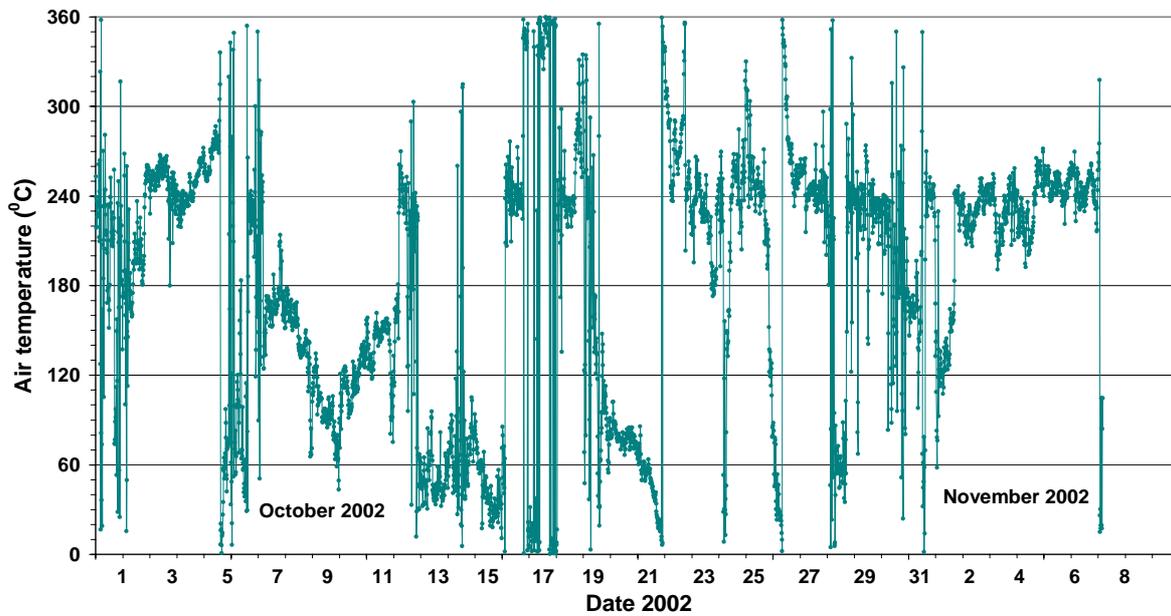


Figure A11: Mean wind direction (15 minute averages), measured at Whim Moss during the course of the present bioindicator exposure period.

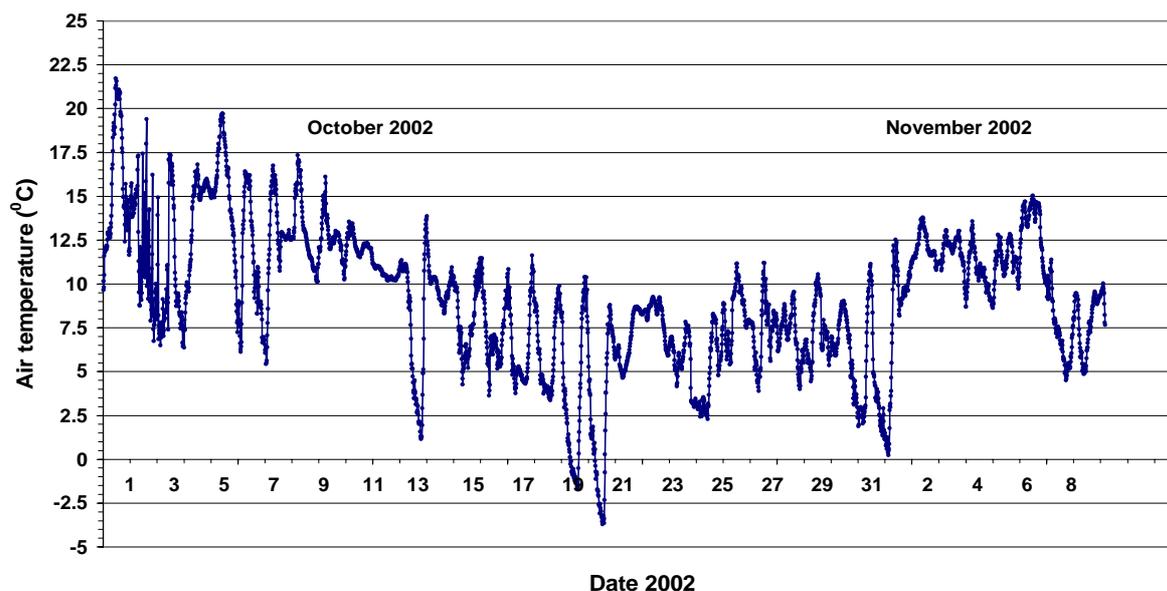


Figure A12: Mean air temperature (15 minute averages), measured at Whim Moss during the course of the present bioindicator exposure period.

6. Ammonia concentrations

Ammonia concentrations were continuously monitored at 1.5 m above ground level at each site using either passive ammonia diffusion samplers (site 1) or Alpha samplers (Adapted low cost passive high absorption samplers: sites 1- 6). Tang *et al* (2001) report the details of sampler specifications and the chemical analysis. Due to the potentially high NH₃ concentrations at site 1, (which are potentially above the operating range for the Alpha samplers), both diffusion tubes with their longer path length (allowing sampling of higher NH₃ concentrations) and Alpha samplers were used. There were 3 replicate samplers at each of the 6 sites. The diffusion and Alpha samplers were exposed from 1 October 2002 until 7 November 2002, with 2 sampling periods (1 - 24 October 2002 and 24 October – 7 November 2002).

The mean ambient concentrations (1 October –7 November 2002) of NH₃ decrease approximately 100 fold over the length of the transect (Table A2, Figure A14) The NH₃ concentrations range from 69.6 µg m⁻³ at 30 m to 0.58 µg m⁻³ at 276 m from the units. These values are very similar to the annual mean concentrations for 1995/96 shown in Figure A3.

Table A2: Mean ambient NH₃ concentrations along 300 m transect from intensive Poultry units

Site No.	1	2	3	4	5	6
Distance from Poultry units (m)	30	16	46	76	126	276
Mean NH ₃ Concentration (µg m ⁻³)	69.60	29.13	12.82	8.01	3.37	0.58

Site 1 is east of the poultry farms. The other 5 sites are north of the farm in woodland (see site map).

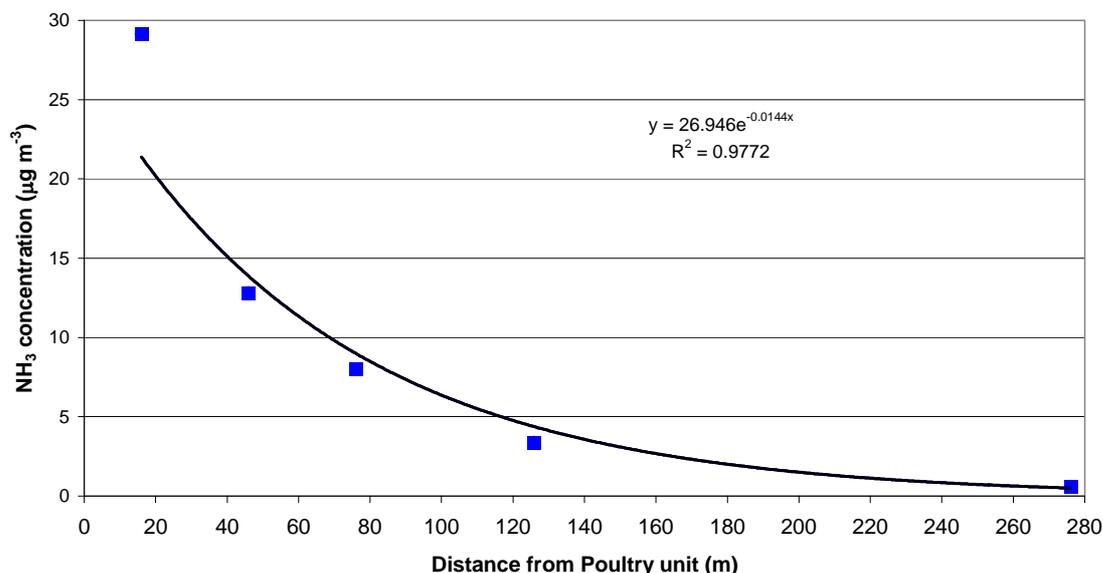


Figure A13: Mean ammonia concentration for exposure period (1 Oct – 7 Nov 2002)

7. Standard Grass transplants - *Lolium perenne*

7.1. Background

The extensive use of standardised plants as bioindicators of atmospheric pollution has been developed by Eurobionet (European Network for the Assessment of Air quality by the use of Bio-indicator Plants), a multinational European Communities study funded as part of the Life programme. Bioindicators are defined by Eurobionet as “organisms or communities of organisms which react to environmental effects by changing their vital functions and / or their chemical composition thus making it possible to draw conclusions on the state of their environment”. (Eurobionet web site).

Eurobionet assessed and evaluated the effects of air pollution by using bio-indicator plants within a network of 11 European cities. The study used the following plants as bio-indicators for a range of urban pollutants; Tobacco (*Nicotiana tabacum* :Bell W 3) and Poplar (*Populus nigra* : clone “Brandaris” as bioindicators of Ozone pollution; Standardised grass culture (*Lolium multiflorum* LAM spp) as bio-indicators of sulphurous compounds and heavy metals/trace elements; *Brassica oleracea acephala* (Curly Kale) as indicators of polycyclic aromatic hydrocarbons (PAH) and *Tradescantia* (clone # 4430) for mutagenic substances.

Although nitrogen was not part of the assessment programme the protocols and procedures used in this large scale study are very useful for any bio-indicator study. Eurobionet states that “ A prerequisite for the use of bio-indicators and particularly for the comparability of results is a high degree of standardisation of the methods of cultivation, exposure, and assessment and evaluation of effects.” Based on these studies and protocols, *Lolium perenne* (L.) was tested as a potential bioindicator of N deposition at the poultry farm site.

7.2. Methods

Plant material

Lolium perenne seed (supplied by Herbiseed, The Nurseries, Billingbear Park, Wokingham, Berkshire, RG40 5RY.) was sown at a rate of 1.22 g per pot on 22 August 2002 into round black pots (volume 3.6 litres) with 4 drainage holes in the bottom. The compost was a peat-loam and grit (4:1:1) mixture with no added fertilizer. After sowing a thin layer of gravel was placed over the seeds in each pot. During the 38 days NH₃ exposure period, the sites would only be visited twice. Therefore it was necessary to use a wicking system to provide irrigation (see EuroBioNet instruction manual for details). Four pieces of glass-fibre cord (300 mm) were placed in each pot, with the wicks running from just below the soil surface to the water reserve tray.



Figure A14: Site 4 with *L. perenne* pots and Alpha sampler post, with detail of protective wire cage.



Figure A15: 6 pots of *L. perenne* in water reserve tray, and detail of wicking system.

The pots were then placed in a heated glasshouse (25 °C) for 7 days to encourage germination. Once the seeds had germinated and the young tillers had reached a height of 3-4 cm, the pots were moved to a sheltered location outside. There was a 100% germination rate for the pots i.e. all 45 pots successfully germinated. During this period (29 August –1 October 2002) the pots received rainfall but were also watered on 3 occasions.

Pre-treatment harvests

On 17 September 2002, two weeks prior to exposure, all the tillers were cut to a height 2-3 mm above the rim of the pot. This was done to prevent the grasses becoming too elongated and also encourage growth during the 38-day exposure period. On 1 October 2002 pre-treatment samples were taken for %N from 5 pots.

Experimental procedure (Poultry Farm NH₃ Transect)

A transect of sampling sites was set up at Heathery Poultry Farm near Earlston in the Scottish Borders on 1 October 2002. Six sites were located in the managed mixed-coniferous woodland at 16, 30, 46, 76, 126 and 276 m to the north of the Poultry farm (for individual site details see Figure A9).

***L. perenne* destructive harvest**

After 38 days of field exposure all pots were collected on 7 November 2002 and returned to CEH Edinburgh for destructive harvest. To provide sufficient sample for both foliar and soluble N analysis three replicate samples were taken by bulking two replicate pots (2 pots x 3 replicates). Using disposable gloves the tillers were cut at 2 mm above the level of the pot.

***L. perenne* tiller, biomass, foliar %N and soluble N**

The tillers were immediately weighed and a fresh weight determined. A 1.5 g fresh weight sample (approximately 50 tillers) was washed in de-ionised water to remove any surface dry N deposition then immediately placed in a plastic bag and stored in a -18°C freezer for soluble N determination. The remainder of the sample was washed, oven dried at 70°C for 3 days then dry weighed. To calculate the total biomass per replicate, the fresh to dry weight ratio was determined and the 1.5 g fresh weight sample incorporated. The dried samples were then ground using a hammer mill (sieve size 0.8mm) and digested and analysed for $\text{NH}_4\text{-N}$ by the CNS method (Grimshaw *et al.* 1989).

7.3. Results: standard grass transplants

Because of the necessity to conduct the field study at the end of the growing season, the growth potential of the experimental *L. perenne* plants will have been affected by the combination of early autumn frosts and high rainfall events. It is very likely that the plants would have shown a much greater response to N deposition had the study been carried out during a period of more favourable conditions. However, even with this potential constraint, very clear results were demonstrated.

Site 1, situated 30 m east of the poultry farm is not on the woodland transect line which runs south to north. Although the site tends to receive more ammonia deposition than sites to the north, it is not in woodland and is more exposed to adverse weather conditions. Consequently, it has not been included in the final analysis of the biomass and foliar N concentrations.

Biomass

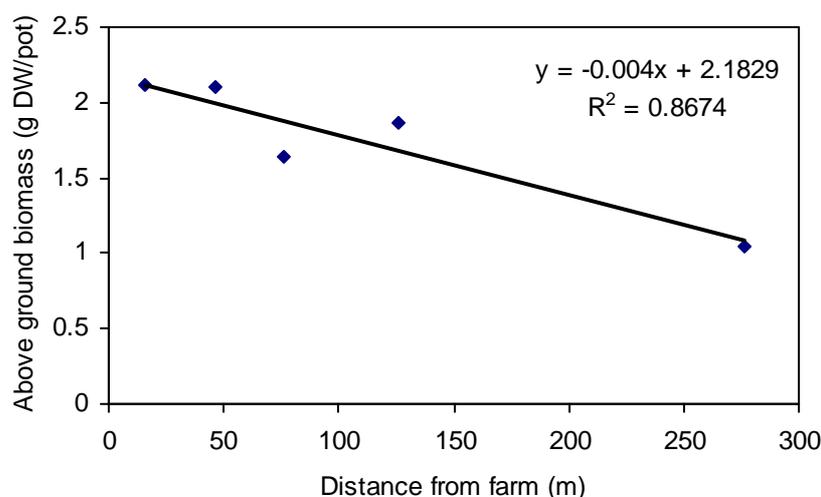


Figure A16: *Lolium perenne* biomass after 37 days exposure to poultry farm emissions

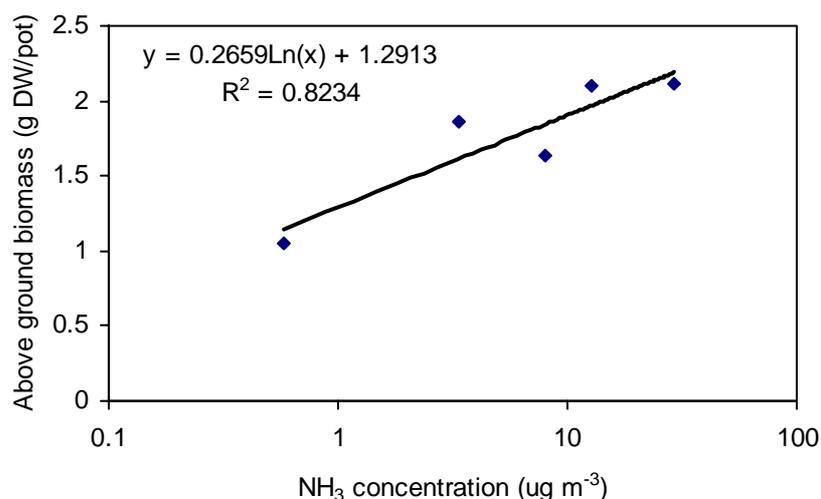


Figure A17: Linear increase in *L. perenne* biomass with increasing ambient NH₃ concentration.

There was a strong linear decrease ($R^2 = 0.86$) in above ground biomass with distance from the poultry farm (Figure A16). The biomass decreased significantly ($P=0.05$) from 2.11 g at 16 m away from the point source to 1.04 g at 270 m. The same significant linear relationship ($R^2 = 0.82$) was found for biomass and ambient NH₃ concentration (Figure A17).

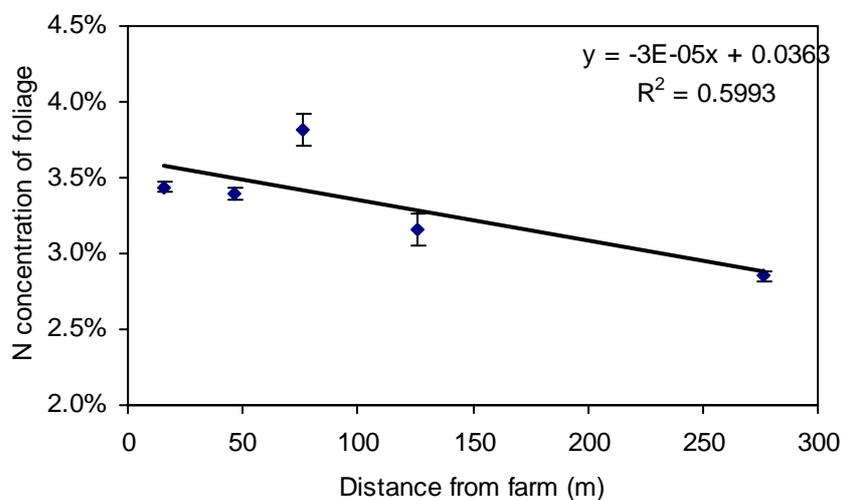


Figure A18: Relationship between tissue N in shoots of *Lolium perenne* and distance from the poultry farm

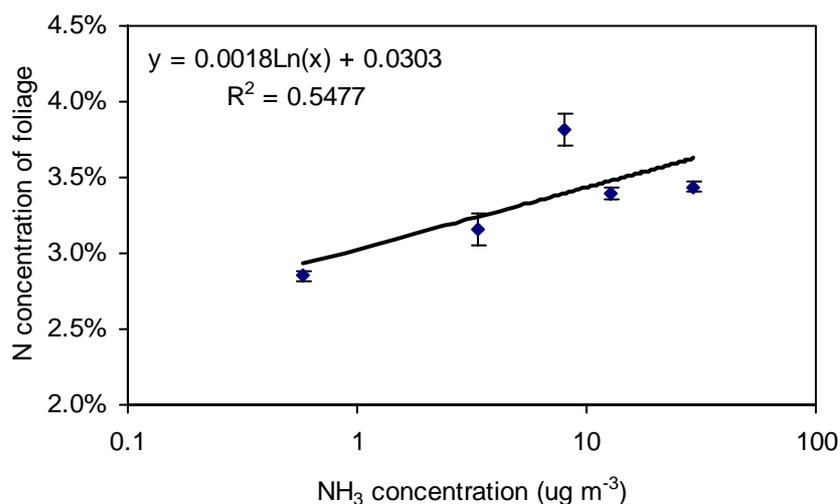


Figure A19: Relationship between tissue N in shoots of *Lolium perenne* and log NH₃ concentrations

Tissue N concentrations

There were reasonably good linear relationships between %N concentration of *L. perenne* and both distance from the poultry farm ($R^2 = 0.60$) and log NH₃ concentration ($R^2 = 0.55$) (Figures A18 and A19). The tissue N concentration at 76 m away from the source appears to be relatively high in comparison with the other values, which is consistent with this site having a denser overstorey and lower light levels (see discussion).

Total above foliar N inventory

It was found that while foliar %N was higher and biomass lower at the more shaded site, this effect of shading was largely cancelled out when the total above ground N inventory of the plants was considered. The result was a close relationship between foliar N and distance from the farm (Figure A20) and NH₃ concentration (Figure A22), with correlation coefficients of $R^2 = 0.98$ and 0.94 , respectively.

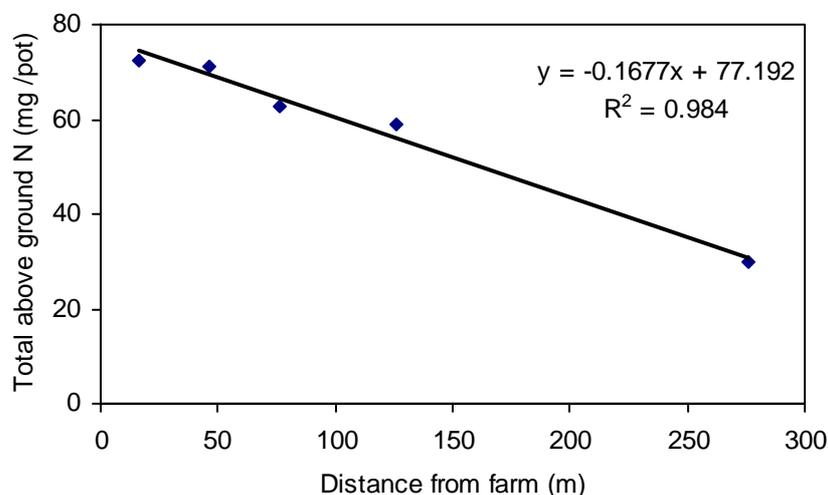


Figure A20: Relationship between total N per pot (mg N) and distance from the poultry farm

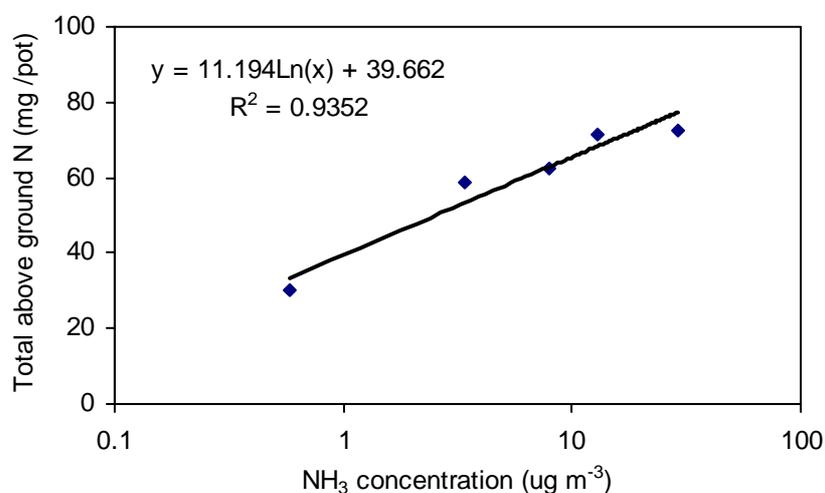


Figure A21: Relationship between total N per pot (mg N) and log NH₃ concentration

8. Biochemical analysis

8.1. Methods

Two moss species, *Rhytidiadelphus triquetrus* and *Hypnum cuppressiforme*, were sampled from 5 sites, and only *Eurynchium praelongum* was sampled from site 1, due to the absence of other species at this location. In addition, due to limited material of *R. triquetrus* and *H. cuppressiforme* occurring at site 2, *R. squarrosus* was also sampled at this location. Disposable gloves were used for all parts of the protocol, and samples were stored in polythene bags at 4 °C prior to sorting. In the laboratory, samples were ‘cleaned’ to obtain a pure sample of the desired species by removing unwanted material (plant, soil and litter), then washed quickly with deionised water to remove any surface contamination without leaching ions from the cells. The sample was then split, half was frozen for soluble N determinations and the other half was oven dried at 70°C for total tissue N content. In selected samples, part of the frozen portion was freeze-dried for amino acid analysis.

8.1.1. Total foliar Nitrogen

Dried samples were hammer-milled to < 0.8 mm and sent to CEH Merlewood for analysis. The ground powder was analysed for total N at CEH Merlewood using the CNS Analyser (Elementar Model: Vario EL).

8.1.2. Soluble substrate nitrogen and ammonium

For analysis of foliar ammonium the method of Hill (1999) and Loubet et al. (2002) was

applied, and this was extended for analysis of total soluble nitrogen. Collected samples were stored in the freezer at -18°C . Small portions of leaf or moss shoot were blotted dry with thin clean laboratory tissues, cut into small segments and placed into the cup of a grinder (10 cm diameter ceramic mortar). The segments were then frozen with liquid nitrogen (approx 10 ml) while quickly grinding the leaves into a thin powder with a ceramic pestle. Two samples of the ground (powdered) plant material were taken (each approximately of 0.1 g) and put into small plastic tubes (1.5 ml Eppendorf). Having previously established the weight of an empty tube, the filled tubes were then weighed precisely, to establish the mass of leaf material. Then 1 ml of de-ionised water was added in each tube, which was closed, shaken and quickly frozen. Samples were stored in the freezer (-18°C) until analysis.

After defrosting samples the solid parts of ground leaf were settled by letting the samples in a tray for few minutes. The supernatant (clear solution on top) was then taken off by pipette and filtered to remove any remaining plant material (using $0.45\mu\text{m}$ Puradisk 25PP syringe filters). The filtered solution was then analysed for total ammonium (the AMFIA membrane dialysis system) and total soluble nitrogen (ANTEK, nitrogen analyser).

The foliar ammonium extraction was performed both on the collected moss samples and on the foliage of the standardized grass transplants.

8.2. Results: biochemical analysis

8.2.1. Foliar N Content

Tissue N content of the 2 moss species, *Rhytidiadelphus triquetrus* and *Hypnum cupressiforme* sampled from the woodland transect (sites 2 to 6), declined sharply with increasing distance from the poultry houses (Figure A23, $R^2 = 0.92$). When plotted against $\log [\text{NH}_3]$, the relationship was even closer, indicating that the response to distance is in fact a response to ammonia concentrations (Figure A24, $R^2 = 0.95$). The slope of the line was steeper for *Rhytidiadelphus triquetrus*, illustrating the benefits of selecting a very responsive species as a bioindicator. The steeper the response, the more suited the species is to indicate narrow deposition ranges.

In all previous sampling campaigns of *R. triquetrus* at this site (1995, 1996, 1999, 2000, 2001, 2002), tissue N data were closely related to NH_3 concentrations and estimated N deposition.

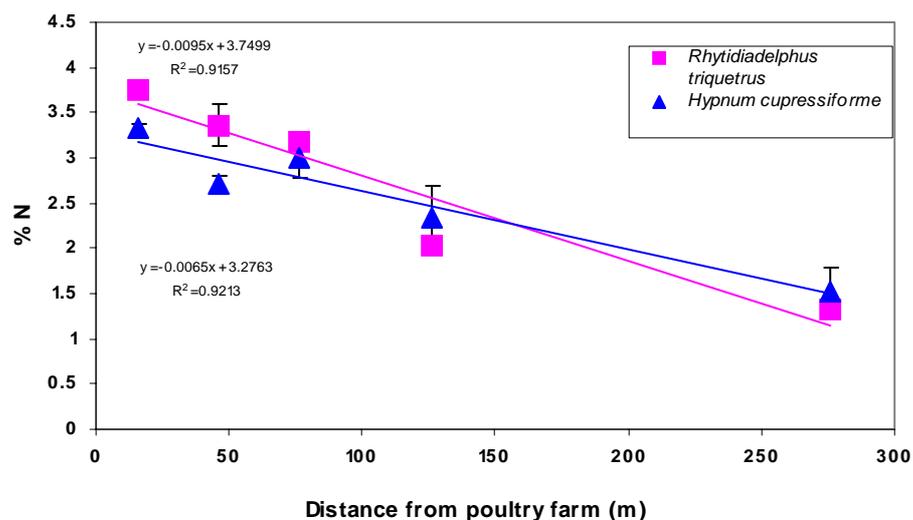


Figure A23: Relationship between tissue N concentration in the moss species, *Rhytidiadelphus triquetrus* and *Hypnum cupressiforme* and distance from the poultry farm.

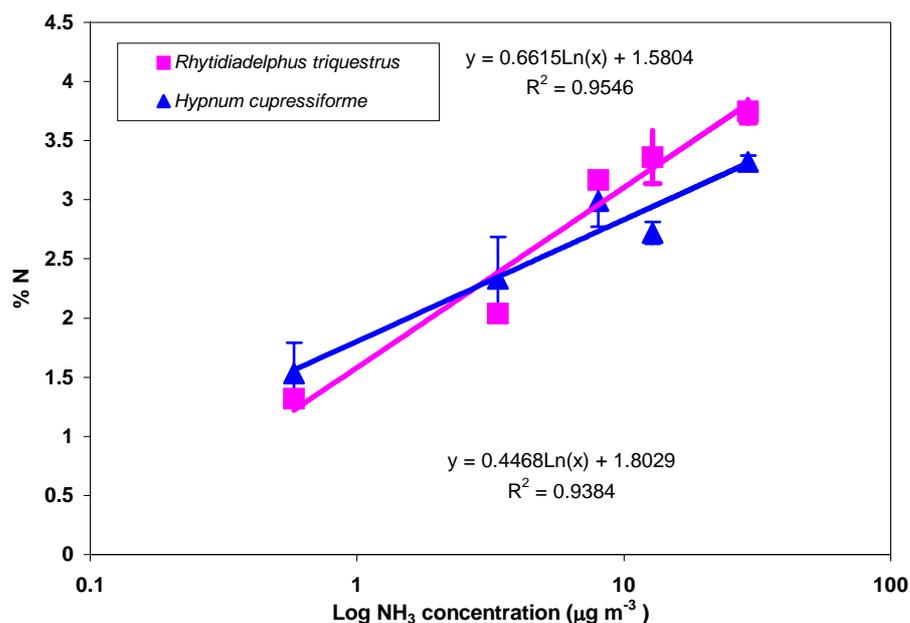


Figure A24: Relationship between tissue N concentration in the moss species, *Rhytidiadelphus triquetrus* and *Hypnum cupressiforme* and log ammonia concentrations.

8.2.2. Foliar soluble nitrogen and ammonium

The measured concentrations of foliar soluble N and foliar ammonium are shown in Figure A25 and A26, respectively. Based on the datasets available, a similar increase of both parameters was seen for each of *H. cupressiforme* and *R. triquetrus*. In addition, although data for *R. squarrosus* and *E. praelongum* were only available at single distances, the values

measured were consistent with the overall increase in concentrations with increasing NH_3 concentrations (and hence nitrogen deposition).

The overall magnitude of soluble N concentrations was larger than that of foliar ammonium by around a factor of 10. In addition, a steeper response to atmospheric NH_3 concentration was seen for foliar ammonium than total soluble N concentrations. A further difference was seen between both these parameters and the measured total tissue N concentration, as can be seen from Figure A27. It is notable that the smaller the concentration of the N component, the larger was its response to NH_3 . At $1 \mu\text{g m}^{-3}$ of NH_3 the values for foliar NH_4^+ , soluble N and total foliar N were 35, 900 and $11000 \mu\text{g g}^{-1}$, while the response across the full range of NH_3 exposure was a change by a factor of 21, 5.5 and 3.5, respectively. These differences are consistent with the idea that a smaller pool size is likely to show larger response to perturbation in nitrogen input.

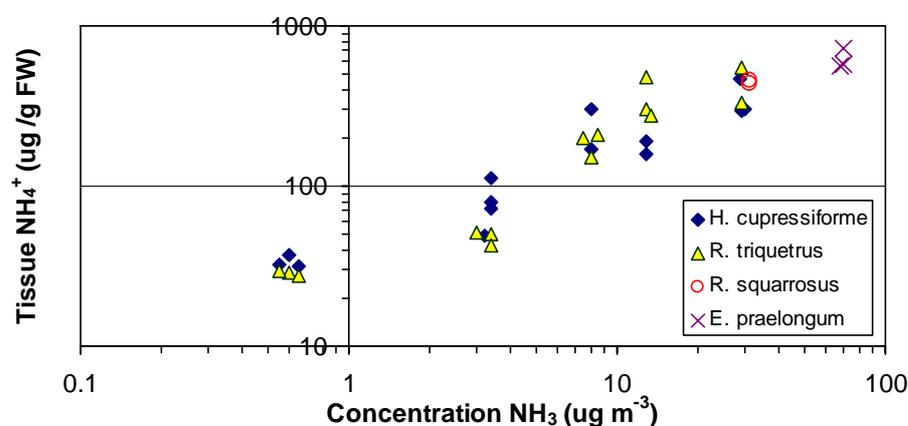


Figure A25: Foliar ammonium concentrations of moss measured at different distances from the poultry farm.

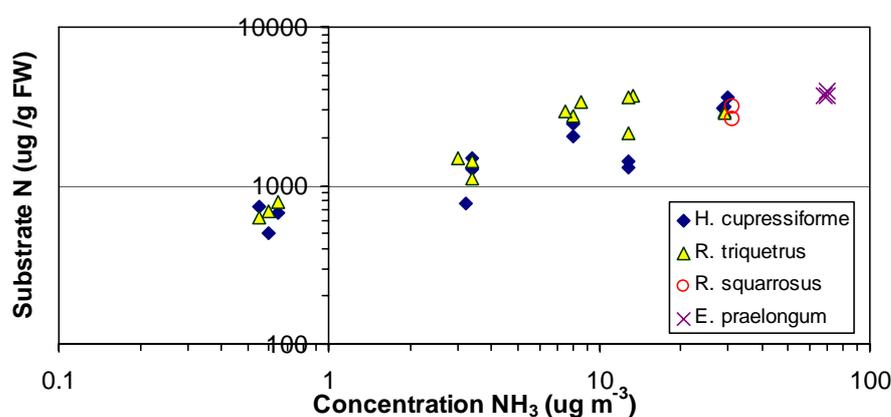


Figure A26: Foliar soluble nitrogen (substrate N) concentrations of moss measured at different distances from the poultry farm.

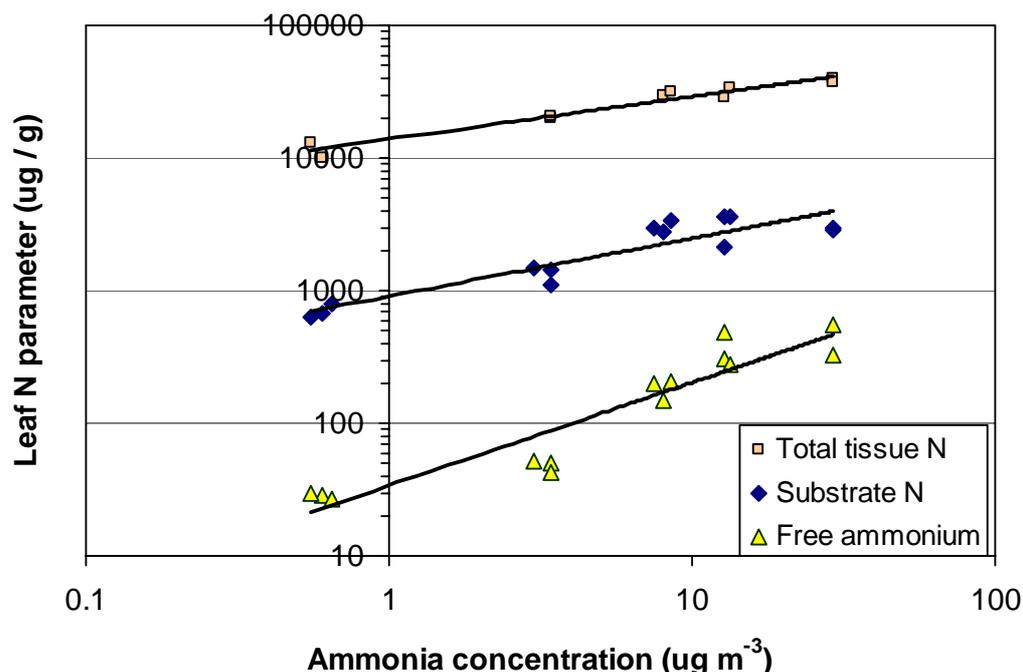


Figure A27: Comparison of three bioindicator methods for nitrogen for the moss *Rhytidiadelphus triquetrus*: total tissue nitrogen, substrate N and foliar NH_4^+ . The largest relative response to NH_3 is shown for foliar NH_4^+ , which is consistent with it representing the smallest nitrogen pool of the three measures and more direct perturbation by atmospheric nitrogen.

Foliar ammonium concentrations were also measured for the standardized grass plants that were set out at different distances from the farm. A clear positive response was observed Figure A28, although at concentrations much lower than those observed in the mosses collected from the site. This may be because of the rather short exposure period of the standard grasses, and the high potential for the grass plants to dilute the absorbed N by growth.

It is worth to noting, however, that foliar ammonium in the grass plants provided a substantially better correlation than of total tissue N in the standard grass plants. It may be recalled that at one site ($\text{NH}_3 = 8 \mu\text{g m}^{-3}$), there was less growth of the plants (low light, Figure A17), together with a higher total tissue N concentration (Figure A19). This effect was cancelled out in the inventory of total above ground foliar N (Figure A21). It appears that this sensitivity to light levels was not so apparent for foliar ammonium concentrations, as shown in Figure A28. It may be added, that Figure A28 also shows the results from the grass plants at Site1 to the east of the poultry house ($69 \mu\text{g m}^{-3}$), and even though this represents a significantly more exposed location, the results are consistent with the main trend from the other sites.

The foliar ammonium analysis was originally developed in the context of field grasses and woodrush (Loubet *et al.* 2002, Hill 1999) and the present test represented the first time that it had been applied to standardized grass plants and bryophytes. It was therefore of interest to test the repeatability of the analysis. For each plant sample replicate the chemical analysis was therefore conducted two times. The results showed a good repeatability of the method at high concentrations, but more scatter at low ammonium concentrations (Figure A29). This may reflect some instability in the extractant-solution at low ammonium levels. This

variability demonstrated the importance of replicating the samples, resulting in the very close relationship to NH_3 concentrations shown in Figures A25 and A28.

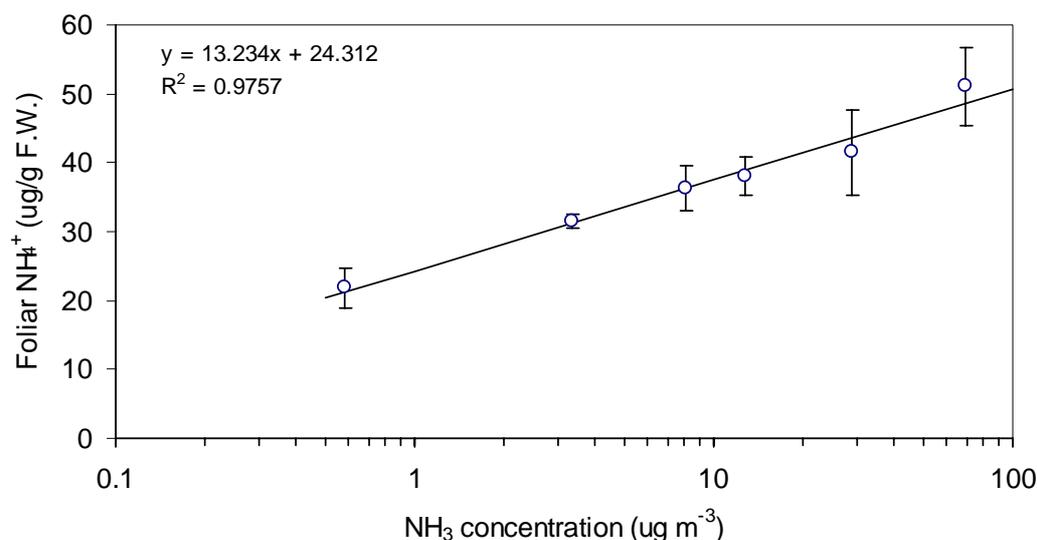


Figure A28: Foliar ammonium concentration in leaves of *Lolium perenne*, exposed for 6 weeks downwind of a poultry farm. These results may be compared with Figure A19 for total tissue N. Results are the means \pm 1 standard deviation of three replicate plant samples. Each of the replicates represents the average of two repeat chemical determinations of the plant samples.

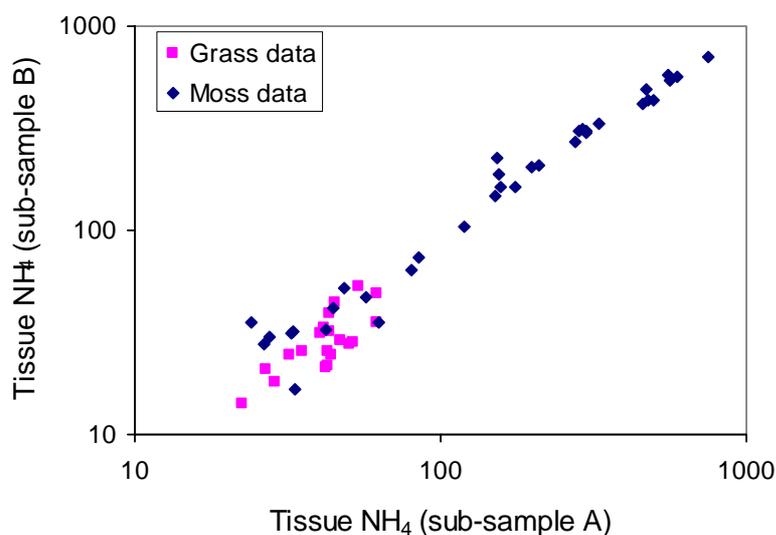


Figure A29: Reproducibility of foliar ammonium analyses for standard grass bioindicator plants and moss samples collected from the field site at different distances. Each vegetation sample was extracted and analyzed twice, so that this graph represents the repeatability of the protocol rather than variation in plant material. Variation at low foliar ammonium concentrations may be due to instability of the extracted solution. (units are $\text{ug NH}_4^+ \text{g}^{-1}$ F.W.)

8.2.3. Application of bioindicator data to estimate nitrogen deposition

The data on foliar ammonium may be used to further investigate the processes of atmospheric N deposition. In the previous graphs, the bioindicator response is gauged against the

measured NH_3 concentration. However, for bryophytes nitrogen concentrations the response is almost certainly related to total N deposition rather than NH_3 concentration. This differs from NH_3 concentration in two ways. Firstly, a background (non NH_3) deposition also needs to be included. Secondly, deposition is largely proportional to NH_3 concentration (through the deposition velocity), but at high concentrations the deposition velocity may be reduced due to a tendency for uptake on plant cuticles to saturate. Two approaches may be taken to deal with this. The first is to estimate from independent studies the background deposition and cuticular saturation response. The data collected here, however, allow for an alternative approach. Based on the hypothesis that the log: log response of foliar ammonium in pleurocarpous mosses to N deposition is linear, the values of background N deposition and cuticular saturation may be identified that best match this hypothesis (see Figure A30). In this way, independent estimates of these parameters may be derived from the bioindicator measurements, based on the availability of the measured NH_3 concentrations for reference.

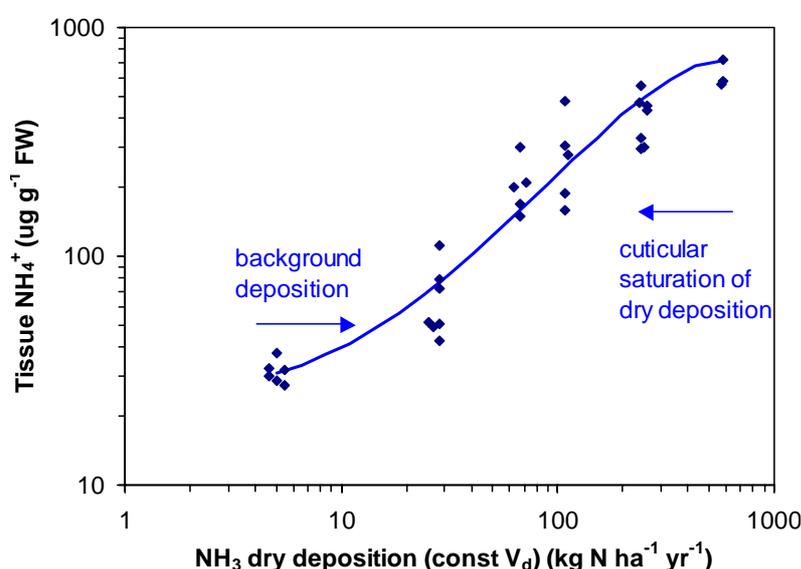


Figure A30: Response of leaf tissue ammonium of pleurocarpous mosses to estimated N dry deposition. In this graph, N deposition is estimated purely from the measured NH_3 concentrations setting background N deposition to zero and assuming that there is no-cuticular saturation of deposition at large NH_3 concentrations. The non-linearity of response may be attributed at low deposition rates to background deposition (wet deposition and oxidized nitrogen) and at high deposition rates to cuticular saturation (see Figure A31).

The stages of this second approach are as follows. Firstly, the value of background atmospheric N deposition is identified that would linearize the response to N deposition at low input rates. Secondly, a saturation parameter to modify the canopy resistance (R_c) is identified that linearizes the response to N deposition at very high input rates. By applying this linearization approach, the background (non- NH_3) deposition to the woodland site of Pitcairn *et al.* (2004a) was fitted as $11 \text{ kg N ha}^{-1} \text{ yr}^{-1}$, while a saturation parameter (S) was fitted to be a value of 1, where canopy resistance (R_c) in s m^{-1} is calculated as $10 + \chi_a^S$ and $R_a + R_b$ are taken as 20 s m^{-1} .

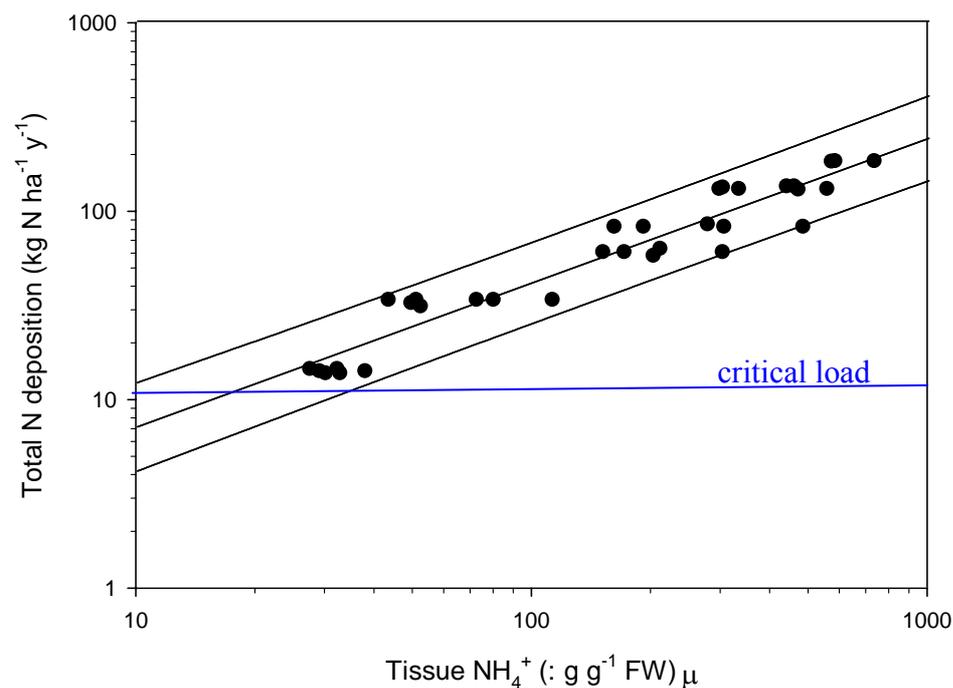


Figure A31: Relationship between estimated total N deposition and measured leaf tissue ammonium of several pleurocarpous moss species under a woodland canopy in the vicinity of a poultry farm. 95% confidence limits of the population are shown, and demonstrate how a measured estimate of moss foliar ammonium can be used to estimate whether the atmospheric deposition at a site is significantly greater than the critical load. The estimate of total N deposition includes a tendency for the deposition velocity (V_d) to reduce at large NH_3 air concentrations. The regression estimates total N deposition as $F_N = 1.5106 (L_{\text{NH}_4^+})^{0.7398}$, $R^2 = 0.922$.

Based on the dataset linearized to reflect a response to total N deposition, it becomes possible to compare the bioindicator results with estimated critical loads. In this case it is of particular interest to be able to estimate the uncertainty in the deposition for a given measured value of foliar N. This may be done by plotting N deposition as a function of the measured bioindicator values. This is done in Figure A31, which also shows the 95% confidence limits of the population, thereby providing a means to see whether a measured foliar ammonium value suggests that atmospheric deposition is significantly above or below the critical load. Based on an indicative critical load for woodland of $12.5 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ (as a mid point of values reported by Achermann and Bobbink, 2003), of foliar ammonium values of $>40 \mu\text{g g}^{-1} \text{ FW}$ would be equivalent to atmospheric N deposition significantly larger than the critical load (95% confidence limits).

The results for the grass biomonitors may also be used to estimate atmospheric N deposition rates and investigate the saturation of N deposition at high NH_3 concentrations. The N accumulation rates of transplanted grass pots were converted to be expressed as $\mu\text{g m}^{-2} \text{ s}^{-1}$ fluxes of nitrogen, and can be also compared with the measured NH_3 air concentrations to estimate dry deposition velocities. In order to estimate the deposition flux, the initial N in the plants must be subtracted, estimated at 35 mg N /pot . The total N deposition rates estimated to the transplanted grasses lie in the range $0.2 - 0.4 \text{ mg N m}^{-2} \text{ s}^{-1}$, except for the plants located at the site most distant to the farm (lowest NH_3 concentrations) where the plants lost N by an estimated $0.05 \text{ mg N m}^{-2} \text{ s}^{-1}$ (Figure A32). This loss of N may be due either to emission of NH_3 from the plants at these low concentrations or due to translocation of N to the roots in a situation with low N supply.

By subtracting the estimated background N deposition from wet deposition and oxidized N dry deposition the NH₃ dry deposition flux may be estimated. Background deposition was taken as 11 kg N ha⁻¹ yr⁻¹ (based on the moss bioindicator estimates above). The estimated NH₃ dry deposition can then be used with measured NH₃ air concentrations to estimate the deposition velocity (V_d). Apart from the site most distant from the farm (where the estimated V_d is necessarily negative, due to the loss of N from the plants), Figure A32 shows that V_d decreases with increasing NH₃ concentration. This saturation of V_d at high NH₃ concentration is broadly consistent with that derived from the moss data above, which are also shown in Figure A32.

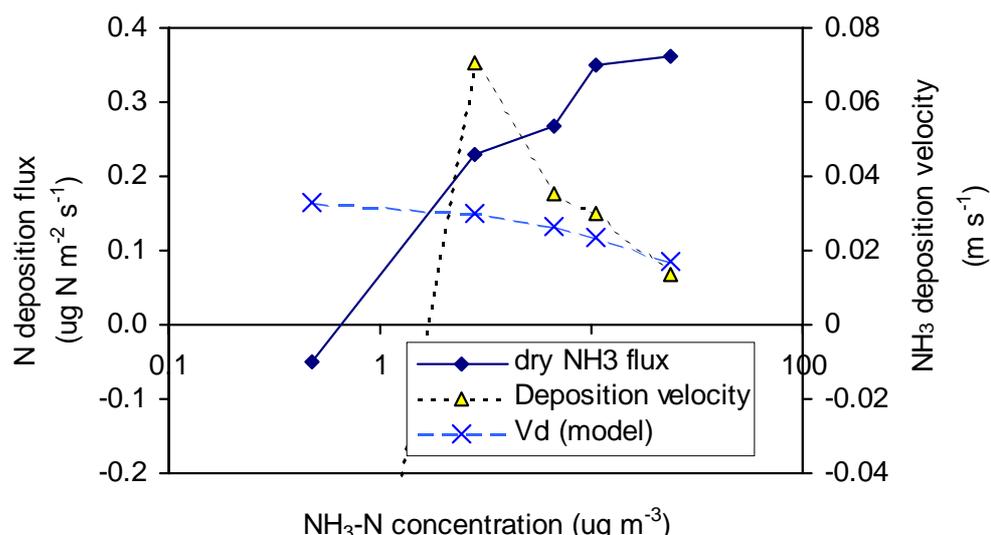


Figure A32: Estimation of atmospheric N deposition flux to standardized grass plants in relation to NH₃ concentration at the exposure location at different distances from the poultry farm. The deposition velocity (V_d) is also shown, calculated from the estimated N deposition flux (minus estimated background N deposition, wet deposition plus dry NO_y deposition, of 11 kg N ha⁻¹ yr⁻¹) and from the measured NH₃ air concentrations. Also shown for comparison is the modelled NH₃ dry deposition velocity derived from the moss NH₄⁺ bioindicator data, showing the tendency to saturate at high NH₃ concentrations.

This section has shown that both the N bioassays of mosses and the standard grass transplants have the potential not just to indicate responses to atmospheric deposition, but to provide quantitative estimates of atmospheric N inputs. In particular, both also provide evidence of the saturation of dry deposition at large NH₃ concentrations. Figure A33 provides an overall comparison of the total N deposition estimates derived from the foliar NH₄⁺ of mosses and from the standardized grass plants. The overall agreement is rather encouraging, with the differences reflecting differences of processes for each. E.g. the moss data are an estimate of the total N deposition to the canopy, while strictly the standard grass data are an estimate to the grass plants themselves. The advantage of the grass plants is that these values are derived without need to monitor NH₃ air concentrations, while their disadvantage is the possibility of negative values related to either emission from the grass plants or re-allocation of the N between roots and shoots in the grasses.

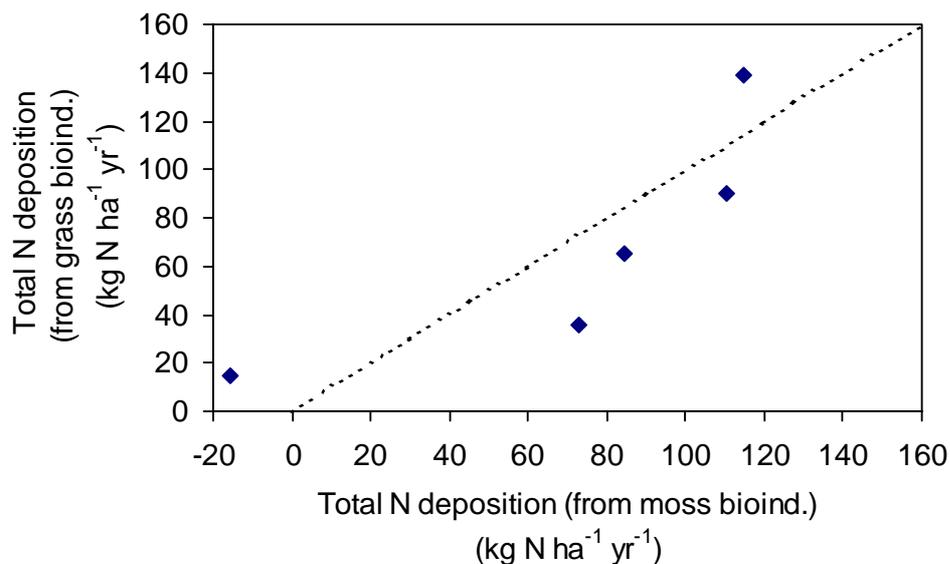


Figure A33: Comparison of N deposition estimates derived from foliar ammonium of mosses and from N accumulation by standard grass transplants at different distances downwind of the farm. Strictly, the moss bioindicator estimates total N deposition, while the grass method estimates deposition to the grass transplants themselves. The negative deposition the grass method at the cleanest site may be due to either N reallocation between root and shoot or to NH₃ emission from the grass plants. $R^2 = 0.69$.

9. Lichen diversity indicators

9.1. Background

Lichens have been widely used as bio-monitors of atmospheric pollution due to sulphur dioxide and heavy metal accumulation across Europe, and for this purpose lichens on corticolous substrata have been widely applied. Lichen diversity was strongly correlated with sulphur dioxide deposition, so that in situations of high deposition there were few lichens present and only toxic-tolerant species were present such as *Lecanora conizaeoides*. The recent reduction in atmospheric SO₂ has led to rapid changes in the lichen communities many of which are associated with increased numbers of nitrophytes.

Ellenberg (1988) in his analysis of European vegetation had already distinguished the effects of toxic substances from the effects of nitrogen as a nutrient. This approach was applied to lichens by Wirth (1992). Subsequent work in the Netherlands showed a strong correlation between nitrophyte species and ammonia deposition, and an accompanying loss of acidophyte species. Scales for nitrophytes (NIW) and acidophytes (AIW) were used to map air pollution due to ammonia in the Netherlands (van Herk 1999). In France, Lallement *et al.* (1999) used selected macro-lichens to map zones in large areas of countryside where agricultural intensification was occurring. The German method (Verein Deutscher Ingenieure (VDI)) was used in the mapping of zones of pollution around industrial and urban conurbations. The VDI was based on frequency of selected species known to be sensitive to air pollution within a prescribed area of the trunk where diversity was highest. Following the lowering of SO₂ levels and the increase in lichens in urban areas a Lichen Diversity Value

(LDV) was devised, based on total diversity and frequency within grids of quadrats on 4 aspects of each tree trunk.

All methods for collection of data and for use of indicator values were tested downwind of the poultry farm, in parallel with measurements of NH₃ concentrations in order to evaluate appropriate methods with which to assess lichen responses to reactive atmospheric nitrogen. This aspect of the field study had the following objectives:

- To test appropriate methods for assessing lichens as bioindicators of atmospheric nitrogen
- To prepare a data set that can be used to compare and contrast methods in current use
- To make recommendations on the current state and future development needs for recording of lichens as bioindicators of the effects of atmospheric nitrogen deposition.

9.3. Field site and methods

The lichen recording was conducted by Wolseley and James (16 - 19th September 2002) at the same sampling locations as those used for the grass bioindicators (see Figure A9). Ammonia concentrations were measured in parallel at the same sites, with the results reported in Table A2 and Figure A13. It is important to note that these NH₃ monitoring locations in the middle of the plantation were well shaded and did not conform to sampling conditions as defined by European methods such as VDI or LDV (Asta *et al.* 2002), or van Herk (1999) where a condition of sampling procedure requires well-lit trees. Therefore, the present sampling provides a test of the robustness of the methods under a range of light conditions.

The site is surrounded by *Picea* trees in close proximity to the sheds and a c.40 year old *Pinus sylvestris* plantation to the north and west. There were large *Fagus sylvatica* trees along the tracks, but the only native tree frequent on the site was *Betula pubescens*. Saxicolous habitats were restricted to a few walls either in deep shade or on the site boundaries and terricolous lichens were rare in this plantation habitat. These habitats were not included in the survey.

Due to the limitation of available tree species on site, conifer trees were used for all lichen sampling on trunks, and twigs of *Betula* spp were sampled at all sites. In order to trial several methods in use for bio-monitoring, sampling was undertaken on 5 trees of conifer and 5 trees of *Betula* spp at each site. Girth of all tree species was recorded. Bark was collected from all trees sampled for lichens, and twig lengths were also cut from birch trees sampled. All specimens were dried and stored in paper bags prior to pH determination.

The whole site at Earlston is surrounded by agricultural land and there is no immediately adjacent site of natural vegetation. In order to assess deviation from naturalness of the site for lichens (Loppi, 2002), a nature reserve at Gordon Moss, 4.7 km east of the site dominated by *Betula pubescens* was included in the field survey of lichens on twigs. No ammonia data exist for this site but modelled values are 0.3 µg m⁻³ (estimated uncertainty +/- 0.15).

In order to conform to data collection by a range of methods, lichens were recorded as follows:

Nitrophyte/Acidophytes and LDV

All lichens recorded on a trunk and nitrophyte and acidophytes species were assigned frequency values as outlined by van Herk (1999), while full lichen species composition was used to assess the Lichen Diversity Value (LDV). In both cases, measurements were made at 1.5 m on the trunk. On each tree 4 quadrats (50 cm x 10 cm with 10 cm grids) were placed at compass points and lichen presence recorded in each 10 cm square for each compass point.

Lichens on twigs

Birch trees (*Betula* spp) were widely distributed throughout the site and were used to assess lichens on twigs adapted from the method outlined by Wolseley and Pryor (1999). Five birch trees were selected at each site, where possible with a girth of > 20 cm and lichen presence recorded on accessible twigs and branches up to 2 cm diameter on each tree.

Measuring bark and twig pH

Samples collected of bark and twigs, were placed in paper packets and air-dried. The pH on tree trunks was measured in the lab as follows (Farmer *et al.* 1990):

- At least 3 samples of tree bark were collected with as flat a surface as possible at least 1 cm in diameter, placed in paper packets and dried at room temperature.
- The surface was wetted with 25 mM KCl for 5 minutes and the pH measured in the lab with a Gelpas surface electrode.

The method used to measure bark pH of twigs was modified from Kermit and Gauslaa (2001). Twigs were cut in lengths and placed in paper packets and dried. In the lab 6 cm twigs 5-7 mm thick were cut and the cut ends sealed with paraffin wax, then placed in a tube containing 6ml of 25 mM KCl. Samples were shaken and incubated for 1 hr at room temperature. Samples were shaken before removing small amounts with a pipette and recording pH with the same electrode. Samples were left overnight and recording repeated.

9.5. Results: Lichen diversity approaches

LDV and VDI Diversity Assessment

Mean species diversity was calculated per site and per aspect for the LDV approach and the results compared with the VDI method for lichen biodiversity assessment. Sampling of trees was restricted to 5 trees for each ammonia monitoring station, as the greatest distance from source was 276 m. At site 1 only *Picea* was available for sampling together with some *Betula pubescens* scrub. In other sites *Pinus sylvestris* was available in a plantation. However tree trunks were not equally exposed to light sources across the site so that in sites 3, 4, 5 and 5a *Pinus* trunks were exposed to very low light levels. In this heavily shaded situation lichen sampling did not fulfil guidelines outlined by LDV (or NIW/AIW see below) that only exposed trees should be sampled. Where possible trees were selected in different compass

quarters, but in practice well-lit trees were chosen in as homogeneous conditions as possible in order to avoid unduly shaded trees.

The LDV and VDI lichen biodiversity scores (mean for all compass quarters) are shown in Figure A34. Neither of the diversity indices correlate with ammonia concentrations, due to the replacement of acidophyte diversity by nitrophyte diversity in sites influenced by high ammonia concentrations. However in sites close to the source exposed to very high levels of ammonia, the low diversity of nitrophytes suggests that many species are facultative nitrophytes and that nitrogen at high levels is damaging except to species that are accumulators such as *Xanthoria parietina*.

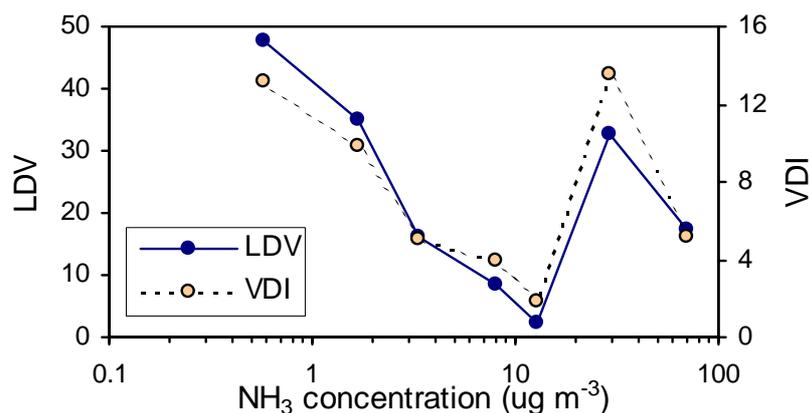


Figure A34: Lichen diversity on trunks of trees recorded at different distances from the poultry farm. VDI, Verein Deutscher Ingenieure (German standard method) and the LDV Lichen Diversity Value (European method).

From these results it would appear that there is a range of sensitivities to critical ammonia among nitrophytic species. It is also notable that the LDV and VDI methods give rather similar responses across the transect, although the absolute values on these scales differ.

Bark pH

The results of the bark pH measurements showed very clear results, and it is useful to show these in advance of the results from the NIW/AIW and Ellenberg (Wirth) scales, as the pH data affect their interpretation. Bark pH of both tree trunks and twigs increased with NH₃ concentration, apart from the closest site to the farm (Site 1) where there was a slight decrease in bark pH (Figure A35). It is notable that the overall increase in bark pH with ammonia was larger for trunks than twigs, so that while trunk and twig pH values were similar at the highest NH₃ concentrations, in clean conditions the twig pH was higher than trunk pH.

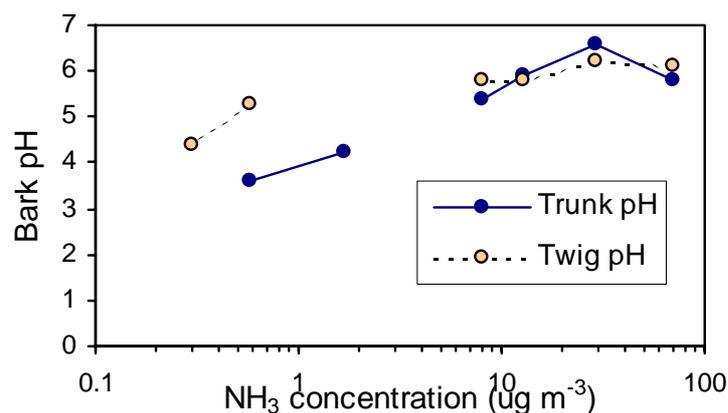


Figure A35: Bark pH of trunks and twigs measured at different distances from the poultry farm on acid barked trees (trunks: pine, except highest NH₃ site, where spruce was sample; twigs: birch).

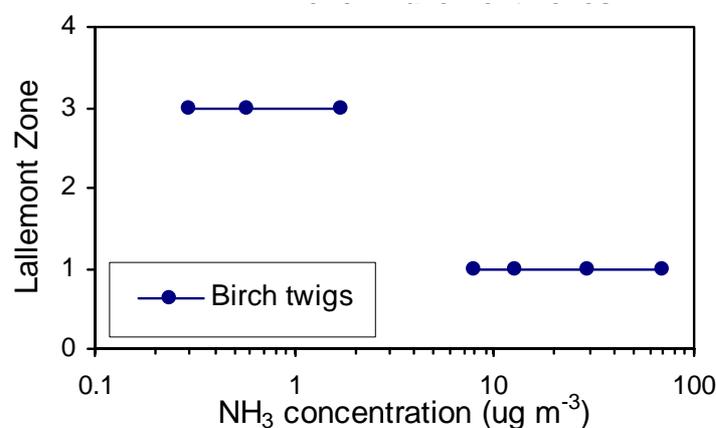


Figure A36: Response to lichens to NH₃ concentration according to the Lallemond system, measured on the twigs of spruce downwind of the poultry farm.

Lallemond Method

The simplest lichen nitrogen index that was applied is that developed by Lallemond et al. (1999) for western France to define zones of agricultural pollution. In this scheme, each location is scored with an integer value between 1 and 7, depending on the species present. The results using this assessment for the study site here are shown in Figure A36. These demonstrate a decrease in the score with increased NH₃ concentrations. By contrast, the method gave little resolution in the values, with values of either 1 or 3 for each of the sampling locations.

Ellenberg (Wirth) Scale

The results of the Ellenberg scoring system developed by Wirth (1992) are shown in Figure A37. The normal application of the method (that is shown in Figure A37a) is to record all species present and calculate the average score of the site. In this study, only presence/absence was recorded, and no account of cover taken. This may be contrasted with the approach of van Herk, where abundance is considered. This approach is therefore simpler than that of van Herk. However, it does still require the identification of all species present.

The Ellenberg method showed a consistent increase in score with increasing NH_3 concentration for twigs that was also seen (although with more scatter) for lichens on trunks. The lowest NH_3 concentration at which an effect could be seen (i.e. a higher score than the cleanest location) was around $2 \mu\text{g m}^{-3}$.

A simplified variant of the method was also tested, by filtering the collected dataset to include only well known/ easily identifiable macrophyte lichens (foliose and fruticose species) in the average Ellenberg score for each site. The results of this “simplified Ellenberg” method are shown in Figure A37b. These demonstrate that clear indicative results can be obtained by this much simpler scheme that only requires the identification of well-known species, and can therefore be applied by people with a basic training in lichen identification. On the other hand, Figure A37b shows that the specificity of the results is lower than the analysis using all the species present, with the lowest NH_3 concentration at which an effect was clearly seen being between $2\text{-}8 \mu\text{g m}^{-3}$.

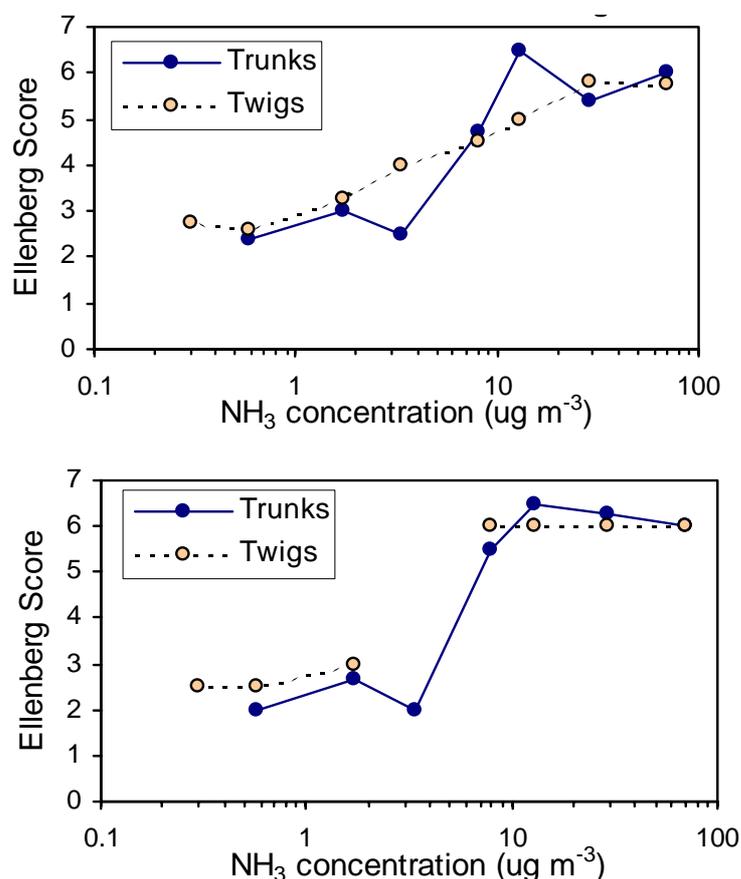


Figure A37: Responses of lichens to NH_3 concentration recorded according to mean Ellenberg score (according to Wirth, 1992) on trunks (pine, except highest NH_3 site, where spruce was sample) and twigs (birch) of acid barked trees downwind of the poultry farm. The first graph represents the standard application based on all species present. The second graph represents the mean score including only well known foliose and fruticose species.

At one location for the twig application of the simplified Ellenberg method, no score was obtained. This was because at this site none of the easily identifiable species were present.

Nitrophyte/Acidophyte Method

The results from applying the van Herk (1999) method on tree trunks are shown in Figure A38. In addition, the application of the scoring method for twigs of Wolseley and Pryor (1999) to the Nitrophyte/Acidophyte scale of van Herk (1999) is also shown (Figure A39). In the normal application of the Acidophyte (AIW) and Nitrophyte (NIW) scales by van Herk (1999) for trunks, the two separate scores are given. In Figures A38 and A39, however, an overall indication of eutrophication is also provided by AIW minus NIW. In this combined scale, a positive value indicates a lichen flora dominated by acidophytes, while a negative value indicates a lichen flora dominated by Nitrophytes.

A strong shift is detected from high NIW values in close proximity to the poultry sheds to low NIW values, and from low AIW values in the vicinity of the farm to high AIW values 276 metres. The highest AIW score on trunks occurred for the 'background' site (6 km distant) being at Gordon Moss SSSI.

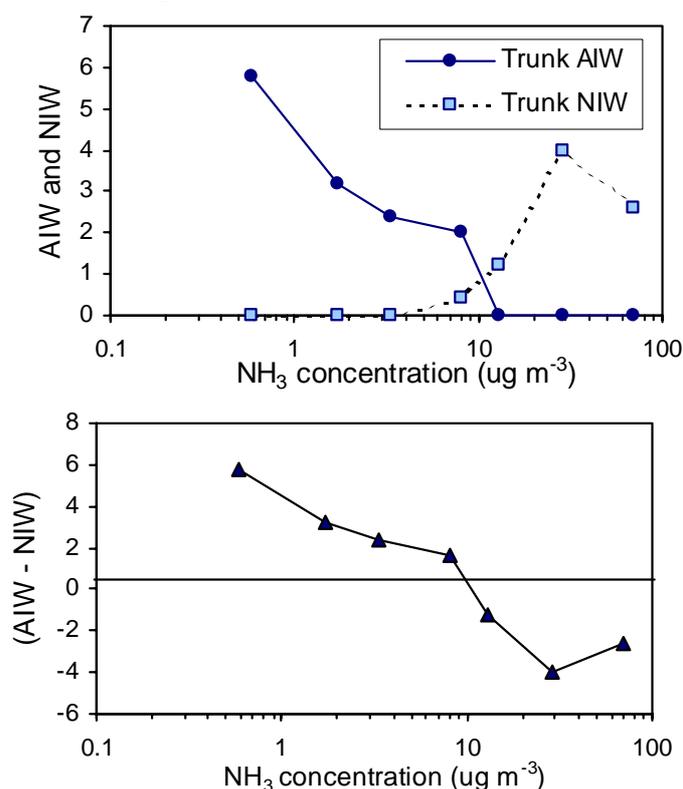


Figure A38: Responses of Acidophyte (AIW) and Nitrophyte (NIW) lichens to NH₃ concentration measured on the trunks (pine, except highest NH₃ site, where spruce) and twigs (birch) downwind of the poultry farm.

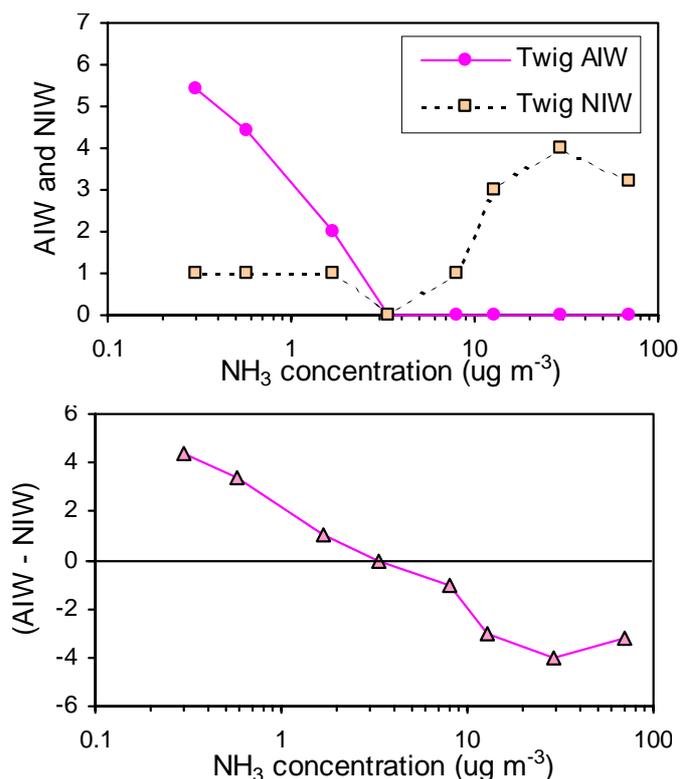


Figure A39: Responses of Acidiphyte (AIW) and Nitrophyte (NIW) lichens to NH₃ concentration measured on the twigs (pine, except highest NH₃ site, where spruce) and twigs (birch) downwind of the poultry farm.

Comparison of the AIW, NIW and (AIW-NIW) scores shows that the decrease in acidophytes occurred at lower NH₃ concentrations than the main increase in Nitrophytes. This is relevant, since it suggests that the presence of nitrophytes is not necessary to indicate that significant losses of acidophyte lichens have occurred.

The results of the twig survey on *Betula* spp for the NIW/AIW scale show a similar correlation of nitrophytes with NH₃ concentrations to the trunks, but with the decrease in acidophyte lichens and increase in nitrophyte lichens occurring at lower NH₃ concentrations than for trunks. This suggests that lichen communities of twigs are more sensitive to NH₃ of trunks. In addition, conditions within the forest were very varied and sites in the shaded part of the forest show low mean NIW and AIW values.

The trunks were covered with green algae and remnants of infertile *Scoliciosporum chlorococcum* where lichen establishment was limited. This community may have been influenced by the significant pollen deposition from the pine canopy above. Site 1(2) is on exposed *Picea* trees planted as a shelter belt adjacent to, and east of the source. Sites 2(3) and 6(9), on the edge of the *Pinus sylvestris* plantation where trunks were exposed to higher light levels had similar species diversity on trunks, but in site 2 this was composed of nitrophyte species with an absence of acidophytes whereas in site 6 acidophytes were numerous and only 1 van Herk nitrophyte present. The use of nitrophyte and acidophyte species as defined by van Herk allows a comparison between the sites which corresponds to recorded ammonia concentrations except at site 1 where *Xanthoria parietina* covers all exposed *Picea* twigs and trunks but contributes to a lower NIW than at site 2 where other nitrophytes are present.

There is a considerable overlap between species found at Earlston with van Herk's definition of NIW and AIW species and very little overlap with Lallement's indicators in western France, most of these species not being present at Earlston. The difficulty in using indicator species to which values are assigned is that regional differences then affect the indices calculated. Further analysis of data collected in a range of sites is needed in the UK in order to assess the relationship between species distribution and atmospheric nitrogen.

Comparison of the results with the pH data shows that the response of the lichens is correlated with an increase in bark pH, and this is also consistent with the differences between twig and trunk pH (Figure A40). This increase in pH indicates that the basic effect of NH_3 is much larger than any possible nitrification effect on the bark. Given the interaction with bark pH, it is believed that the effects on the lichens represent more of a response to NH_3 concentrations than total N deposition. Hence effects of atmospheric NH_3 on lichens should be considered using the critical levels rather than the critical loads approach.

Finally, it may be noted that total foliar N of Lichens was also measured at selected locations and these gave results consistent with those for the mosses (Figure A40).

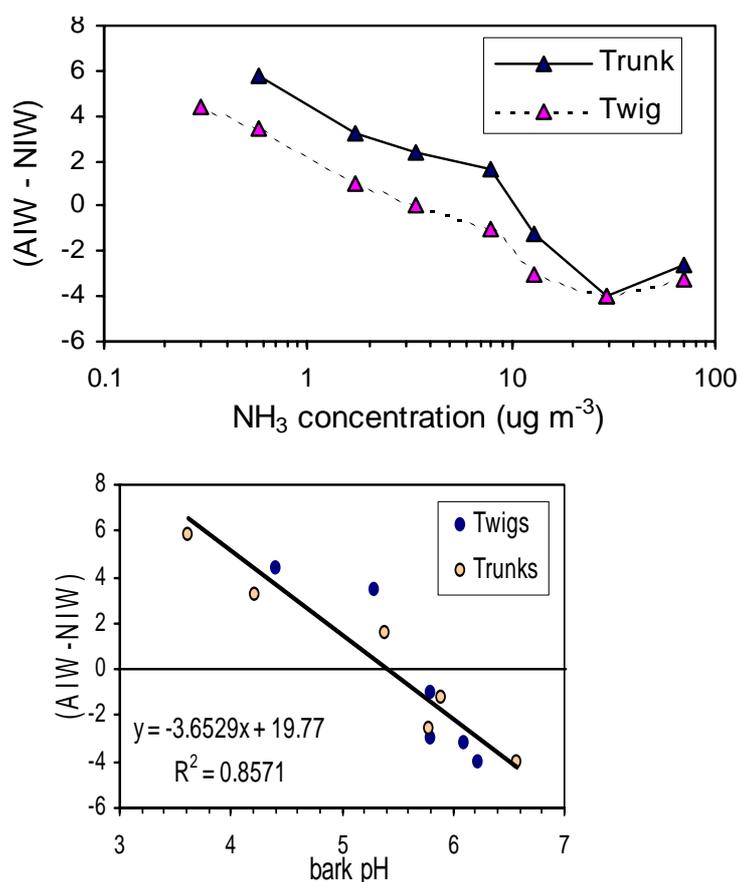


Figure A40: Effect of NH_3 concentration and bark pH on net eutrophication of epiphyte lichen flora as expressed by the difference between the acidophyte and nitrophyte scores (AIW-NIW) for trunks and twigs. Twig data lichen scores and pH are for birch, while trunk values are for pine (except for the trunk data at the highest NH_3 concentration which are for spruce).

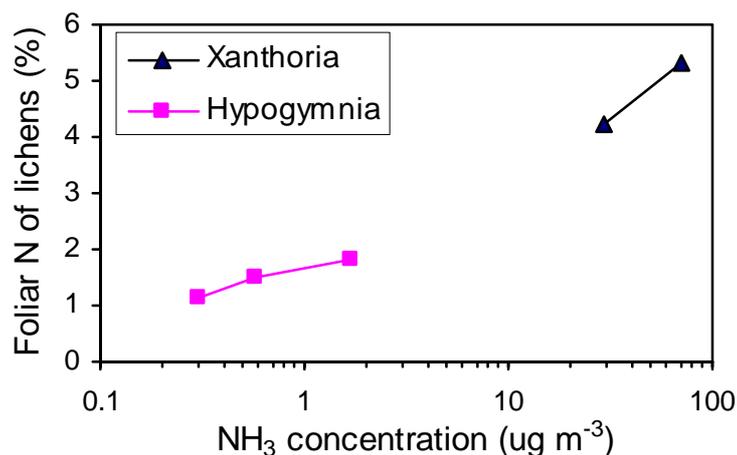


Figure A41: Total tissue nitrogen concentration of foliose lichens in relation to measured NH₃ concentrations at different distances from the poultry farm. For the two cleanest location

10. Discussion

10.1. Standardized plant bioindicators

The result of the present study show a clear increase in % N and above ground biomass closer to the farm, where the plants were exposed to larger NH₃ concentrations. Some scatter in the results may be attributed to variable shading, and this effect was largely cancelled out in the total above ground N inventory of the plants. This suggests that although % N may be diluted by additional growth (under high light conditions) the total N accumulated by the plants is little affected by shading.

The present study was designed not to include the measurement of root biomass and N concentration in the *L. perenne* plants. Root washing /extraction would have been difficult and time consuming with the loam/peat compost used, and was outwith the remit of this study. However, in any future studies, consideration should be given to the type of growing medium used and to the possibility of including root biomass / N concentration to provide important additional information. In a transplant study with *L. multiflorum*, Sommer and Jensen (1991) found that much of the additional N deposited was reallocated to the roots.

Although the experiment took place at the end of the growing season when meteorological conditions were often not favourable to growth, there was a strong relationship between both foliar N concentration, biomass and ambient NH₃ along the 270 m transect. It can be concluded that this field transplant method is relatively inexpensive, simple to set up, requires virtually no maintenance/checks and appears to be a good bio-monitor of N deposition over a relatively short time period (37 days). To find a response to N deposition after less than 6 weeks exposure, makes *Lolium* an attractive potential bio-indicator species for direct biological demonstration of the effects of atmospheric N deposition.

Further studies using standardised grass cultures including *Lolium* species and possibly native species, at a range of point and line sources (intensive livestock units, roads, industrial plants)

and during the growing season are required to further refine and develop the method. In addition, there is scope for calibrating this method by controlled exposure of the standard plants to ammonia or ammonium in open-top chambers and also in the field. The calibration could then be validated under different conditions of climate and pollutant concentration at a range of UK monitoring stations.

10.2. Biochemical methods

The results of the present field test support the application of total tissue N concentrations in pleurocarpous mosses as a bioindicator for atmospheric N deposition. These results are consistent with those obtained previously at this site and with results from other locations (see main report).

This field test represents the first time that soluble foliar N and foliar ammonium have been analyzed for mosses. The test showed extremely promising results, with these lower concentration parameters showing a stronger response to NH₃ concentrations than total tissue nitrogen. In particular, the largest response was seen for foliar ammonium in pleurocarpous mosses, representing a factor 20 increase across the range of conditions in this study.

The results of foliar ammonium in the standardized grass plants were curiously different from those of total foliar N concentration. While the more usually measured total foliar N appeared to be sensitive to growth dilution effects (for example induced by differences in shading along the transect) the foliar ammonium concentration appeared to be insensitive to these effects. Further tests are obviously required to investigate these interactions.

The foliar ammonium results provided the opportunity for a more advanced analysis, leading to the estimation of N deposition and saturation of deposition at high NH₃ concentrations. These results demonstrate the power of this new bioindicator method, indicating the need for refinement of the method.

By comparison of the available data from this study, foliar ammonium appears to be the better bioindicator than soluble N. This is because of the larger response (factor 20 rather than factor 5), and better correlation coefficient.

The scale of the foliar ammonium response in the present study has shown that it is amenable to a more advanced analysis to estimate both background atmospheric N deposition and the saturation of the NH₃ deposition velocity at high NH₃ concentrations. This can only be done where NH₃ air concentrations are measured in parallel and the dataset is sufficiently large to allow these subtleties to be detected. In this respect, this particular analysis remains a research rather than operational task. However, based on improved quantification of the cuticular saturation rate, and with estimates of background N deposition, it becomes possible to apply these results to provide an indication of whether or not critical loads are exceeded. This has already been shown by Pitcairn *et al.* (2002) for total foliar N, but the present analysis extends this to apply the approach for foliar ammonium and to show how confidence limits of the population may be used to estimate uncertainties of the extent to which a bioindicator measurement reflects exceedance of the critical load.

The main challenges for improvement of the foliar ammonium bioassay are improving the speed of the extraction-procedure, collecting further data in different situations to better characterize the response function to N deposition. However, already it is possible to indicate how this bioassay can be used to estimate whether or not a certain critical load is exceeded.

10.3. Lichen diversity methods

The present results are the first time in the UK that the full range of lichen diversity methods have been compared to assess the impact of atmospheric N alongside other bioindicator methods. They also show the usefulness of comparing analysis of trunk lichens with lichens occurring on twigs, with the evidence of this study pointing to an increased sensitivity of twig acidophyte lichens to NH₃ compared with trunk acidophytes. There may be several causes of this difference. Firstly, other recent work has shown that lichen communities of formerly acidified old trunks may be slow to change, whereas lichen communities of younger trunks and of new substrata available as twigs are influenced by prevailing environmental conditions and by availability of propagules. Secondly, the higher pH of twigs compared with trunks may indicate naturally higher pH of twig bark under clean conditions (or a lower buffering capacity).

Although it was not possible to sample the twig flora of pine trees, *Betula pubescens* and *B. pendula* were widely distributed across the site so their twigs could be sampled. In addition it is a native species found widely in natural areas outside the site so could be used to compare conditions across a wider geographic area. Although nitrophytes are absent from the trunk from sites over 125 m from the source (5, 5a and 6), they are present on twigs at all sites except 6. This may reflect the influence of nitrogen compounds other than ammonia at a background level in surrounding agricultural land even at Gordon Moss. Lichen communities colonising newly available bark substrata on twigs appear to provide the most reliable estimate of present environmental conditions.

The present test of the lichen sampling methods has shown excellent comparisons along the ammonia transect, using both the nitrophyte/acidophyte and Ellenberg approaches. However, it is recognized that the site criteria did not match to those normally applied for lichen survey (e.g. well lit trees), but still gave clear results. This points to a limitation of the current protocols, which are restricted to rather exacting site conditions, which may not be met in practice in the field. Further work needs to improve on these sampling procedures, both to make them more easy to use (also for non specialists) and as widely applicable to use in different field conditions. As part of this, further characterization of lichen species preferences is needed, since the original methods (van Herk 1999, Wirth 1992, Lallement et al. 1999) were developed for continental locations in Europe rather than the UK.

The following key conclusions may be drawn with regard to the lichen methods:

- The use of an index of lichen diversity based on frequency alone (VDI and LDV) to assess ammonia levels around point sources does not appear to be appropriate, due to the replacement of acidophyte lichen diversity by nitrophytic diversity in sites with high concentrations of ammonia.
- The use of aspect to detect direction of atmospheric nitrogen does not correspond to lichen diversity in this site. Diversity remains highest on south and west aspects (as

expected) except in close proximity to the source where diversity of nitrophytic species is low.

- There is a need to assess UK nitrophyte and acidophyte indicator species and to test these against corresponding ammonia or ammonium values in order to accommodate a range of regional climatic and atmospheric conditions.
- Standardised recording methods as outlined in the German VDI and European protocols are not always appropriate for use in a range of ecological conditions especially in humanly altered habitats.
- Lichens colonising newly available surfaces on twigs appear to provide the most reliable estimate of present environmental conditions whereas trunks may carry remnants of cryptogamic communities from former environmental conditions.
- The response of lichens to additional NH₃ near the farm appears to be related to the increase in bark pH due to the basic action of NH₃, and this difference also partly explains the difference between lichens on trunks and twigs. The effect of NH₃ on lichens therefore needs to be considered as an issue relevant for critical levels rather than critical loads of total N deposition.

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APPENDIX II

Datasheets of bio-indicator methods for
atmospheric nitrogen concentrations,
deposition and impacts.

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Method	Total Tissue Nitrogen
Basis of the approach	When N availability exceeds demands for plant growth, N may accumulate in the tissues, most commonly in the actively growing regions. In species typical of N poor habitats and those with little or no attachment/dependence on the soil for N uptake, atmospheric inputs are an important source of nutrients especially N. Both slow growing plants with a low N demand and partial dependence on atmospheric deposition, i.e. ericaceous shrubs such as <i>Calluna vulgaris</i> , and plants wholly or partly dependent on atmospheric deposition, i.e. ectohydric mosses and certain lichens are closely coupled to the atmosphere and its supply of N. Based on these close links, tissue N content of certain plant species has been shown to be roughly proportional to atmospheric inputs of N (Bobbink <i>et al.</i> , 1993; Baddeley <i>et al.</i> 1994; Hyvarinen & Crittenden, 1998; Pitcairn <i>et al.</i> , 1995, 1998).
Previous experience	The foliar N content of trees (van Dobben, Pitcairn <i>et al.</i> 1998) ericaceous shrubs (Pitcairn <i>et al.</i> , 1995, 2001; Hicks <i>et al.</i> , 2001; Leith <i>et al.</i> , 2000) herbs (Pitcairn <i>et al.</i> 2002), mosses (Baddeley <i>et al.</i> 1994; Pitcairn <i>et al.</i> 1995, 1998, 2002; Woolgrove <i>et al.</i> 1995) and lichens (Hyvarinen & Crittenden, 1998; Gaio-Oliveira <i>et al.</i> 2001) have been shown to be related to atmospheric N inputs suggesting that tissue N could be a useful indicator of N deposition in a range of habitats.
Suitability to indicate atmospheric concentrations	The method is suitable for indicating atmospheric concentrations of ammonia. However there is less evidence for the suitability of the method to distinguish between concentration and deposition of N in rainfall or cloud.
Suitability to indicate atmospheric deposition	This method is well suited to indicate enhanced N deposition. There is a strong relationship between tissue-N and N deposition. % N ranges of many species are well known and available from the literature.
Suitability to indicate environmental impacts of N	There is less direct evidence of links between tissue N and impacts on vegetation. However indirect evidence suggest that enhanced tissue N indicates the potential for perturbation to plant health and ecosystem composition, and also susceptibility to disease from pests and pathogens. The method is best suited to a broad range of N deposition loads (i.e. <8 to >30 kg N ha ⁻¹ y ⁻¹) or to very high N levels (> 20 kg N ha ⁻¹ y ⁻¹). It is less likely to be effective when applied to sites with a low level of N deposition.
Sensitivity to other factors	<ol style="list-style-type: none"> 1. Any factor that reduces growth can potentially increase foliar N. For some species, concentrations of tissue N can be restricted by phosphorus and potassium availability in the soil. 2. Light levels may also modify tissue N levels 3. High rainfall may lead to leaching of N from the tissues. 4. Drought leading to low water table in mires can affect N uptake and retention by some moss species.
Time constant	This method can be used to assess long-term or short-term change in deposition. However, care must be taken in selecting species suitable for the type of change. Tree species can be used for long-term change, especially if 2 age classes of needle are used. Whereas mosses may be more suitable for response to short-term changes. For some sites, in high rainfall areas, <i>Calluna</i> may be more suitable than mosses.
Limitations to applicability	The method is not limited to particular habitats or species types, but is most suitable for woodland and moorland ecosystems. Ideally several common species should be sampled to obtain values that could be compared with published values. At least 3 replicate samples should be collected at several points in the site. Sampling also needs to take account of adjacent vegetation as this strongly influences the N composition through competition. Approximately 3 g fresh weight of material should be collected to provide a minimum of 0.5 g of dried, ground material for chemical analysis.
Expertise in field	Sampling requires training in species identification particularly of bryophytes or lichens, which many Conservation agency staff may have already received. When age or growth phase of the plant or foliage is important, these must be

	<p>identifiable by staff.</p> <p>Ideally, samples should be collected, stored in cold conditions, cleaned (removal of litter, other species etc) and oven dried before being transported to an analytical laboratory by post. When suitable ovens are not available, samples can be air-dried before transportation to the laboratory.</p>
Expertise in laboratory	<p>Determination of total tissue N in plants can be carried out by digestion (with strong acids in a fume cupboard) and subsequent analysis using specialist, but well proven laboratory techniques. However most laboratories currently use a CNS analyser to determine total N.</p>
Cost	<p><i>Cost per unit of sampling (e.g. one SAC sampled at the edge, centre and an intermediate location at one time). Number of person days of required (Conservation agency + External specialists) in field and laboratory. Indicative cost as of 2003-2004.</i></p> <p>For 3 species, 3 replicates per species and 3 locations per site</p> <p><i>Conservation agency Staff</i></p> <p>Sample collection, cleaning, drying and packaging for 27 samples – 4 days</p> <p><i>Analytical Laboratory costs</i></p> <p>Preparation - milling (at least 0.7 mm sieve size, or ball mill), mixing, tubing (and supply of sample tubs), labelling tubs etc... £10* per sample + VAT (if applicable); Analysis - Total N - CNS analyser (Combustion; Manufacturer: Elementar Model: Vario EL) ~£11 per sample + VAT (if applicable).</p> <p>Total per sample - ~£21 (+ VAT if applicable).</p> <p>Total per 27 samples - ~£567* (+ VAT if applicable).</p> <p>*Discounts may be available for specific research work e.g. collaborative research projects part funded with CEH and/or NERC, or linked to NERC Research Grants or Ph D studentships. Extra costs may be incurred if less than 20 samples are submitted for analysis at any one time.</p>
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	<p>deposition, species composition and foliar nitrogen concentrations in woodland flora in the vicinity of livestock farms. <i>Environ. Pollut.</i>, 102(S1), 41-48.</p> <p>van Dobben, H. F. (1993). Vegetation as a monitor for deposition of nitrogen and acidity. Ph.D. Thesis, Wageningen</p> <p>Woolgrove CE & Woodin SJ. (1996) Ecophysiology of a snow-bed bryophyte <i>Kiaeria starkei</i> during snowmelt and uptake of nitrate from meltwater. <i>Canadian Journal of Botany-Revue Canadienne De Botanique</i>, 74, 1095-1103</p>
Basis of the approach	<p>When N availability increases, N may be taken up by plants in surplus and accumulated rather than being used in biomass production. At a cellular level, if protein is not synthesized, NH_4^+ is assimilated into specific N metabolites of which free amino acids are particularly important. Thus accumulation of amino acids could potentially be used to monitor N impacts on vegetation. Concentrations of a few specific amino acids, (which are species specific), are particularly well correlated with N deposition. Species typical of N- poor habitats accumulate arginine, whereas those more typical of N-rich habitats tend to accumulate asparagine (Nordin and Nasholm 1997). Changes in the composition of the amino acid pool may also occur in response to enhanced N deposition (Nordin <i>et al.</i> 1998; Pitcairn <i>et al.</i> 2003).</p>
Previous experience	<ol style="list-style-type: none"> 1. In early studies in the Netherlands, damaged trees were found to contain high concentrations of the free amino acid arginine (van Dijk and Roelofs 1988). 2. Several experiments have confirmed the link between enhanced N deposition and arginine concentrations. For example, when N deposition to the forest floor was reduced to 1-2 kg N ha⁻¹ y⁻¹ in the NITREX plot in Speuld forest, arginine concentrations in coniferous needles declined 3 fold within 4 years (Boxman <i>et al.</i> 1995). Another example is the very large reduction in arginine concentrations with increasing distance from a fur farm in Finland (Pietila <i>et al.</i> 1991). 3. Näsholm <i>et al.</i> (1994) found that exposure to large concentrations of N increased the accumulation of amino acid N (as arginine, asparagine, glutamine and others) in a range of forest plants from trees to bryophytes, and suggested that concentrations of specific amino acids in a range of plants may be used to indicate atmospheric N deposition. 4. Baxter <i>et al.</i> (1992) showed a rapid response in amino acid concentrations in two populations of <i>Sphagnum cuspidatum</i> following addition of 0.1 and 1.0 mM NH_4^+ in the laboratory. 5. Downwind of a poultry farm in Scotland, UK, Appendix I determined amino acid concentrations in 3 moss species (<i>Rhytidiadelphus triquetrus</i>, <i>Brachythecium rutabulum</i> and <i>Pseudoscleropodium purum</i>) in late autumn and showed a strong relationship with distance from the poultry buildings and hence with NH_3 concentrations. Arginine was the dominant amino acid at high concentrations close to the buildings in all 3 species. The linear regression of arginine concentration with log distance from the poultry farm gave values for r^2 of > 0.95 for the 3 species demonstrating the potential for arginine accumulation in moss as an indicator of enhanced N deposition.
Suitability to indicate atmospheric concentrations	<p>There is insufficient evidence to determine whether this method is suitable to indicate atmospheric N concentrations, although amino acid content of 'pool' <i>Sphagna</i> responded to increased concentrations of ammonium in the laboratory.</p>
Suitability to indicate atmospheric deposition	<p>Although the positive relationship between amino acid concentrations and N deposition is well proved, the fact that different species may accumulate <i>different</i> amino acids in response to enhanced N deposition reduces the robustness of the method. It is therefore necessary to assess the changes of each of the main amino acids. There is insufficient evidence to determine whether this method is more suited to a wide range of N deposition levels or to low or very high levels. There is evidence to suggest that addition of NH_4^+-N may lead to larger increases in amino acid N (and also total N) than with NO_3^--N.</p>
Suitability to indicate environmental impacts of N	<p>Although there is good evidence of a relationship between amino acid concentrations and impacts of N deposition, the fact that different species may accumulate different amino acids in response to enhanced N deposition reduces the robustness of the method.</p>

Sensitivity to other factors	<p>1. Concentrations of amino acids tend to change seasonally. Late autumn is the best season for sampling for this method for most species as it is most stable at this time.</p> <p>2. Accumulation of amino acids can occur in response to growth limiting conditions where nutrients other than N are restricting growth e.g. amino acids can accumulate in response to SO₂ pollution. Thus amino acid accumulation might be regarded as a non-specific indicator of perturbation of nutrient availability, which does affect the robustness of the method. Changes in the composition of the amino acid pool may overcome this factor, but there is insufficient data available at present to evaluate this.</p> <p>3. Concentrations of free amino acids are affected by several environmental factors including nutrient deficiencies and the form of the nitrogen deposition and the sites of storage also vary between species.</p>
Time constant	<p>As amino acids are a storage compound, with elevated values reflecting surplus N, the method will reflect directly the time constant of excess N in the plants. Typically, this may result from gradual N accumulation in the ecosystem over several years, however, experimental work (e.g. Baxter <i>et al.</i> 1992) shows that the effects of new exposure can be determined in a few days.</p>
Limitations to applicability	<p>The method is not limited to habitat or species. However, storage amino acids tend to be species dependent and not all species respond to enhance N by storage in the same amino acid. Species typical of N- poor habitats accumulate arginine, whereas those more typical of N-rich habitats tend to accumulate asparagine.</p> <p>Large quantities of material are not required. Approximately 3 g fresh weight of material should be collected to provide a minimum of 0.5 g of dried, ground material for chemical analysis.</p>
Expertise in field	<p>Specific species sampling requires training in species identification particularly of bryophytes or lichens, which many conservation agency staff may have already received. When age or growth phase of the plant or foliage is important, these must be identifiable by staff. Ideally, samples should be collected, cleaned (removal of litter, other species etc) and frozen and freeze-dried before being transported to an analytical laboratory by post, and specific conditions for storage and time between collection and freeze drying must be observed. When suitable equipment is not available for freeze-drying, samples can be frozen and delivered to the analytical laboratory by car in a 'cool'box. Alternatively they could be transported in the fresh state as quickly as possible (by courier or special delivery) for sample preparation at the analytical laboratory.</p>
Expertise in laboratory	<p>Determination of amino acids requires extraction of the freeze dried sample and subsequent analysis using specialist expensive laboratory equipment, HPLC (prone to break-down) and skilled operators. Data analysis and interpretation also require experience.</p>
Cost	<p><i>Cost per unit of sampling (e.g. one SAC sampled at the edge, centre and an intermediate location at one time). Number of person days of required (Conservation agency + External specialists) in field and laboratory. Indicative cost as of 2003-2004.</i></p> <p>For 3 species, 3 replicates per species and 3 locations per site</p> <p><i>Conservation agency Staff</i></p> <p>Sample collection, cleaning, freezing and packaging for 27 samples – 4 days</p> <p><i>Analytical Laboratory costs</i></p> <p>Per sample: Preparation -£10; Analysis - £11; Total - £21</p> <p>Per 27 samples - £567</p> <p>(VAT may be chargeable)</p>
References	<p>Baxter, R., Emes, M.J. & Lee, J.A. 1992. Effects of experimentally applied increase in ammonium on growth and amino-acid metabolism of <i>Sphagnum cuspidatum</i> Ehrh. Ex. Hoffm. from differently polluted areas. <i>New Phytol.</i> 120, 265-274.</p> <p>Boxman, A.W., van Dijk, H.F.G., van der Ven, P.J.M. & Roelofs, J.G.M. 1995. Effects of reduced nitrogen and sulphur inputs to two coniferous ecosystems in The Netherlands. <i>Proc. Int. Symp.on Ecosystem Manipulations, Windermere</i></p>

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Method	Free Amino Acids
Basis of the approach	<p>The nitrogen present in plant tissues may be broadly distinguished into two pools: substrate N and structural N (Riedo <i>et al.</i> 1997). Substrate N is the nitrogen available for plant growth, existing in the form of a range of soluble compounds, such as amino acids, ammonium, amines (and to a much lesser extent nitrate). Following the photosynthetic production of substrate C (i.e. sugars etc), substrate C and N are used to build the plant structure, consisting of insoluble compounds. Initial interest in substrate N originated in the development of process-based models of plant C / N cycling and ammonia exchange with the atmosphere (Riedo <i>et al.</i> 1997, 2002). This led to the development of the first protocols to determine substrate N – as total soluble plant N (Loubet <i>et al.</i> 1999, Hill 1999). In parallel, work has analysed total foliar ammonium as an indicator of both substrate N and apoplastic ammonium (Sutton <i>et al.</i> 2001, 2002). [Note: Apoplastic (intercellular) ammonium is a separate parameter of interest to determine the ammonia emission potential of plants – the “compensation point”, but it is much more complex to measure and therefore not well suited as a simple N bioassay.]</p> <p>The same principles that lead to accumulation of total tissue N and amino acids apply to substrate N. The substrate N method is expected to be more sensitive than total tissue N, since it is the substrate (available) N pool that undergoes the primary variation with N supply. Secondly, by considering all forms of soluble N, the substrate N approach is more robust than the analysis of amino acids, since different plant species accumulate different amino acids.</p> <p>Free ammonium is a basic precursor for amino acid production, and is therefore expected to be directly related to substrate N.</p>
Previous experience	<ol style="list-style-type: none"> 1. The first measurements of free ammonium were made as a simple indicator of apoplastic (intercellular) ammonium. Hill <i>et al.</i> (2001) demonstrated a positive correlation between the two parameters in the native woodland plant wood rush (<i>Luzula sylvatica</i>), with both increasing with elevated N supply (Hill 1999). 2. Foliar ammonium was shown by Loubet <i>et al.</i> (2002) to increase following cutting and fertilization of rye grass (<i>Lolium perenne</i>) in Scotland. 3. Substrate N and foliar ammonium increased following N fertilization of a mixed grass sward in Germany (Sutton <i>et al.</i> 2002). 3. Analysis of foliar ammonium in standardized rye grass biomonitors set out in a transect from a poultry farm, showed a linear increase in the range 30-80 ug /g FW with the log of NH₃ concentration (Appendix I). 4. Analysis of foliar ammonium in pleurocarpous mosses occurring downwind of a poultry farm, showed an exponential increase with NH₃ concentration from background values of 20-40 ug /g FW up to values > 500 ug /g FW (Appendix I). The values for different moss species were not significantly different. An increase for substrate N was also measured and found to be intermediate between that for foliar ammonium and total tissue N.
Suitability to indicate atmospheric concentrations	<p>The values downwind of the poultry farm studied by Pitcairn <i>et al.</i> (2003) show clear relationships to NH₃ concentration. However, this is probably an indirect relationship, with the plants actually responding to N deposition. The method appears to be sensitive over a wide range of NH₃ with an increase occurring between 0.6-3 ug m⁻³ NH₃ and a response still occurring between 30-70 ug m⁻³ NH₃, with the response flattening at high concentrations. The response to NO_y concentrations has yet to be studied.</p>
Suitability to indicate environmental impacts of N	<p>The evidence of Pitcairn <i>et al.</i> (2003) suggests that foliar NH₄⁺ and substrate N respond strongly to atmospheric deposition. The method is most sensitive at low N supply, and tends to saturate to maximum values at very high N deposition. The method has so far only been tested for NH_x deposition. Following the available information for amino acids and total N, there is the suggestion that responses for NO_y deposition will be smaller.</p>

Suitability to indicate impacts of N	As with total tissue N and amino acids, the method reflects a direct response of the plants to N and is therefore by definition measuring an impact. The altered nutrient balances will be expected to link to altered competition between plant species.
Sensitivity to other factors	1. As with amino acids, concentrations of free ammonium and substrate N are expected to vary seasonally. Values are expected to be smallest during the main growth period, assuming that atmospheric inputs are constant throughout the year. Values are expected to be most stable in autumn and winter. 2. The magnitude of enhancement of foliar NH_4^+ and substrate N for bryophytes in the vicinity of a poultry farm (up to a factor of 10 increase) cannot easily be due to anything other than excess N supply.
Time constant	The method will reflect directly the time constant of excess N response of the plants. Typically, this may result from gradual N accumulation in the ecosystem over several years (where deposition inputs are gradual). However, experimental work (e.g. Mattsson <i>et al.</i> 2002) for tissue ammonium shows that the effects of sudden exposure can be detected in a few hours/days.
Limitations to applicability	The methods are not limited to habitat or species. However, the experience to date has been with different grass species (Loubet <i>et al.</i> 2001, Sutton <i>et al.</i> 2001, Appendix I), wood rush (Hill <i>et al.</i> 2001) and pleurocarpous mosses (Appendix I). At least 1 g fresh weight of material should be collected to provide a minimum of 0.2 g ground fresh material for chemical analysis.
Expertise in field	Specific species sampling requires training in species identification particularly of bryophytes or lichens, which many conservation agency staff may have already received. When age or growth phase of the plant or foliage is important, these must be identifiable by staff. Ideally, samples should be collected, cleaned (removal of litter, other species etc) and frozen and before being transported to an analytical laboratory by post, and specific conditions for storage and time between collection and freezing. Alternatively they could be transported in the fresh state as quickly as possible (by courier or special delivery) for sample preparation at the analytical laboratory.
Expertise in laboratory	Aqueous ammonium may be analysed on a range of detectors, the most suitable being membrane dialysis at high pH (AMFIA) or reaction with o-phthalaldehyde. Aqueous phase analysis of soluble N is performed with an ANTEK analyser. The current protocol for sample preparation requires grinding the leaf sample in liquid nitrogen. However, further methods development should further simplify the procedure.
Cost	<i>Cost per unit of sampling (e.g. one SAC sampled at the edge, centre and an intermediate location at one time). Number of person days of required (Conservation agency + External specialists) in field and laboratory. Indicative cost as of 2003-2004.</i> For 3 species, 3 replicates per species and 3 locations per site Conservation agency Staff Sample collection, cleaning, freezing and packaging for 27 samples – 4 days Analytical Laboratory costs Per sample: Total - ~£20 Per 27 samples - ~£540 (VAT may be chargeable)
References	Hill P.W. (1999) Physiological aspects of the exchange of gaseous ammonia between <i>Luzula sylvatica</i> (Huds.) Gaud. And the atmosphere. University of Dundee, 216 pp. Hill P.W., Raven J.A., Loubet B., Fowler D., Sutton M.A. (2001) Comparison of gas exchange and bioassay determinations of the ammonia compensation point in <i>Luzula sylvatica</i> (Huds.) Gaud. <i>Plant Physiology</i> 125 , 476-487. Loubet B., Hill P.W., Sutton M.A., Tang Y.S and Cellier P. (1999) Apoplastic and bulk ammonia in leaves of <i>Lolium perenne</i> L. and <i>Zea mays</i> L. effects of management and fertilization. Report INRA, Thiverval Grignon / Institute of Terrestrial Ecology, Edinburgh Research Station. Loubet B., Milford C., Hill, P.W. Tang Y.S., Cellier P., and Sutton M.A. (2002)

	<p>Seasonal variability of apoplastic NH_4^+ and pH in an intensively managed grassland. <i>Plant and Soil</i> 238, 97-110.</p> <p>Mattsson M. and Schjoerring JK (2002) Dynamic and steady state responses of inorganic nitrogen pools and NH_3 exchange in leaves of <i>Lolium perenne</i> and <i>Bromus erectus</i> to changes in root N supply. <i>Plant Physiol.</i> 128, 742-750.</p> <p>Riedo M, Grub A, Rosset M and Fuhrer J (1997) A pasture simulation model for dry matter production and fluxes of carbon, nitrogen, water and energy. <i>Ecological Modelling</i> 105, 141-183.</p> <p>Riedo, M., Milford, C., Schmid, M. and Sutton, M.A. (2002) Coupling soil-plant-atmosphere exchange of ammonia with ecosystem functioning in grasslands. <i>Ecological Modelling</i> 158, 83-110.</p> <p>Sutton M.A., C. Milford, E. Nemitz, M.R. Theobald, K.J. Hargreaves, D. Fowler, J.K. Schjørring, M.E. Mattsson, S. Husted, J.W. Erisman, R. Otjes, A. Hensen, J. Mosquera, P. de Bulk, P. Jongejon, H. Mols, P. Cellier, B. Loubet, M. David, R. Roche, B. Durant, P. Laville, A. Neftel, A. Blatter, B. Hermann, S.K. Jones, M. Riedo, L. Horvath, T. Weidinger, R. Meszaros, J. Raso, M., Rajkai K., Gallagher, J. Dorsey, M. Flynn, B. Lehmann, J. Burkhardt and U. Dämmgen (2002) Biosphere atmosphere exchange of ammonia with grasslands across Europe: GRAMINAE. Final synthesis report to the EU. CEH Edinburgh.</p> <p>Sutton M.A., Milford C., Nemitz E., Theobald M.R., Hill P.W., Fowler D., Schjoerring J.K., Mattsson M.E., Nielsen K.H., Husted S., Erisman J.W., Otjes R., Hensen A., Mosquera J., Cellier P., Loubet B., David M., Genermont S., Neftel A., Blatter A., Herrmann B., Jones S.K., Horvath L., Führer E., Mantzanas K., Koukoura Z., Gallagher M., Williams P., Flynn M. and Riedo M. (2001) Biosphere-atmosphere interactions of ammonia with grasslands: experimental strategy and results from a new European initiative. <i>Plant and Soil</i> 228 (1): 131-145.</p>
Basis of the approach	<p>The nitrogen present in plant tissues may be broadly distinguished into two pools: substrate N and structural N (Riedo <i>et al.</i> 1997). Substrate N is the nitrogen available for plant growth, existing in the form of a range of soluble compounds, such as amino acids, ammonium, amines (and to a much lesser extent nitrate). Following the photosynthetic production of substrate C (i.e. sugars etc), substrate C and N are used to build the plant structure, consisting of insoluble compounds. Initial interest in substrate N originated in the development of process-based models of plant C / N cycling and ammonia exchange with the atmosphere (Riedo <i>et al.</i> 1997, 2002). This led to the development of the first protocols to determine substrate N – as total soluble plant N (Loubet <i>et al.</i> 1999, Hill 1999). In parallel, work has analysed total foliar ammonium as an indicator of both substrate N and apoplastic ammonium (Sutton <i>et al.</i> 2001, 2002). [Note: Apoplastic (intercellular) ammonium is a separate parameter of interest to determine the ammonia emission potential of plants – the “compensation point”, but it is much more complex to measure and therefore not well suited as a simple N bioassay.]</p> <p>The same principles that lead to accumulation of total tissue N and amino acids apply to substrate N. The substrate N method is expected to be more sensitive than total tissue N, since it is the substrate (available) N pool that undergoes the primary variation with N supply. Secondly, by considering all forms of soluble N, the substrate N approach is more robust than the analysis of amino acids, since different plant species accumulate different amino acids.</p> <p>Free ammonium is a basic precursor for amino acid production, and is therefore expected to be directly related to substrate N.</p>
Previous experience	<ol style="list-style-type: none"> 1. The first measurements of free ammonium were made as a simple indicator of apoplastic (intercellular) ammonium. Hill <i>et al.</i> (2001) demonstrated a positive correlation between the two parameters in the native woodland plant wood rush (<i>Luzula sylvatica</i>), with both increasing with elevated N supply (Hill 1999). 2. Foliar ammonium was shown by Loubet <i>et al.</i> (2002) to increase following cutting and fertilization of rye grass (<i>Lolium perenne</i>) in Scotland. 3. Substrate N and foliar ammonium increased following N fertilization of a mixed grass sward in Germany (Sutton <i>et al.</i> 2002).

	<p>3. Analysis of foliar ammonium in standardized rye grass biomonitors set out in a transect from a poultry farm, showed a linear increase in the range 30-80 ug /g FW with the log of NH₃ concentration (Appendix I).</p> <p>4. Analysis of foliar ammonium in pleurocarpous mosses occurring downwind of a poultry farm, showed an exponential increase with NH₃ concentration from background values of 20-40 ug /g FW up to values > 500 ug /g FW (Appendix I). The values for different moss species were not significantly different. An increase for substrate N was also measured and found to be intermediate between that for foliar ammonium and total tissue N.</p>
Suitability to indicate atmospheric concentrations	The values downwind of the poultry farm studied by Pitcairn <i>et al.</i> (2003) show clear relationships to NH ₃ concentration. However, this is probably an indirect relationship, with the plants actually responding to N deposition. The method appears to be sensitive over a wide range of NH ₃ with an increase occurring between 0.6-3 ug m ⁻³ NH ₃ and a response still occurring between 30-70 ug m ⁻³ NH ₃ , with the response flattening at high concentrations. The response to NO _y concentrations has yet to be studied
Suitability to indicate environmental impacts of N	The evidence of Pitcairn <i>et al.</i> (2003) suggests that foliar NH ₄ ⁺ and substrate N respond strongly to atmospheric deposition. The method is most sensitive at low N supply, and tends to saturate to maximum values at very high N deposition. The method has so far only been tested for NH _x deposition. Following the available information for amino acids and total N, there is the suggestion that responses for NO _y deposition will be smaller.
Suitability to indicate impacts of N	As with total tissue N and amino acids, the method reflects a direct response of the plants to N and is therefore by definition measuring an impact. The altered nutrient balances will be expected to link to altered competition between plant species.
Sensitivity to other factors	<p>1. As with amino acids, concentrations of free ammonium and substrate N are expected to vary seasonally. Values are expected to be smallest during the main growth period, assuming that atmospheric inputs are constant throughout the year. Values are expected to be most stable in autumn and winter.</p> <p>2. The magnitude of enhancement of foliar NH₄⁺ and substrate N for bryophytes in the vicinity of a poultry farm (up to a factor of 10 increase) cannot easily be due to anything other than excess N supply.</p>
Time constant	The method will reflect directly the time constant of excess N response of the plants. Typically, this may result from gradual N accumulation in the ecosystem over several years (where deposition inputs are gradual). However, experimental work (e.g. Mattsson <i>et al.</i> 2002) for tissue ammonium shows that the effects of sudden exposure can be detected in a few hours/days.
Limitations to applicability	The methods are not limited to habitat or species. However, the experience to date has been with different grass species (Loubet <i>et al.</i> 2001, Sutton <i>et al.</i> 2001, Appendix I), wood rush (Hill <i>et al.</i> 2001) and pleurocarpous mosses (Appendix I). At least 1 g fresh weight of material should be collected to provide a minimum of 0.2 g ground fresh material for chemical analysis.
Expertise in field	Specific species sampling requires training in species identification particularly of bryophytes or lichens, which many conservation agency staff may have already received. When age or growth phase of the plant or foliage is important, these must be identifiable by staff. Ideally, samples should be collected, cleaned (removal of litter, other species etc) and frozen and before being transported to an analytical laboratory by post, and specific conditions for storage and time between collection and freezing. Alternatively they could be transported in the fresh state as quickly as possible (by courier or special delivery) for sample preparation at the analytical laboratory.
Expertise in laboratory	Aqueous ammonium may be analysed on a range of detectors, the most suitable being membrane dialysis at high pH (AMFIA) or reaction with o-phthalaldehyde. Aqueous phase analysis of soluble N is performed with an ANTEK analyser. The current protocol for sample preparation requires grinding the leaf sample in liquid nitrogen. However, further methods development should further simplify the procedure.
Cost	<i>Cost per unit of sampling (e.g. one SAC sampled at the edge, centre and an</i>

	<p><i>intermediate location at one time). Number of person days of required (Conservation agency + External specialists) in field and laboratory. Indicative cost as of 2003-2004.</i></p> <p>For 3 species, 3 replicates per species and 3 locations per site Conservation agency Staff Sample collection, cleaning, freezing and packaging for 27 samples – 4 days Analytical Laboratory costs Per sample: Total - ~£20 Per 27 samples - ~£540 (VAT may be chargeable)</p>
References	<p>Hill P.W. (1999) Physiological aspects of the exchange of gaseous ammonia between <i>Luzula sylvatica</i> (Huds.) Gaud. And the atmosphere. University of Dundee, 216 pp.</p> <p>Hill P.W., Raven J.A., Loubet B., Fowler D., Sutton M.A. (2001) Comparison of gas exchange and bioassay determinations of the ammonia compensation point in <i>Luzula sylvatica</i> (Huds.) Gaud. <i>Plant Physiology</i> 125, 476-487.</p> <p>Loubet B., Hill P.W., Sutton M.A., Tang Y.S and Cellier P. (1999) Apoplastic and bulk ammonia in leaves of <i>Lolium perenne</i> L. and <i>Zea mays</i>. L. effects of management and fertilization. Report INRA, Thiverval Grignon / Institute of Terrestrial Ecology, Edinburgh Research Station.</p> <p>Loubet B., Milford C., Hill, P.W. Tang Y.S., Cellier P., and Sutton M.A. (2002) Seasonal variability of apoplastic NH_4^+ and pH in an intensively managed grassland. <i>Plant and Soil</i> 238, 97-110.</p> <p>Mattsson M. and Schjoerring JK (2002) Dynamic and steady state responses of inorganic nitrogen pools and NH_3 exchange in leaves of <i>Lolium perenne</i> and <i>Bromus erectus</i> to changes in root N supply. <i>Plant Physiol.</i> 128, 742-750.</p> <p>Riedo M, Grub A, Rosset M and Fuhrer J (1997) A pasture simulation model for dry matter production and fluxes of carbon, nitrogen, water and energy. <i>Ecological Modelling</i> 105, 141-183.</p> <p>Riedo, M., Milford, C., Schmid, M. and Sutton, M.A. (2002) Coupling soil-plant-atmosphere exchange of ammonia with ecosystem functioning in grasslands. <i>Ecological Modelling</i> 158, 83-110.</p> <p>Sutton M.A., C. Milford, E. Nemitz, M.R. Theobald, K.J. Hargreaves, D. Fowler, J.K. Schjørring, M.E. Mattsson, S. Husted, J.W. Erisman, R. Otjes, A. Hensen, J. Mosquera, P. de Bulk, P. Jongejon, H. Mols, P. Cellier, B. Loubet, M. David, R. Roche, B. Durant, P. Laville, A. Neftel, A. Blatter, B. Hermann, S.K. Jones, M. Riedo, L. Horvath, T. Weidinger, R. Meszaros, J. Raso, M., Rajkai K., Gallagher, J. Dorsey, M. Flynn, B. Lehmann, J. Burkhardt and U. Dämmgen (2002) Biosphere atmosphere exchange of ammonia with grasslands across Europe: GRAMINAE. Final synthesis report to the EU. CEH Edinburgh.</p> <p>Sutton M.A., Milford C., Nemitz E., Theobald M.R., Hill P.W., Fowler D., Schjoerring J.K., Mattsson M.E., Nielsen K.H., Husted S., Erisman J.W., Otjes R., Hensen A., Mosquera J., Cellier P., Loubet B., David M., Genermont S., Neftel A., Blatter A., Herrmann B., Jones S.K., Horvath L., Führer E., Mantzanas K., Koukoura Z., Gallagher M., Williams P., Flynn M. and Riedo M. (2001) Biosphere-atmosphere interactions of ammonia with grasslands: experimental strategy and results from a new European initiative. <i>Plant and Soil</i> 228 (1): 131-145.</p>

Method	Enzyme activity
Basis of the approach	<p>Enzyme activity in both soil and plants has been used to indicate N deposition and may provide possibilities as a biomonitor. The activities of a range of enzymes involved in the assimilation of N and other nutrients have been measured in response to N additions.</p> <p>Phosphomonoesterase (PME): By increasing the availability of N, the main growth driving nutrient, enhanced N deposition will also increase the demand for other nutrients such as P, albeit by proportionally smaller amounts. Phosphomonoesterase is the dominant phosphatase enzyme responsible for converting organic to inorganic P in most soils and hence changes in PME activity can potentially be used to monitor P availability in the soils.</p> <p>Nitrate reductase (NR): NR activity has been use as an indicator of N deposition since the early eighties, and the inducibility of NR appears to be one of the most useful indicative responses of bryophytes, especially <i>Sphagnum</i> species, to anthropogenic N (Woodin Press & Lee 1985, Press, Woodin & Lee 1986, Woodin & Lee 1987). Bryophytes respond to and assimilate nitrate very rapidly and efficiently in small quantities but continuous exposure to large N inputs suppresses this response. <i>Sphagnum</i> is capable of assimilating NO_3^- immediately, showing no lag phase unlike higher plants. In mosses the ability to induce NR activity has thus been used to assess their nitrate exposure history with the inability to induce activity being seen to signify N saturation. The <i>in vivo</i> methodology was developed by Woodin & Lee (1987).</p>
Previous experience	<p>Johnson et al (1999) found that short-term (18 month) N additions to grassland sites had no effect on root-surface PME activity whereas long-term (7 years) additions significantly increased enzyme activity. Addition of P also significantly reduced PME activity. In the long-term plots, PME activity was closely related to extractable soil ammonium. NH_4^+ extracted in 2M KCl accounted for 67% of the variation in PME activity of <i>Plantago lanceolata</i> in calcareous grassland and 86% of the variation in PME activity of <i>Agrostis capillaries</i> in acid grassland. Activity of PME was an order magnitude lower in <i>P. lanceolata</i> than in <i>A. capillaries</i> possibly reflecting the difference in root surface.</p> <p>Bryophytes respond to and assimilate nitrate very rapidly and efficiently in small quantities but continuous exposure to large N inputs suppresses this response. The ability of mosses to induce NR activity has thus been used to assess nitrate exposure history with the inability to induce activity being seen to signify N saturation . (Woodin Press & Lee 1985, Press, Woodin & Lee 1986, Woodin & Lee 1987). <i>Sphagnum</i> is capable of assimilating NO_3^- immediately, showing no lag phase unlike higher plants.</p>
Suitability to indicate atmospheric concentrations	Insufficient evidence
Suitability to indicate atmospheric deposition	Some evidence with scope for development
Suitability to indicate environmental impacts of N	Unsuitable
Sensitivity to other factors	Sensitive to a range of factors affecting enzyme activity e.g. temperature, moisture and season.
Time constant	Insufficient evidence, although available literature suggest that the enzyme activity represents a long-term response.
Limitations to applicability	Limited by soil, horizon type, pH and factors affecting plant photosynthesis.
Expertise in field	Ideally, samples should be collected, cleaned (removal of litter, other species etc) and frozen before being transported to an analytical laboratory by post, and specific conditions for storage and time between collection and freezing. Alternatively they could be transported in the fresh state as quickly as possible (by courier or special delivery) for sample preparation at the analytical laboratory.

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Expertise in laboratory	Specialist chemical analysis of samples is necessary.
Cost	Insufficient information available.
References	<p>Johnson, D., Leake, J.R. and Lee, J.A. (1999). The effects of quantity and duration of simulated pollutant nitrogen deposition on root-surface phosphatase activities in calcareous and acid grasslands: a bioassay approach. <i>New Phytologist</i> 141, 433-442.</p> <p>Woodin, S.J. & Lee, J.A. (1987) The fate of some components of deposition in ombrotrophic mires. <i>Environ. Pollut.</i>, 45, 61-72.</p> <p>Woodin, S.J., Press, M.C. & Lee, J.A. (1985) Nitrate reductase activity in <i>Sphagnum fuscum</i> in relation to wet deposition of nitrate from the atmosphere. <i>New Phytol.</i> 99, 381-388.</p> <p>Press, M.C., Woodin, S.J. & Lee, J.A. (1986) The potential importance of an increased atmospheric supply to the growth of ombrotrophic Sphagnum species. <i>New Phytol.</i> 103, 45-55.</p> <p>Campbell, W.H. (1999) Nitrate reductase structure, function and regulation: Bridging the gap between biochemistry and physiology. <i>Annu. Rev. Plant Physiol. Plant Mol. Biol.</i> 50, 277-303.</p>

Method	N:P ratio in leaf tissues
Basis of the approach	Where plant growth may be limited by P availability or when enhanced N deposition may lead to reduced availability and/or uptake, measurements of the N:P ratios in selected species may provide a better indication of N saturation. Optimum N:P ratios for plant growth range from 10-14 (van den Driesshe, 1974; Ingestad, 1979). Low ratios (<10) indicate N limited growth and high ratios (>14) indicate P limitation (Koerselma & Meuleman, 1996).
Previous experience	<ol style="list-style-type: none"> 1. In areas of southern Sweden with high inputs of atmospheric N, <i>Sphagnum</i> growth was found to be P limited and N:P ratios of around 34 were measured in <i>Sphagnum capitula</i> (Aerts <i>et al.</i> 1992). Conversely in northern Sweden where N inputs are small, <i>Sphagnum</i> growth was limited by N availability and N:P ratios were very small (6). 2. Long term increases in atmospheric N deposition has led to increased foliar N levels and reduced concentrations of P, K and Mg, in beech stands in north eastern France Duquesnay <i>et al.</i> (2000), leading to increases in ratios of N:P, N:K and N:Mg of 42, 19 and 77, respectively. 3. Biodiversity and species richness have also been related to N:P ratios (Ertsen 1998). In selected Dutch grassland and heathland sites a N:P ratio >16 reduced biodiversity (Roem & Berendse, 2000).
Suitability to indicate atmospheric concentrations	There is insufficient evidence available
Suitability to indicate atmospheric deposition	The evidence available is not adequate to support a robust relationship between N:P and N deposition. (However when determining tissue N as a bioindicator, the added determination of P at relatively modest extra cost, provides valuable information in the form of N:P).
Suitability to indicate environmental impacts of N	There is some evidence of an ability to indicate species richness but more research is needed.
Sensitivity to other factors	The ratio is sensitive to changes in available P in the soil such as may occur close to urban areas.
Time constant	This method can be used to assess long-term or short-term change in deposition. However, care must be taken in selecting species suitable for the type of change. Tree species can be used for long-term change whereas mosses may be more suitable for response to short-term changes. For some sites, in high rainfall areas, <i>Calluna</i> may be more suitable than mosses.
Limitations to applicability	The method is not limited to particular habitat or species types, but is most suitable for woodland and moorland ecosystems. Ideally several common species should be sampled to obtain values that could be compared with published values. At least 3 replicate samples should be collected at several points in the site. Sampling also needs to take account of adjacent vegetation as this strongly influences the N composition through competition. Approximately 2 g fresh weight of material should be collected to provide a minimum of 0.5 g of dried, ground material for chemical analysis.
Expertise in field	Sampling requires training in species identification particularly of bryophytes or lichens, which many conservation agency staff may have already received. When age or growth phase of the plant or foliage is important, these must be identifiable by staff. Ideally, samples should be collected, stored in cold conditions, cleaned (removal of litter, other species etc) and oven dried before being transported to an analytical laboratory by post. When suitable ovens are not available, samples can be air-dried before transportation to the laboratory.

Bioindicator and biomonitoring methods for assessing the effects of atmospheric nitrogen on statutory nature conservation sites

Expertise in laboratory	Determination of total tissue N and P in plants requires sample digestion (with strong acids in a fume cupboard) and subsequent analysis using specialist, but well proven laboratory techniques.
Cost	<p><i>Cost per unit of sampling (e.g. one SAC sampled at the edge, centre and an intermediate location at one time). Number of person days of required (Conservation agency + External specialists) in field and laboratory. Indicative cost as of 2003-2004.</i></p> <p>For 3 species, 3 replicates per species and 3 locations per site Conservation agency Staff Sample collection, cleaning, drying and packaging for 27 samples – 4 days Analytical Laboratory costs Preparation - milling (at least 0.7 mm sieve size, or ball mill), mixing, tubbing (and supply of sample tubs), labelling tubs etc... £10* per sample + VAT (if applicable).; Analysis - Total N - CNS analyser (Combustion; Manufacturer: Elementar Model: Vario EL) £11* per sample + VAT (if applicable). Total P - £9* per sample + VAT (if applicable). Total per sample - £30 (+ VAT if applicable). Total per 27 samples - £810 (+ VAT if applicable). *Discounts may be available for specific research work e.g. collaborative research projects part funded with CEH and/or NERC, or linked to NERC Research Grants or Ph D studentships. Extra costs may be incurred if less than 20 samples are submitted for analysis at any one time.</p>
References	<p>Duquesnay, A., Dupouey, J.L., Clement, A., Ulrich, E. & LeTacon, F. 2000. Spatial and temporal variability of foliar mineral concentration in beech (<i>Fagus sylvatica</i>) stands in northeastern France. <i>Tree Physiol.</i> 20, 13-22.</p> <p>Ingestad, T. 1979. Nitrogen stress in birch seedlings. II. N, K, P, Ca and Mg nutrition. <i>Physiol. Plant.</i> 45, 149-157.</p> <p>Koerselman, W., Meuleman, A.F.M. 1996. The vegetation N:P ratio: a new tool to detect the nature of nutrient limitation. <i>J. Appl. Ecol.</i> 33, 1441-1450.</p> <p>Roem, W.J.& Berendse, F. 2000. Soil acidity and nutrient supply ratio as possible factors determining changes in plant species diversity in grassland and heathland communities. <i>Biol. Conserv.</i> 92, 151-161.</p> <p>Van den Driessche, R. 1974. Prediction of mineral nutrient status of trees by foliar analysis. <i>Botanical Review</i>, 40, 347-394.</p>

Method	Ellenberg N Index (Higher plants and bryophytes)
Basis of the approach	Ellenberg N values estimate the position along a productivity/macro-nutrient availability gradient at which a species reaches peak abundance. The Ellenberg N Index consists of allocating a N score to each plant species, so that the overall mean score for the community lies on a scale of nutrient poor (1) to nutrient rich (10) Calculating mean values for sampled vegetation allows spatial or temporal changes in productivity to be inferred. Many calibration studies support the reliability of these values in signal detection, but attributing change to a specific cause is difficult because the N values integrate a range of effects.
Previous experience	There is an extensive published literature on the application of Ellenberg N values in Britain and Europe. These include: 1) Quantification of vegetation change and inferring shifts in productivity or/and macro-nutrient availability; Smart (2000), Ejrnaes et al (2003), Lameire et al (2000). 2) Correlative studies linking vegetation change and N deposition; (Pitcairn <i>et al.</i> 2002, Diekmann & Dupre 1997, Falkengren-Grerup 1996, van Dobben 1992). The index has been used at a local scale (e.g. Pitcairn <i>et al.</i> 2002;) and also at a national scale (Haines-Young <i>et al.</i> 2000). 3) Calibration studies that validate N values or modify them for specific regions; Schaffers & Sykora (2000), Hill et al (2000), Thompson <i>et al.</i> (1993), Diekmann (1995), Ertsen <i>et al.</i> (1998).
Suitability to indicate atmospheric concentrations	No inherent discriminatory power between different N forms, unsuited.
Suitability to indicate atmospheric deposition	No inherent discriminatory power to quantify deposition. However, there is clear evidence of a relationship between the Ellenberg index and atmospheric N deposition (See Pitcairn <i>et al.</i> , 2002).
Suitability to indicate environmental impacts of N	By definition the method represents the status of a plant community, with differences attributed to the impact of varying N availability. It is therefore well suited to indicate N deposition impacts. The corresponding limitation is that the method cannot discriminate between the different drivers of eutrophication.. The method has been used to indicate species change in relation to enhanced N inputs from both fertilisation and atmospheric deposition.
Sensitivity to other factors	Spatial and temporal change in mean N values can be influenced by a range of additional factors. These include; nutrient limitation, pH, disturbance, climate, light levels, presence of responsive species, frequency distribution of values within plots and within local species pools.
Time constant	The method reflects the time constant of colonization and species change. While differences as small as 3 years have been examined, in general the method reflects the responses occurring over several decades.
Limitations to applicability	The method is applicable to 1791 taxa in GB flora, which includes 239 introduced species, all native, non-critical taxa. Indicator values for bryophytes and lichens can be obtained from Siebel (1993) and Wirth (1991), respectively. The method has a fine spatial resolution, depending on the averaging scale of the plant community being considered. For forest ground flora the approach has been applied across transects of <100 m.
Expertise in field	The method can be applied by conservation agency staff skilled in botanical identification. The simplicity depends on species diversity and/or survey of cover vs presence/absence. There is no requirement for plant sample collection and transport.
Expertise in laboratory	Mean Ellenberg scores (frequency weighted or unweighted) are extremely simple to compute. Analyses of spatial or temporal change need to take account of standard statistical issues not unique to the manipulation of N values.

Cost	<p><i>Cost per unit of sampling (e.g. one SAC sampled at the edge, centre and an intermediate location at one time). Number of person days of required (Conservation agency + External specialists) in field and laboratory. Indicative cost as of 2003-2004.</i></p> <p>The method is difficult to cost because it is dependant on the biodiversity of the site. Species identification and recording can be very time consuming in species rich sites. e.g. for typical mixed woodland ground flora.</p>
References	<p>Diekmann, M (1995) The use and improvement of Ellenbergs indicator values in deciduous forests of the boreo-nemoral zone in Sweden. <i>Ecography</i> 18, 178-189.</p> <p>Diekmann, M, Dupre, C (1997) Acidification and eutrophication of deciduous forests in northwestern Germany demonstrated by indicator species analysis. <i>J.Veg.Sci.</i> 8, 855-864.</p> <p>Ejrnaes, R, Hansen, DN, Aude, E (2003) Changing course of secondary succession in abandoned sandy fields. <i>Biol.Cons.</i> 109, 343-350. .</p> <p>Ertsen, ACD, Alkemade, JRM, Wassen, MJ (1998) Calibrating Ellenberg indicator values for moisture, acidity, nutrient availability and salinity in the Netherlands. <i>Plant Ecol.</i> 135, 113-124. .</p> <p>Hill, MO, Roy, DB, Mountford, JO, Bunce, RGH (2000). Extending Ellenbergs' indicator values to a new area: an algorithmic approach. <i>J.Appl.Ecol.</i> 37, 3-15. .</p> <p>Lamiere, S, Hermy, M, Honnay, O (2000) Two decades of change in the ground vegetation of a mixed deciduous forest in an agricultural landscape. <i>J.Veg.Sci.</i> 11, 695-704.</p> <p>Pitcairn C. E. R., Skiba U. M., Sutton M. A., Fowler D., Munro R. & Kennedy, V. K. (2002). Defining the spatial impacts of poultry farm ammonia emissions on species composition of adjacent woodland groundflora using Ellenberg indicators, nitrous oxide and nitric oxide and foliar nitrogen as marker variables. <i>Environ. Pollut.</i>, 119, 9-21.</p> <p>Schaffers, AP, Sykora, KV (2000) Reliability of Ellenberg indicator values for moisture, nitrogen and soil reaction: a comparison with field measurements. <i>J.Veg.Sci.</i> 11, 225-244.</p> <p>Smart, SM (2000) Ecological assessment of vegetation data from a nature reserve using regional reference data and indicator scores. <i>Biodiversity and Conservation</i> 9, 811-832.</p> <p>Thompson, K, Hodgson, JP, Grime, JP, Rorison, IH, Band, SR, Spencer, RE (1993) Ellenberg numbers revisited. <i>Phytoceonologia</i> 23, 277-289. .</p> <p>van Dobben, H. F. (1993). Vegetation as a monitor for deposition of nitrogen and acidity. Ph.D. Thesis, Wageningen</p>

Method	Other N Indicators
Basis of the approach	<p>1. FNIS is a functional N index for each species and is based on mineralization rates of various forms of soil N (Diekmann and Falkengren-Grerup, 1998). Species N indices for nitrate and ammonium are calculated as the weighted averages of mineralised N in plots where the species are present. The species indices for ammonium and nitrate are then combined to produce the Functional N Index for each Species (FNIS).</p> <p>2. N_{dev} was developed by Diekmann and Falkengren-Grerup (2002) to reflect soil N status in respect to N and pH at which individual species occurred, and hence to predict response to enhanced N deposition. N_{dev} was calculated for all species at the study sites of southern Sweden, based on their observed versus expected nitrification rates at a given soil pH, and was then used to predict changes in species abundance.</p> <p>3. Life history traits, which may be affected by disturbance, have been used to classify species into groups. Traits include life form, phenology, plant height, anatomy, growth rate, foliar N content and others (Lavorel <i>et al.</i> 1997, 1999).</p>
Previous experience	<p>1. Diekmann and Falkengren-Grerup (1998) found that FNIS explained more floristic variability than Ellenberg N values and was a reliable expression of species response to N availability in deciduous forest soil of southern Sweden.</p> <p>2. N_{dev} was shown to be related to species frequency change in Swedish study sites and other areas of central Europe and able to predict species response to short-term fertilizer experiments (Diekmann and Falkengren-Grerup, 1998).</p> <p>3. Life history traits such as plant height, leaf anatomy, foliar N and phenology were significantly correlated with species change in Swedish forest sites, but were not as good predictors as N_{dev} (Diekmann and Falkengren-Grerup, 1998). Species likely to increase in abundance in response to enhanced N deposition are those favoured by a high soil nitrification rate at a given soil pH, tall stature, a hydro or helomorphic anatomy, large foliar N content and late phenological development.</p>
Suitability to indicate atmospheric concentrations	Unsuitable
Suitability to indicate atmospheric deposition	Current evidence is not adequate to support a robust relationship between FNIS, N_{dev} or life history traits and N deposition, although these parameters represent responses to atmospheric deposition.
Suitability to indicate environmental impacts of N	By definition each of these indices represent changes in the ecosystem related to varying nitrogen supply. There is some evidence for all 3 indicators, of an ability to indicate species richness, but more research is needed
Sensitivity to other factors	More research is needed, particularly on the interaction with different soil types.
Time constant	These methods are more suited to the assessment of long-term change in deposition (several years/decades).
Limitations to applicability	The available evidence is confined to forest ecosystems. More research is needed
Expertise in field	Staff with specialist plant identification skills, and specialist soil identification and sampling skills.
Expertise in laboratory	Specialist soil processing skills and equipment are needed. Soils should be sent to specialist laboratories. Measurement of mineralisation is highly empirical (dependent on moisture levels) and samples must be stored under specific conditions (preferably in deep freeze)
Cost	<p><i>Cost per unit of sampling (e.g. one SAC sampled at the edge, centre and an intermediate location at one time). Number of person days of required (Conservation agency + External specialists) in field and laboratory. Indicative cost as of 2003-2004.</i></p> <p>Insufficient information. The combination of soil analysis and plant survey likely to require > 4 days survey work and 5 days analytical time. Processing of many sites in parallel may reduce costs.</p>

References	<p>Diekmann, M. & Falkengren-Grerup, U. (1998) A new species index for forest vascular plants: development of functional indices based on mineralization rates of various forms of soil nitrogen. <i>Journal of Ecology</i>, 86, 269-283.</p> <p>Diekmann M. & Falkengren-Grerup U. (2002) Prediction of species response to atmospheric nitrogen deposition by means of ecological measures and life history traits. <i>Journal of Ecology</i>, 90, 108-120.</p> <p>Lavorel, S., McIntyre, S. & Grigulis, K. 1999. Plant response to disturbance in a Mediterranean grassland : how many functional groups? <i>Journal of Vegetation Science</i>, 10, 661-672.</p> <p>Lavorel, S., McIntyre, S., Landsber, J. & Forbes, R.D.A. (1997) Plant functional classifications: from general groups to specific groups based on response to disturbance. <i>Trends in Ecology and Evolution</i>, 12, 474-478.</p>
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Method	Transplant methods: Standardized plants
Basis of the approach	<p>The extensive use of standardised plants as bioindicators of atmospheric pollution, has been developed recently by Eurobionet (European Network for the Assessment of Air quality by the use of Bio-indicator Plants), a multinational European Communities study funded as part of the Life Programme. Standardised grass culture (<i>Lolium multiflorum</i> LAM spp) was used to indicate sulphurous compounds and heavy metals/trace elements. However, there is a longer history of using plants to indicate ammonia concentrations and deposition in the field (Sommer 1988, Appendix I, this report). Trays or pots of the standardised plants are placed in the field for a fixed period of time and then later assessed for growth, nitrogen uptake etc.</p> <p>In principle this method is highly attractive for application to statutory nature conservation sites as it a) provides an estimate of N deposition, b) demonstrates over a short a direct ecological response of N deposition.</p>
Previous experience	<p><i>Examples of application of the methods, including key references.</i></p> <ol style="list-style-type: none"> 1. Sommer (1988) used barley (<i>Hordeum vulgare</i> var. Harry) as a bio-indicator of ammonia deposition around a dairy farm. The N content of the green biomass was found to reflect N deposition along a 300 m transect from a dairy farm. 2. Sommer and Jensen (1991) assessed foliar absorption of NH₃ by ryegrass <i>Lolium multiflorum</i> Lam (Italian ryegrass) along a transect using ¹⁵N. They found that foliar %N concentration decreased with distance from the point source. 3. The results of Sommer and Jensen (1991) were subsequently re-analysed by Sutton <i>et al.</i> (1993), who showed that the grass biomonitors could be used to demonstrate a saturation of the NH₃ deposition velocity at very high NH₃ concentrations. 4. Recent application of the Eurobionet growth system for <i>Lolium perenne</i> applied for N accumulation downwind of a poultry farm showed a very close relationship to NH₃ concentrations in each of dry matter production, tissue %N content and total above ground N (Appendix I). A similar saturation of deposition velocity with NH₃ concentration was implicated.
Suitability to indicate atmospheric concentrations	Suitable to indicate concentrations of NH ₃ in the atmosphere. Further study is needed to establish the robustness of the method and its sensitivity to N level.
Suitability to indicate atmospheric deposition	Suitable to indicate N deposition. Further study is needed to establish the robustness of the method and its sensitivity to N level.
Suitability to indicate environmental impacts of N	Well suited to demonstrating biological impact of N deposition on a site in a controlled manner (e.g. for stakeholders). However, by contrast it does not monitor the actual plants occurring at a site, unless standard plants native to the site investigated are used
Sensitivity to other factors	The method is sensitive to seasonal effects and meteorological conditions, which govern growth. However, these effects may be avoided where the focus is local spatial comparisons conducted at one time.
Time constant	The method is well suited to detecting short-term changes in N deposition and biological response (e.g. 4-8 weeks).
Limitations to applicability	<p>As standardized biomonitors are used, these may be applied to any terrestrial habitat. Typically, 6 replicate plants are required for exposure at each site. Fast growing species should be selected which can be readily grown from seed. There is scope for use of native species especially native grass species.</p>
Expertise in field	<p>The culture of the standard plants in sufficient quantities and under uniform conditions should take place in a suitable laboratory or institution. After setting out in the field, maintenance of uniform water regime for the introduced plants in trays or pots is essential. This can be automated with only the need for weekly checks by conservation agency staff.</p> <p><i>(Given the correct facilities, conservation agency staff could be trained in these procedures with only limited supervision by specialist staff)</i></p>

Bioindicator and biomonitoring methods for assessing the effects of atmospheric nitrogen on statutory nature conservation sites

Expertise in laboratory	The harvesting and preparation of samples for further analysis should also be done in a specialist laboratory.
Cost	Although this method does not require sophisticated equipment, it does assume the presence of basic laboratory and glasshouse equipment. Culture of the plants takes 3-4 weeks and plants must be checked and cared for over this time. However this involves only short periods of time. The most cost effective way of using this method would be to assess several sites at a time. Further work is required before a cost can be specified.
References	<p>Eurobionet. 2003. www.uni-hohenheim.de/eurobionet</p> <p>Sommer, S.G.& Jenson, E.S. 1991. Foliar absorption of atmospheric Ammonia by Ryegrass in the field. <i>Journal of Environmental Quality</i>. 20: 153-156. .</p> <p>Sommer, S.G. 1988. A simple biomonitor for measuring ammonia deposition in rural areas. <i>Biol. Fertil. Soils</i>. 6:61-64.</p> <p>Sutton M.A., Pitcairn C.E.R. and Fowler D. 1993. The exchange of ammonia between the atmosphere and plant communities. <i>Adv. Ecol. Research</i>. 24, 301-393.</p>

Method	Transplantation: Native transplants
Basis of the approach	<p>N transplant experiments have mainly been used to assess the impact of N pollution on a given species, rather than provide a direct measure of N pollution levels <i>per se</i>. Transplant experiments involve moving a group of individuals from relatively unpolluted sites to sites receiving high levels of pollution or vice versa and involve a range of methods (Brodo 1961; Ferry & Coppins, 1979; Kauppi, 1976). Ideally all environmental and physical variables between the two sites are the same except the pollution level. This allows an assessment of the impact of the pollutant on a given species to be made. While there have been many transplant experiments involving lower plants and SO₂, few transplant experiments have involved N pollution. Because bryophytes and lichens are so closely coupled to the atmosphere, most transplant experiments use these groups of plants. However some transplant experiments have taken place using vascular plants (Spinks and Parsons, 1988; Hicks, 1996). While turfs can be moved, epiphytic species attached to bark, twigs or stones are more easily moved without disturbing the plants.</p> <p>The responses of that may be measured are either physiological or the accumulation of pollutants in the tissues (Pearson, 1993) and some calibration of the response.</p>
Previous experience	<ol style="list-style-type: none"> 1. Bakken (1995b) transplanted <i>Dicranum majus</i> from two Norwegian areas with different nitrogen deposition rates and concluded that both genetic and environmental factors determined the concentrations of total nitrogen, protein and chlorophyll concentrations in the moss tissue. 2. Transplantation studies both between regions and between sites within a mountain system in the UK linked the deterioration of <i>Racomitrium</i> heath in Britain during the last 50 years with increased atmospheric N deposition (Baddeley <i>et al.</i> 1994) by showing deposition related changes in tissue N content of <i>Racomitrium lanuginosum</i>. 3. When four epiphytic species of bryophytes (<i>Isothecium myosuroides</i>, <i>Dicranum scoparium</i>, <i>Frullania tamarisici</i> and <i>Ulota crispa</i>) were transplanted from Atlantic oak woods in Britain between sites of low (12 kg N ha⁻¹ y⁻¹), and high (54 kg N ha⁻¹ y⁻¹) atmospheric N inputs (Mitchell <i>et al.</i>, in prep), detrimental impacts of increased N deposition on the growth of the bryophyte species were demonstrated with the possibility of slow recovery in growth rate following a decrease in atmospheric N.
Suitability to indicate atmospheric concentrations	<p>Transplant of epiphytic species may be suitable for assessing N concentrations in stemflow. However, the method is unsuited to indicating atmospheric concentrations, unless a transect approach is used.</p>
Suitability to indicate atmospheric deposition	<p>Limited available evidence, but may prove a reasonable indicator of N deposition, especially when used in transects with distance from source. However, the deposition estimate is that to the biomonitor rather than the canopy.</p>
Suitability to indicate environmental impacts of N	<p>Well suited to determine impacts of N deposition on a species in near natural conditions. In addition, the reciprocal transplanting has the advantage that it can be used to assess recovery following transplant from high N exposure to low N exposure conditions. This may provide useful information to support implementation of local N abatement policies. Recovery experiments are only possible where the species concerned still survives at the high N deposition location.</p>
Sensitivity to other factors	<ol style="list-style-type: none"> 1. Transplant experiments are not conducted under controlled conditions and therefore there are a variety of other factors that may influence the results. 2. Responses of epiphytic bryophytes to changes in atmospheric N may vary according to how they obtain their nutrients, the chemistry of the bark and the roughness of the bark and how it traps dust particles (Brown, 1982). 3. Species that grow on acid and basic barked tree species may respond differently to N levels. 4. Climatic differences between sites may also confound results especially since they influence growth /nutrient dilution.

	5. Bryophytes and lichens may present a memory for their exposure history, which fades out in time. Allowance (calibration) must be made for such an effect.
Time constant	The method can be used to indicate short term and long term changes in N deposition. A typical minimum period would be 1 year.
Limitations to applicability	<i>Habitat types, species groups. Required quantities of survey vegetation or sampling material.</i> Because of the absence of a root system for nutrient uptake and dependence on the atmosphere for the acquisition of major nutrients bryophytes and lichens are more suited for transplantation. While turfs can be moved, epiphytic species attached to bark, twigs or stones are more easily moved without disturbing the plants. However, epiphytic bryophytes tend to be better suited to transplants than lichens, with the latter being sensitive to changes in climatic conditions. Transplant experiments are time consuming and must be left for sufficient time for any lag effects to be minimal. Most transplant experiments on lower plants run for at least a year as this includes all seasons and therefore minimizes any seasonal effects on the results.
Expertise in field	<i>Need for specialist staff to take samples (either specialist equipment or identification skills), and for specialist sample transport.</i> Staff need to be trained in plant identification, particularly of bryophytes and lichens and in good scientific practice in transplantation methods. They also need training in monitoring responses of transplanted material (e.g. growth and sampling for chemical analysis).
Expertise in laboratory	Need for specialist processing of samples or data, in order to calculate results. Specialist interpretation of physiological data Specialist processing of samples for chemical analysis (cf tissue N)
Cost	1 species, 10 clumps transplanted per site therefore 30 clumps, each clump tagged with 8 shoots - 1.3 person days . Collecting in and measuring - 1 day. (Obviously how long it takes depends on the species as some species are more fiddly than others for tagging etc. the other cost involved is travel time between sites, but this would vary). Cleaning 30 samples for chemical analysis - 2 days Total costs @ £300 per day - £1200 Analytical Laboratory costs Preparation - milling (at least 0.7 mm sieve size, or ball mill), mixing, tubing (and supply of sample tubs), labelling tubs etc... £10* per sample + VAT (if applicable).; Analysis - Total N - CNS analyser (Combustion; Manufacturer: Elementar Model: Vario EL) £11* per sample + VAT (if applicable). Total per sample - £21 (+ VAT if applicable). Total per 27 samples - £567 (+ VAT if applicable). *Discounts may be available for specific research work e.g. collaborative research projects part funded with CEH and/or NERC, or linked to NERC Research Grants or PhD studentships. Extra costs may be incurred if less than 20 samples are submitted for analysis at any one time.
References	Baddeley JA, Thompson DBA, & Lee JA. (1994) Regional and historical variation in the nitrogen-content of <i>Racomitrium-lanuginosum</i> in Britain in relation to atmospheric nitrogen deposition. <i>Environmental Pollution</i> , 84, 189-196. Bakken S. (1995a) Effects of nitrogen supply and irradiance on growth and nitrogen status in the moss <i>Dicranum majus</i> from differently polluted areas. <i>Journal of Bryology</i> , 18, 707-721. Brodo IM. (1961) Transplant experiments with corticolous lichens using a new technique. <i>Ecology</i> , 42, 838-841. . Brown DH (1982). Mineral Nutrition. In <i>Bryophyte Ecology</i> (ed Aje Smith), pp. 383-344. Chapman & Hall, London. Ferry BW & Coppins BJ. (1979) Lichen transplant experiments and air pollution studies. <i>Lichenologist</i> , 11, 63-73.

	<p>Hicks, W.K., Leith, I.D., Woodin, S.J. & Fowler, D. 2000. Does atmospheric nitrogen deposition determine the foliar nitrogen concentration of upland vegetation? Evidence from field surveys. <i>Environ. Pollut.</i> 107, 367-376. .</p> <p>Kauppi M. (1976) Fruticose lichen transplant technique for air pollution experiments. <i>Flora, BD.</i>, 165, 407-414.</p> <p>Leith, I.D., Hicks, W.K., Fowler, D. & Woodin, S.J. 1999. Differential responses of UK upland plants to nitrogen deposition. <i>New Phytol.</i> 141, 277-289. .</p> <p>Mitchell RJ, Sutton MA, Truscott A-M, Leith ID, Cape JN, Pitcairn CER, & van Dijk N. (in prep) Growth and tissue N of epiphytic bryophytes: effects of increased and decreased atmospheric N deposition.</p> <p>Pearson L (1993). Active monitoring. <i>In Lichens as bioindicators of air quality</i> (ed L Huckaby), pp. 89-95. US department of agricultural, Colorado. .</p>
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Method	Stable isotope - $\delta^{15}\text{N}$
Basis of the approach	<p>Nitrogen exists as the 2 stable isotopes ^{14}N and ^{15}N. (For detailed descriptions of the use of stable isotopes in ecology, see Hogberg, 1997; Handley and Raven, 1992). Isotopic composition is expressed in terms of δ values – parts per thousand (per mil ‰) difference from the standard, which is N_2 in air: $\delta^{15}\text{N} = [(15/14 \text{ sample} - 15/14 \text{ standard}) / 15/14 \text{ standard}] * 10^3$.</p> <p>$\delta^{15}\text{N}$ values are measures of the amounts of heavy and light N isotopes in a sample ($^{15}\text{N}/^{14}\text{N}$), a positive value indicating ^{15}N enrichment and a negative value the reverse. Stable isotope studies can help to identify the source and fate of N added to the environment by anthropogenic activities. There is evidence that the 2 major forms of atmospheric N, NO_x and NH_y have different $\delta^{15}\text{N}$ signatures, reflecting their different origins. Ammonia emissions from volatilisation processes tend to ^{15}N negative, while NO_x emissions from vehicles tend to be ^{15}N positive. (NH_3 emissions from vehicles may also be positive, though further research is required on this). Monitoring of the ^{15}N signal has therefore be used to provide an indication of the emission source of (combustion processes or volatilisation) of the nitrogen.</p>
Previous experience	<p>Studies have shown a close correlation between $+\delta^{15}\text{N}$ and traffic exposure, for higher plants (pers comm. S. Power and T Collins) and mosses (Pearson <i>et al.</i> 2000). Moss samples collected near motorways or busy roads had mainly positive signatures ranging from +6 to -1‰. Power and Collins demonstrated a significant relationship between $\delta^{15}\text{N}$ signature in <i>Calluna vulgaris</i>, distance from London and from nearest 'A' trunk roads and also background NO_2 in London. Signatures were more negative, ranging from just 0 to -9‰.</p> <p>Ammonia emitted from agricultural practices has very negative $\delta^{15}\text{N}$ values, as the ammonia volatilised is preferentially enriched with the lighter ^{14}N. Hence $\delta^{15}\text{N}$ values of moss species sampled upwind and downwind of a poultry farm in southern Scotland, were shown to reflect the deposition of negative ammonia and its uptake into plant tissue (Harrison <i>et al.</i> 1999) with values from -6.8‰ upwind to -11.5‰ downwind.</p>
Suitability to indicate atmospheric concentrations	Not suited
Suitability to indicate atmospheric deposition	Although the evidence produced is limited, the relationships demonstrated between $\delta^{15}\text{N}$ and N deposition are strong and can be used for a range of species. However, the method is best suited to very high levels of nitrogen supply, where a particular source (with given isotopic signature) dominates.
Suitability to indicate environmental impacts of N	Insufficient evidence available, but unlikely to be suitable
Sensitivity to other factors	A wide range of other factors affect isotopic fractionation of nitrogen species. Wet and dry deposition may also be fractionating processes.
Time constant	Long term (typically >1 to several years).
Limitations to applicability	Insufficient information is available on practical aspects such as sampling season etc., but it is likely that constraints similar to those governing total tissue N would also apply here. In general the method has not been adequately used or tested to provide any uncertainty measure.
Expertise in field	Sampling requires training in species identification particularly of bryophytes or lichens, which many conservation agency staff may have already received. When age or growth phase of the plant or foliage is important, these must be identifiable by staff.

	Ideally, samples should be collected, cleaned (removal of litter, other species etc) dried (either by oven or in the air) and transported to an analytical laboratory.
Expertise in laboratory	Measurement of $\delta^{15}\text{N}$ requires highly expensive equipment and specialist technicians and costs per sample can be high. Interpretation of results would require the services of an expert.
Cost	<p><i>Cost per unit of sampling (e.g. one SAC sampled at the edge, centre and an intermediate location at one time). Number of person days of required (Conservation agency + External specialists) in field and laboratory. Indicative cost as of 2003-2004.</i></p> <p>For 3 species, 3 replicates per species and 3 locations per site Conservation agency Staff Sample collection, cleaning, drying and packaging for 27 samples – 4 days <i>Analytical laboratory</i> Preparation - milling (at least 0.7 mm sieve size, or ball mill), mixing, tubing (and supply of sample tubs), labelling tubs etc... £10* per sample + VAT (if applicable). Analysis - N-15 - LN2 milling, Mass Spec £17* per sample + VAT (if applicable) Total per sample - ~£27 Total per 27 samples - ~£729 *Discounts may be available for specific research work eg collaborative research projects part funded with CEH and/or NERC, or linked to NERC Research Grants or Ph D studentships. Extra costs may be incurred if less than 20 samples are submitted for analysis at any one time.</p>
References	<p>Handley, L.L. & Raven, J.A. 1992. The use of natural abundance of nitrogen isotopes in plant physiology and ecology. <i>Plant, Cell and Environment</i>, 15, 965-985.</p> <p>Harrison, A.F., Sleep, D., Pearson, J & Woodall, J. 1999. Trends in ^{15}N signatures of nitrogen deposition along woodland transects from a chicken farm and a motorway as indicated by vegetation and soil analyses. Report to the Department of the Environment, Food and Rural Affairs. (DEFRA Contract EPG1/3/52).</p> <p>Hogberg, P. 1997. ^{15}N natural abundance in soil-plant systems. <i>New Phytol.</i> 137, 179-203.</p> <p>Pearson, J., Wells, D.M., Seller, K.J., Bennett, A., Soares, A., Woodall, J. & Ingouille, M.J. 2000. Traffic exposure increases natural ^{15}N and heavy metal concentrations in mosses.</p> <p>Wania, R., Hietz, P. & Wanek, W. 2002. Natural ^{15}N abundance of epiphytes depends on the position within the forest canopy: source signals and isotope fractionation. <i>Plant, Cell and Environment</i>, 25, 581-589. .</p>

Method	Chlorophyll Fluorescence
Basis of the approach	When plant leaves are illuminated, part of the light energy is trapped by the chlorophyll molecule, which becomes 'excited' and undergoes a shift in energy state. The energised state is unstable and the energy is rapidly released (re-emitted) via 3 competing pathways. Fluorescence describes the energy that is re-emitted as a low energy high wavelength photon of red and far red light. The remaining energy is used to drive photosynthesis or dissipated as heat. Damage to photo-system II (PSII) and reductions in photosynthetic rate will reduce the amount of fluorescence released in response to light. Thus fluorescence measurements provide information on inhibition or damage to transfer of electrons from PSII and photochemical quantum yield.
Previous experience	Measurement of fluorescence has been used as an early indicator of physiological stress. Reductions in fluorescence usually precede visible damage. Different parameters associated with fluorescence kinetics have been related to a range of stresses such as freezing, drought and high photo-illumination following cold nights. Changes in fluorescence kinetics may be linked to N through effects on water use and light harvesting. However, changes in fluorescence are considered to reflect increased sensitivity to environmental stress resulting from N deposition, rather than as a direct effect of N deposition.
Suitability to indicate atmospheric concentrations	The relationship between chlorophyll fluorescence and N concentrations/deposition and or N impact is not direct. The method provides a general indicator of stress that is not specific to cause and data may be difficult to interpret. Insufficient evidence is available to review the sensitivity of the method.
Suitability to indicate atmospheric deposition	The relationship between chlorophyll fluorescence and N concentrations/deposition and or N impact is not direct. The method provides a general indicator of stress that is not specific to cause and data may be difficult to interpret. Insufficient evidence is available to determine whether the method is better suited to a wide range of N levels or to low or very high N levels.
Suitability to indicate environmental impacts of N	The relationship between chlorophyll fluorescence and N concentrations/deposition and or N impact is not direct. The method provides a general indicator of stress that is not specific to cause and data may be difficult to interpret.
Sensitivity to other factors	Different parameters associated with fluorescence kinetics have been related to a range of stresses such as freezing, drought and high photo-illumination following cold nights. The method is strongly affected by moisture content of the plant, particularly in cryptogams, where photoassimilation is determined by H ₂ O availability. However, it is not very sensitive to temperature within the range of normal outside conditions in UK (Wulf <i>et al.</i> , 1994).
Time constant	Available evidence suggests that the method is more suited for assessing short-term direct effects (e.g. days to months).
Limitations to applicability	The method does not appear to be limited to particular habitat or species types.
Expertise in field	The method is simple and rapid and many measurements can be undertaken in a single day, providing sufficient dark adaptation clips are available. The equipment is portable, relatively inexpensive and could be operated by conservation agency staff following suitable training.
Expertise in laboratory	Data can be difficult to interpret without the help of an experienced researcher.
Cost	<i>Cost per unit of sampling (e.g. one SAC sampled at the edge, centre and an intermediate location at one time). Number of person days of required (Conservation agency + External specialists) in field and laboratory. Indicative cost as of 2003-2004.</i> Sufficient data can be collected by trained conservation agency staff, (or an expert) in 1 day. However interpretation of the data may take several days and may involve help from a experienced researcher. Daily costs for expert help would costs around £300 per day.

References	Wulff, A., Sheppard, L.J. & Leith, I.D. 1994. Evaluation of Electrolyte Leakage, Chlorophyll Fluorescence and Ultrastructural Techniques for Detecting Effects of Acid Mist on Frost Hardiness of Sitka Spruce Shoots. 1994. <i>Environmental and Experimental Biology</i> , 34 : 261-73.
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Method	Frost Hardiness
Basis of the approach	<p>The ability of plants to minimise the risk of freezing damage is conferred by synchronising their phenology with the growing environment. The indigenous flora generally has a good safety margin between its frost hardened status and minimum temperatures, unless the growth environment changes. A negative link between enhanced N deposition and reduced frost hardiness was widely suspected to be a casual factor in the observed decline of red spruce in the nineteen eighties (Eagar & Adams 1992).</p> <p>The bioassay is conducted on detached shoots, which are frozen to a range of temperatures in a purpose built frosting chamber using a cooling rate of 5 °C per hour, and kept for 3 hours at the target temperature. The shoots are then thawed at 10 °C increase per hour back to ambient temperature. Damage is assessed by electrolyte leakage, by immersing shoots into de-ionized water and analysing conductivity.</p>
	<p>Nihlgard (1985) postulated that increasing N deposition prolonged the growing season and led to carbon assimilate being diverted away from the production of 'cryoprotectants', which protect from frost damage. Subsequent studies using N fertilizers which have significantly raised foliar N concentrations, failed to support this hypothesis showing that the addition of N to N-deficient trees improved frost hardiness (DeHayes <i>et al.</i> 1990, Klein <i>et al.</i> 1989).</p> <p>Nursery studies with conifers also suggested improved frost hardiness with N fertilisation although the effect was dependent on the timing of the fertilizer application (Benzian and Freeman 1967). Christersson (1973) observed higher levels of freezing damage in field plots treated with N, which he attributed to nutrient imbalances with respect to N.</p> <p>Shoots of spruce taken from three N manipulation experiments showed a positive but non-significant improvement in winter hardiness in response to N additions (Sheppard <i>et al.</i> 2003). When foliar N status is very significantly raised there does appear to be some loss of hardiness.</p>
Suitability to indicate atmospheric concentrations	Recent evidence from the GANE Whim experiment measuring the response of <i>Calluna vulgaris</i> (heather) suggests the plants respond to peak NH ₃ concentrations rather than average NH ₃ concentrations and deposition (L. Sheppard, pers. comm.).
Suitability to indicate atmospheric deposition	While there may be a link between N deposition and increased frost sensitivity the evidence is often inconclusive implying a large N excess may be a prerequisite. Ammonium appears to have a stronger effect than nitrate when applied as wet deposition.
Suitability to indicate environmental impacts of N	Reduction in frost hardiness represents a direct ecological response to high N gaseous concentrations and deposition.
Sensitivity to other factors	Frost hardiness is also sensitive to water availability and sulphur deposition, and expected to depend on ozone exposure. A limitation is that the frost hardiness response depends on local climatic conditions and may vary between years.
Time constant	Available evidence suggests that the method is more suited for assessing short term changes.
Limitations to applicability	Natural variation is high so that effects must be large to be detectable. The analysis is destructive and requires large numbers of samples especially as assessments may need to be made at different times – early autumn, winter and spring. Effects are also difficult to quantify in the absence of controls.
Expertise in field	Collection of material in the field requires trained, experienced personnel
Expertise in laboratory	The assessment of frost hardiness in the laboratory requires specialist equipment and trained, experienced personnel.
Cost	<i>Cost per unit of sampling (e.g. one SAC sampled at the edge, centre and an intermediate location at one time). Number of person days of required (Conservation agency + External specialists) in field and laboratory. Indicative cost as of 2003-2004.</i>

	<p>The method would need to be carried out in a specialist laboratory. The assessment of frost hardiness at one site would probably take 3 days work at the cost £300 per day + equipment running costs. Total costs per site - £920</p>
References	<p>Benzian B. and Freeman S.C.R. (1967) Effect of late season nitrogen and potassium top dressings applied to conifer seedlings and transplants on nutrient concentrations in the whole plant and on growth after transplanting. Report on Forest Research 1967, HMSO, pp 135-140.</p> <p>Christersson (1973) The effect of inorganic nutrients on water economy and hardiness of conifers. <i>Studia Forest. Suecica</i> 103, 1-26.</p> <p>DeHayes, D.H., Ingle, M.A. and Wark, C.E. (1989). Nitrogen fertilization enhances cold tolerance of red spruce seedlings. <i>Canadian Journal of Forest Research</i> 19, 1037-1043.</p> <p>Eager, C., & Adams, M.B. (editors). (1992) Ecology and decline of red spruce in the eastern United States. Ecological Studies Vol. 96. Springer-Verlag, Berlin.</p> <p>Klein R.M., Perkins T.D., Myers H.L. (1989) Nutrient status and winter hardiness of Red Spuce foliage. <i>Canadian J. Forest Research</i> 19, 754-758.</p> <p>Nihlgård, B. (1985) The ammonium hypothesis – an additional explanation to the forest dieback in Europe. <i>Ambio</i>, 14: 2-8.</p> <p>Sheppard L.J., Rosengren U., Emmett B.A. (2003) Do nitrogen additions change the sensitivity of detached shoots from Sitka and Norway spruce to freezing temperatures? Evidence from three field manipulation studies. <i>Scand. J. Forest Res.</i> 18, 487-498.</p> <p>Sheppard, L.J. & Leith, I.D. (2002) Effects of NH₃ Fumigation on the Frost Hardiness of Calluna – Does N Deposition Increase Winter Damage by Frost? <i>Phyton</i>, 42: 183-190.</p>

Method	Soil gas fluxes
Basis of the approach	<p>The gases NO and N₂O are produced in soils by nitrifying and denitrifying bacteria, and the magnitude of the emissions is controlled by the availability of N as ammonium (NH₄) or nitrate (NO₃) and also by certain climatic and soil properties which promote nitrification or denitrification, e.g. temperature, rainfall, organic matter content (Skiba & Smith 2000).</p> <p>Studies of a range of semi-natural ecosystems, which have received various forms of N deposition, suggest that measurements of soil NO and N₂O emissions may be useful indicators of soils where N supply exceeds demand of vegetation (Skiba <i>et al.</i> 1998).</p>
Previous experience	<p>Skiba <i>et al.</i> (1998) showed that nitrogen additions by fertilisation, manure and atmospheric deposition increased emission of N₂O. For example, fluxes of N₂O and NO were closely related to atmospheric N deposition in the vicinity of livestock farms in southern Scotland (Pitcairn <i>et al.</i>, 2002; Skiba <i>et al.</i>, 1998). Emissions of N₂O and NO from woodland soil were large close to the poultry houses and decreased with increasing distance from the poultry houses. Emissions represented around 0.8% of the atmospheric N deposited. Larger losses of N₂O were measured in a sheep grazed pasture (1.7% of N input), and in an experimental sitka spruce plantation which had received acid mist containing N (3.7 % of N input).</p>
Suitability to indicate atmospheric concentrations	Not suitable
Suitability to indicate atmospheric deposition	There is a robust relationship between soil gas emissions and N deposition
Suitability to indicate environmental impacts of N	Emissions of N ₂ O and NO represent a biological response of soils to added nitrogen, so that the method by definition assesses environmental impact.
Sensitivity to other factors	<p>The method is sensitive to climatic and soil properties which promote nitrification or denitrification, e.g. temperature, rainfall, organic matter content (Skiba & Smith 2000).</p> <p>Freeze thaw cycles can influence the availability of soil mineral N and thus can potentially increase emissions. The nitrogen history of a site will strongly modify its capacity for N₂O and NO emissions.</p>
Time constant	More suited for indication of long-term changes (several years to decades).
Limitations to applicability	Emissions depend on the presence of an active nitrifying and denitrifying population and optimal soil aeration, which is controlled by soil moisture and its relationship to the proportion of water filled pore spaces, and soil texture. The method can be assessed on most terrestrial habitats.
Expertise in field	Specialist staff and equipment are needed to obtain gas samples in the field.
Expertise in laboratory	Specialist staff and equipment are needed carry out gas analysis in the laboratory.
Cost	<p><i>Cost per unit of sampling (e.g. one SAC sampled at the edge, centre and an intermediate location at one time). Number of person days of required (Conservation agency + External specialists) in field and laboratory. Indicative cost as of 2003-2004.</i></p> <p>To set up equipment at 3 positions in the field and collect samples, plus gas analysis and data interpretation in lab and would probably take 3 days work at the cost £300 per day + equipment running costs. Total costs per site ~£920</p>
References	<p>Skiba, U. & Smith, K.A. 2000. The control of nitrous oxide emissions from agricultural and natural soils. <i>Chemosphere-Global Change Science</i>, 2, 379-386.</p> <p>Skiba, U., Sheppard, L.J., Pitcairn, C.E.R., Leith, I.D., Crossley, A., van Dijk, S., Kennedy V.J. & Fowler, D. 1998. Soil nitrous oxide and nitric oxide emissions as indicators of the exceedance of critical loads of atmospheric nitrogen deposition in semi-natural ecosystems. <i>Environ Pollut</i>, 102(S1), 457-461.</p> <p>Pitcairn C. E. R., Skiba U. M., Sutton M. A., Fowler D., Munro R. & Kennedy, V. K. (2002). Defining the spatial impacts of poultry farm ammonia emissions</p>

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	on species composition of adjacent woodland groundflora using Ellenberg indicators, nitrous oxide and nitric oxide and foliar nitrogen as marker variables. Environ. Pollut., 119 , 9-21.
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Method	Invertebrates responses
Basis of the approach	<p><i>Insect pests</i>: It is generally thought that the increased infestations of insect pests particularly sucking insects, observed following N addition from the atmosphere or as fertiliser, is a response to increased N content of the plants. While the presence of certain pests may indicate an effect of N deposition, their absence does not indicate the lack of an effect and the introduction of pests in order to observe change is not acceptable.</p> <p><i>Soil invertebrates</i>: The composition of soil fauna may reflect the N status of the soil, although the application of high doses of nitrogen has had mixed results.</p>
Previous experience	<p>For insect pests, the infestation of beech aphid <i>Phyllaphis fagi</i> in a N fertilization experiment, increased significantly with increasing N concentration in leaves and N/P ratios respectively (Flückiger and Braun, 1998). The occurrence of insect damage to pine needles in permanent plots in Scotland was positively correlated with modelled N deposition (range 7-22 kg N ha⁻¹ yr⁻¹). This relationship was associated with a negative relationship between the years of needles retained and modelled N deposition (NEG-TAP, 2001).</p> <p>For soil fauna, the abundance of <i>Nematoda</i>, <i>Oligochaeta</i> and microarthropodes, especially <i>Collembola</i>, had increased in some studies, but decreased in others after adding fertilizers (>150 kg N ha⁻¹ yr⁻¹) (Abrahamsen and Thompson, 1979; Huhta <i>et al.</i>, 1983; Vilkkamaa and Huhta, 1986). A one-time application of 100 kg N ha⁻¹ yr⁻¹ (as NH₄NO₃) in Eastern France, produced an effect on soil micro-organisms which was still significant after 23 years (Deleporte and Tillier, 1999). A reduction in the nitrogen deposition in a <i>Pinus sylvestris</i> stand to preindustrial levels increased the species diversity of microarthropods due to a decreased dominance of some species (Boxman <i>et al.</i>, 1995). In Sweden, a significant decrease of snails over 14-46 years in areas with N deposition of 15-25 kg N ha⁻¹ yr⁻¹ was observed, while in areas with N deposition of 3-6 kg N ha⁻¹ yr⁻¹ no significant changes were found (Gårdenfors <i>et al.</i>, 1995).</p>
Suitability to indicate atmospheric concentrations	Not suitable
Suitability to indicate atmospheric deposition	There is some evidence to suggest a link between abundance of certain species and N deposition. However the relationship is not robust and further work is needed.
Suitability to indicate environmental impacts of N	By definition, changes in invertebrates would represent a biological impact of nitrogen. However, the relationship is not robust and further work is needed.
Sensitivity to other factors	There will be sensitivity to many other factors, which affect the growth of the insect pests and their hosts and the competitive growth of soil invertebrates.
Time constant	No evidence available, likely to be a response over many years.
Limitations to applicability	The method is in principle relevant for most terrestrial ecosystems, but the main concerns are the sensitivity to other factors which limit its practical application.
Expertise in field	Conservation agency staff may be trained to check for and monitor insect pests. Surveys of soil fauna require skilled staff to both develop and apply the methods
Expertise in laboratory	Identification of soil fauna require skilled staff
Cost	Insufficient information
References	<p>Abrahamsen, G. and Thompson, W.N. (1979). A long-term study of the enchytraeid (Oligochaeta) fauna of a mixed coniferous forest and the effects of area fertilization. <i>Oikos</i>, 24, 318-327.</p> <p>Flückiger, W. and Braun, S. (1998). Nitrogen deposition in Swiss forests and its possible relevance for leaf nutrient status, parasite attacks and soil acidification. <i>Environmental Pollution</i> 102, 69-76.</p> <p>NEG-TAP 2001. Transboundary Air Pollution: Acidification, Eutrophication and Ground-Level Ozone in the UK. National Expert Group on Transboundary Air Pollution. DEFRA, London. ISBN 1 870393 619</p> <p>Abrahamsen, G. and Thompson, W.N. (1979). A long-term study of the enchytraeid (Oligochaeta) fauna of a mixed coniferous forest and the effects of area fertilization. <i>Oikos</i>, 24, 318-327.</p>

	<p>Huhta, V., Hyvönen, R., Kaskenniemi, A. and Vilkkamaa, P. (1983). Role of pH in the effect of fertilization on Nematoda, Oligochaeta and microarthropods. In: Lebrun, P. <i>et al.</i> (eds.). <i>New Trends in Soil Biology</i>. Ottignies - Louvain - La Neve, 61-73.</p> <p>Vilkkamaa, P. and Huhta, V. (1986). Effects of fertilization and pH upon communities of Collembola in pine forest soil. <i>Annales Zoologicae Fennici</i> 23, 167-174.</p> <p>Deleporte, S. and Tillier, P. (1999). Long-term effects of mineral amendments on soil fauna and humus in an acid beech forest floor. <i>Forest Ecology and Management</i> 118, 245-252.</p> <p>Boxman, A.W., van Dijk, H.F.G., van der Ven, P.J.M. & Roelofs, J.G.M. 1995. Effects of reduced nitrogen and sulphur inputs to two coniferous ecosystems in The Netherlands. Proc. Int. Symp.on Ecosystem Manipulations, Windermere UK.</p> <p>Gärdenfors, U., Waldén, H.W. and Wäreborn, I. (1995). Effects of soil acidification on forest land snails. <i>Ecological Bulletins</i> 44, 259-270.</p>
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Method	Ectomycorrhizal Fungi (ECM)
Basis of the approach	<p>Many plant species benefit from symbioses with mycorrhizal fungi, which enhance nutrient exploration and uptake and protect roots from pathogens and drought. In ectomycorrhizal (ECM) plants the fungal hyphae enclose the plant root as a fungal mantle and penetrate between the epidermal and cortical cells of the root axis to form the 'Hartig net'. The ECM fungi that produce large above-ground fruitbodies are usually associated with trees. N deposition can affect fruit body formation, the production and distribution of the extra radical mycelium in the soil and the formation of ECM (Wallenda & Kottke 1998).</p> <p>Data from long-term N deposition studies have shown loss of species diversity both above and below-ground. 'Generalist' species, forming a symbiosis with a wide range of tree species are less affected than 'specialist' species. The negative effects on ECM reflect high concentrations of N in the soil.</p>
Previous experience	<p>Observations of the effects of an N manipulation experiment at CEH Edinburgh have shown varying effects depending on whether the N supplied as NH₄NO₃ is also supplied with sulphuric acid. In addition the responses have changed as the stand has aged so that the initial restriction of the ECM species <i>Lactarius rufus</i> and domination by <i>Tylospora fibrillose</i> has become less obvious as the stand has aged (Ingleby pers comm.).</p> <p>Ritter (1990) observed that ECM fruit bodies associated with <i>Pinus sylvestris</i> ceased to occur within a certain distance of a pig farm, i.e. they only occurred at lower levels of N deposition. Lilleskov & Fahey (1996) measured an N gradient around a fertilizer factory in Alaska and found that <i>Laccaria laccata</i>, <i>L. bicolor</i>, <i>Lactarius theiogalus</i> and <i>Paxillus involutris</i> were to be found all along this gradient whereas <i>Cortinarius spp</i> and <i>Russulas</i> declined drastically in both abundance and diversity with increasing N deposition.</p>
Suitability to indicate atmospheric concentrations	Insufficient evidence
Suitability to indicate atmospheric deposition	Some evidence with scope for development
Suitability to indicate environmental impacts of N	Some evidence with scope for development. By definition the method assesses a biological response to nitrogen.
Sensitivity to other factors	The presence of ECM fungi is affected by temperature and moisture levels and site history.
Time constant	The method probably reflects a long term response (several years to decades).
Limitations to applicability	The presence of ECM fungi species depends on the presence of a suitable host plant species. Because of seasonal and annual variations in fruiting, sites must be visited regularly to check for ECM fruiting bodies.
Expertise in field	The method requires skilled personnel to correctly identify fungal fruiting bodies. However training could be provided in fungal identification.
Expertise in laboratory	Further fungal identification may be necessary in the laboratory requiring specialist skills.
Cost	Insufficient information available
References	<p>Lilleskov, E.A. & Fahey, T.J. 1996. Patterns of ectomycorrhizal diversity over an atmospheric nitrogen deposition gradient near Kenai, Alaska. In: Szaro T.M., Bruns, T.D. eds. <i>Abstracts of the First International Conference on Mycorrhizae</i>. Berkely CA, USA.</p> <p>Ritter, G. 1990. Zur Wirkung von Stickstoffeintragen auf das Feinwurzelsystem und Mykorrhizabildung in Kiefernbeständen. <i>Beiträge für die Forstwirtschaft</i> 4, 162-166.</p> <p>Wallenda, T. & Kottke, I. 1998. Nitrogen deposition and ectomycorrhizas. <i>New Phytol.</i> 139, 169-188.</p>

Method	Lichen Diversity Value (LDV) European method
Basis of the approach	<p>Epiphytic lichen diversity may be impaired by air pollution and environmental stress. A combination of lichen diversity and frequency on selected trees is therefore used as a measure of environmental quality. In order to avoid bias over quadrat location, a prescribed method of selecting trees and of sampling trunks is provided which takes into account directional effects on each tree. The resulting LDV value is used to define different zones of environmental quality. All species are recorded apart from some that are difficult to identify and/or are easily overlooked. Multivariate analyses of the sampled data together with physicochemical and other data can be used to interpret environmental gradients, to identify indicator species, and to detect changes in relation to aspect.</p> <p>Lichen diversity and frequency is assessed on trees of a single species or bark type distributed at intersections of a geographical grid across the area being surveyed. Up to 12 trees at each grid intersection are sampled using 4 quadrat segments comprising 5 contiguous 0.1 m x 0.1 m quadrat squares placed at 1.50 m height on the tree trunk at 4 cardinal points (North, South, East, West). All species present within the 0.1 m x 0.1 m quadrats are recorded and frequency is assessed for each aspect to determine total diversity per trunk and diversity with respect to aspect.</p> <p>This method was compiled by a panel of experts to allow comparison of results over a wide geographic region.</p>
Previous experience	<p>This method has now been tested in urban and rural sites in Britain and also in rural sites in Italy. Preliminary investigations show that it is difficult to fulfil the sampling criteria in urban and rural sites where tree species, structure and exposure are variable.</p> <p>In a survey of London parks 5 <i>Fraxinus</i> trees in 6 sites showed that mean lichen diversity per site increased with distance from the centre of London. No correlations with N concentration or deposition or directional analysis were carried out (Davies <i>et al.</i>, 2002).</p> <p>In one large woodland SSSI (220 ha, Burnham Beeches, UK) directional quadrats placed on 17 <i>Quercus</i> trees showed great dissimilarity of lichen communities on oak with greater similarity with location than with aspect. Areas of wood pasture were associated with acidophyte lichen species and low bark pH. More open areas were characterised by mixture of acidophytes and nitrophytes and higher bark pH. (Purvis <i>et al.</i> 2002).</p> <p>Brunialti <i>et al</i> (2002) compared methods of sampling trees and quadrats and demonstrated that this method avoided clustering effects, and provided directional information.</p> <p>In the present study (Appendix I), 5 trunks of <i>Pinus sylvatica</i> and <i>Picea</i> spp were selected in 6 sites along a gradient from a point source of ammonia. Lichen decreased with moderate NH₃ concentrations, but increased with higher levels, due to the replacement of acidophyte species with nitrophyte species.</p>
Suitability to indicate atmospheric concentrations	Not well suited because of the complex relationships with atmospheric N concentrations.
Suitability to indicate atmospheric deposition	Not well suited because of the complex relationships with atmospheric N deposition. Recent work using directional quadrats in an area where ammonia concentration was monitored suggests that in close proximity to the source of NH ₃ there is directional influence due to higher NH ₃ deposition on the source side of the trees.
Suitability to indicate impacts of N	By definition changes in lichen biodiversity represents an impact of N. However, more extensive survey work and further analysis of lichen data are required in relation to atmospheric nitrogen to define appropriate nitrophyte and acidophyte indicator species within this system.
Sensitivity to other factors	Variation in ecological conditions of light, bark structure and chemistry. Presence of other pollutants. Tree age/girth may also be a factor where trees carry a relict lichen flora from former conditions or else have undergone changes to bark chemistry.

Time constant	This method can be used to assess distribution of zones around a point source or for long-term recording of changes over time on marked trees. Lichen biodiversity on tree trunks represents a consequence of the previous pollution climate over several decades. A 3-5 time year period between surveys is recommended.
Limitations to applicability	The method is only applicable where sufficient number of trees of the same species and fulfilling stringent criteria are available for sampling.
Expertise in field	Specialist/trained staff are required for sampling, and for data interpretation.
Expertise in laboratory	Limited chemical analysis of specimens is required for the identification of species & in analysis / interpretation of data
Cost	Setting up of a grid will depend on area to be sampled. Once the grid is established, it takes c. 2 days of external specialist time to locate suitable trees and record lichens on c. 5 trees. A total cost needs to account for travel and data analysis time of external specialists.
References	<p>Asta.J., Erhardt, E., Ferretti, M., Fornasier, F.,Kirschbaum, U., Nimis, P.L., Purvis, O.W., Pitintso, S., Scheidegger, C., van Haluwyn, C, and Wirth, V. (2002) Mapping Lichen Diversity as an indicator of environmental quality. . In <i>Monitoring with Lichens – Monitoring Lichens</i>. Eds Nimis. PL, Scheidegger, C. and Wolseley, P.A. Nato Science Series, Kluwer, Dordrecht. 273-280.</p> <p>Davies, L., James, P.J., Chimonides, J. and Purvis, W. Corticolous lichens in London. In Anon (2002) <i>Effects of NO_x and NH₃ on lichen communities and urban ecosystems</i>. A pilot study. Report for DEFRA by Imperial College and the Natural History Museum. Pp 8-29 Downloadable in pdf format from the National Air Quality Archive, under Effects of Air Pollution on Natural Ecosystems: http://www.airquality.co.uk/archive/reports/reports.php?action=category&section_id=10</p> <p>Wolseley, P.A., and James, P.W. (2002) Assessing the role of Biological Monitoring using lichens to monitor excessive ammonia deposition in the UK. . In <i>Effects of NO_x and NH₃ on lichen communities and urban ecosystems</i>. Report for DEFRA by Imperial College and the Natural History Museum. Pp 1- 22 pp: 50-69.</p> <p>Purvis, O.W., S. Louwhoff & Chimonides, J. (2002) Burnham Beeches Lichen Monitoring Phase 2. Year 3. Report for Corporation of London. Pp 76.</p> <p>Brunialti, G., Giordani, P. & Ferretti, M. (2002) Evaluation of different sampling strategies in the assessment of the Index of Lichen Diversity. Poster presented at Eurobionet 2002 Conference on Urban Air Pollution, Bioindication and Environmental Awareness, University of Hohenheim.</p> <p>Pitcairn C.E.R., Leith I.D., Sheppard L.J., van Dijk N., Tang Y.S., Wolseley P.A., James P.W. and Sutton M.A. (2004) Field inter-comparison of different bio-indicator methods to assess the impacts of atmospheric nitrogen deposition. Appendix I. (This volume).</p>

Method	Lichen Diversity /Deviation from Naturality
Basis of the approach	Lichen Diversity (LD) is a method developed in Italy to assess deviation from natural lichen diversity on trees in sites within a homogeneous bioclimatic area. LD is calculated using the sum of frequencies on trees of the same species within 10 samples of units 15 cm x 10 cm (30 cm x 50 cm) and the mean calculated from the number of releves. A 5-scale class of deviation from naturality is based on % deviation from the natural LD values in the area and can be correlated with climatic, land management and pollution data. Loppi <i>et al.</i> (2003)
Previous experience	The method was developed and applied in the Tyrrhenian area of Italy using <i>Tilia</i> and deciduous <i>Quercus</i> . LD calculated for each phorophyte and a scale of deviation from naturality was worked out for both phorophyte species (Loppi <i>et al.</i> 2002). Field work in Appendix I suggests that on comparison with a local conservation site a woodland near an ammonia source showed a loss of diversity even for the cleanest location at 300 m from the farm.
Suitability to indicate atmospheric concentrations	Uncertain, but not well suited as the relationship with total lichen diversity is complex.
Suitability to indicate atmospheric deposition	Uncertain, but not well suited as the relationship with total lichen diversity is complex.
Suitability to indicate environmental impacts of N	Changes in lichen diversity by definition represent an indication of nitrogen impacts, but methods based on lichen diversity alone unlikely to be suitable for assessing impacts of N due to the complex relationship to N levels.
Sensitivity to other factors	Climate change and temperature may cause shifts in lichen communities towards nitrogen tolerant species. The lichen flora of tree species depends different characteristic bark types.
Time constant	Lichen biodiversity on tree trunks represents a consequence of the previous pollution climate over several decades. A 3-5 year time period between surveys is recommended.
Limitations to applicability	Requires a sufficient number of trees of a single species in a relatively homogeneous environment.
Expertise in field	Specialist/trained personnel are needed for lichen identification
Expertise in laboratory	Limited chemical analysis of specimens is required for the identification of species. Analysis of data only requires the application of an equation to calculate deviation from naturality.
Cost	<i>Cost per unit of sampling (e.g. one SAC sampled at the edge, centre and an intermediate location at one time). Number of person days of required (Conservation agency + External specialists) in field and laboratory. Indicative cost as of 2003-2004.</i> Not well established at present. Indicatively, it may take c. 2 days of external specialist time to locate suitable trees and record releves on c. 5 trees. A total cost needs to account for travel and data analysis time of external specialists.
References	Brunialti, G., Giordani, P., Isocrono, D. and Loppi, S. (2002) Evaluation of data quality in lichen biomonitoring studies: the Italian experience. <i>Environmental Monitoring and Assessment</i> 75 , 271-280. Loppi, S., Giordani, P., Brunialti, G. Isocrono, D. and Piervittori, R. (2003) A new scale for the interpretation of lichen biodiversity values in the Tyrrhenian side of Italy. <i>Bibliotheca Lichenologica</i> (in press) Nimis, P.L. (1999) Linee-guida per la bioindicazione degli effetti dell'inquinamento tramite la biodiversità dei licheni epifiti, in C. Piccini and S. Salvati (eds.), Proc. Workshop "Biomonitoraggio della qualità dell'aria sul territorio Nazionale", Roma, 26-27 th November 1998, ANPA, Rome, pp 267-277.

Method	Lichen Acidophyte-Nitrophyte Diversity /Dutch method
Basis of the approach	<p>Lichen diversity and cover is assessed on trunks of specified trees and weighted according to selected species that are classified as “nitrophytes” (species preferring nitrogen enriched tree bark”) or “acidophytes” (species preferring naturally acidic clean tree bark). The method was developed in the Netherlands based on large-scale monitoring in conjunction with physicochemical measurements (van Herk 1999, 2002).</p> <p>10 acid -barked trees between 1-2.5 m girth are selected in each site and all lichen species on the trunk are recorded up to 2 m. The abundance of lichens at each site is recorded using a 6 point scale from “only one thallus present” to “present on 6-10 trees with more than 10 cm² per tree”. Species are scored as nitrophytes or acidophytes according to van Herk, and the mean number of nitrophyte and acidophyte species found per tree is expressed as NIW and AIW values.</p>
Previous experience	<p>The method has been widely used in the Netherlands to map and monitor spatial patterns of ammonia (NH₃) pollution. AIW and NIW values have been calculated in c. 50% of sites adjacent to sampling points of ambient NH₃ air concentration. In these sites, a strong correlation of high NH₃ with NIW values was found and a negative correlation with AIW values (van Herk 1999, 2002).</p> <p>This method was tested in the UK in a) Norfolk and b) Devon on <i>Quercus</i> species at sites in the vicinity of NH₃ recording stations using species defined as nitrophytes and acidophytes by van Herk. In the vicinity of chicken sheds at a) NIW values showed a strong correlation with NH₃ deposition except at high concentrations of ammonia suggesting critical level exceedances. In the vicinity of stock rearing sheds at b) there was little correlation of NIW values with distance from the sheds. However there was a strong negative correlation with AIW values. (Wolsey, and James, 2002).</p> <p>For the present report (Appendix I), lichens on available trees of <i>Pinus sylvatica</i>, <i>Picea</i> and <i>Betula</i> were sampled with distance from a poultry farm in Scotland. A close response was found with the NH₃ concentration, with the method proving the most robust and sensitive of several methods tested. A variation of the approach applied to twigs (Wolsely and James 2000, see additional record), was shown to be similarly sensitive, but may indicate present N levels more robustly than the tree trunk lichen flora.</p>
Suitability to indicate atmospheric concentrations	<p>A close correlation between NIW and AIW scores and atmospheric NH₃ concentrations has been demonstrated in the Netherlands and preliminary testing in the UK, with the method able to detect around 1 ug m⁻³ NH₃.</p>
Suitability to indicate atmospheric deposition	<p>The close correlation with NH₃ concentrations also implies a response to total nitrogen deposition. However, it is unclear whether the same response occurs for oxidized nitrogen and wet deposited ammonium as for gaseous NH₃. It is expected that lichen response is most sensitive to NH₃ due to the parallel effect on bark eutrophication and pH. (With some exceptions, NH₃ inputs tend to raise bark pH, while NH₄⁺ inputs tend to reduce bark pH).</p>
Suitability to indicate environmental impacts of N	<p>By definition a change in lichen species composition represents an impact of N deposition. The method is very well suited and a robust response to ammonia levels has been shown.</p>
Sensitivity to other factors	<p>This method appears most suitable for temperate climates. In warm and dusty climates (e.g. Mediterranean) nitrophytes are a natural constituent of the flora. Species lists of nitrophytes and acidophytes therefore require adjustment for different climatic regions. Certain species (e.g. <i>Xanthoria</i>) may develop on acid-barked trees as a result of the ‘alkaline dust effect (Gilbert 1976) in the vicinity of quarries.</p> <p>Further work is required regarding the development of the method in UK conditions.</p>
Time constant	<p>Lichen biodiversity on tree trunks represents a consequence of the previous pollution climate over several decades. The twig-based modification is anticipated to reflect conditions over a much shorter period.</p>
Limitations to applicability	<p>The method requires the occurrence of suitable tree species with naturally acidic bark (e.g. oak, birch, spruce), with tree trunks of appropriate diameter and age.</p>

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	While most work in the Netherlands has used hedgerow trees, with good light availability on trunks, the recent UK work has shown that the method can also work in transects through woodland, even where much lower light levels occur.
Expertise in field	Specialist/trained personnel are needed for identification. While the method provides the most robust and sensitive lichen analysis response to nitrogen, it is also one of the most complex in terms of scoring and data analysis.
Expertise in laboratory	Basic chemical tests for species identification may be necessary, and there is specialist analysis required to calculate the NIW and AIW scores.
Cost	<i>Cost per unit of sampling (e.g. one SAC sampled at the edge, centre and an intermediate location at one time). Number of person days of required (Conservation agency + External specialists) in field and laboratory. Indicative cost as of 2003-2004.</i> The work needs to be conducted by external specialists. Location of sites in the vicinity needs 1 day, and sampling of 10 trees per site needs 1.5 days. The total cost also needs to account for travel and data analysis time of external specialists.
References	Van Herk, C.M. (1999) Mapping of ammonia pollution with epiphytic lichens in the Netherlands. <i>Lichenologist</i> 31: 9-20 Van Herk, C.M. (2002) Epiphytes on wayside trees as an indicator of eutrophication in the Netherlands. In <i>Monitoring with Lichens – Monitoring Lichens</i> . Eds Nimis, P.L., Scheidegger, C. and Wolseley, P.A. Nato Science Series, Kluwer, Dordrecht. 285-290. Wolseley, P.A., and James, P.W. (2002) Assessing the role of Biological Monitoring using lichens to monitor excessive ammonia deposition in the UK. . In <i>Effects of NOx and NH3 on lichen communities and urban ecosystems</i> . Report for DEFRA by Imperial College and the Natural History Museum. Pp 1-22 pp: 50-69. Pitcairn C.E.R., Leith I.D., Sheppard L.J., van Dijk N., Tang Y.S., Wolseley P.A., James P.W. and Sutton M.A. (2004) Field inter-comparison of different bio-indicator methods to assess the impacts of atmospheric nitrogen deposition. Appendix I. (This volume).

Method	Lichen Diversity /Verein Deutscher Ingenieure (VDI) method
Basis of the approach	<p>The VDI approach is a standardized German method that was devised to assess changes in lichen communities over wide geographical areas in order to assess the influence of air pollution in Central Europe. The method is based on the frequency of occurrence of selected widespread lichen species within a unit area on selected tree trunks.</p> <p>The VDI method is based on establishing a grid over the area to be surveyed, whose units would vary according to the availability of suitable tree species and the size of the area to be surveyed. 3-6 trees conforming to established criteria are selected in each grid square for recording lichens within a grid of 0.1 m x 0.1 m units measuring 0.2 m x 0.5 m, placed at 1.50 m on the tree trunk where lichens are most frequent. The frequency of selected species is assessed and the air quality value (LGW) expressed as the average of the total sum of frequencies for each grid sample.</p> <p>This highly selective approach has been widely used for producing maps of polluted zones around point sources and in urban areas (VDI, 1995). It has since been replaced by the European method in order to allow further analysis of whole data sets and to detect changes in the lichen flora.</p>
Previous experience	<p>This method has been successfully applied to assess the impacts of SO₂ over large areas where industrial and domestic burning of fossil fuels has occurred in Germany and a similar method of using an Index of Atmospheric Purity has been applied successfully in Italy, where a weighting for toxiphoby was applied (Kirschbaum, 1995,1996, 1998).</p> <p>Loppi <i>et al.</i> (1996) used this method in central Italy on lichen communities of <i>Quercus</i> species in agricultural and protected areas where bark pH and total nitrogen content were determined. They showed that the distribution of nitrophytes (identified according to Wirth and van Dobben) did not correspond to bark pH or %nitrogen. Loppi <i>et al.</i> (2000) investigated the distribution of nitrophytes in the vicinity of limestone and sandstone quarries where results showed a correlation with dust that was independent of its base status.</p> <p>Testing of this method at in the vicinity of a poultry farm in Scotland (Appendix I) showed that this method provided very similar results to the European Lichen Diversity Values (LDV) system. An initial decrease with increasing NH₃, was followed by an increase in lichen diversity by both methods, as nitrophyte species replaced acidophytes.</p>
Suitability to indicate atmospheric concentrations	Not well suited because of the complex relationships with atmospheric N concentrations.
Suitability to indicate atmospheric deposition	Not well suited because of the complex relationships with atmospheric N deposition.
Suitability to indicate environmental impacts of N	By definition changes in lichen biodiversity represent an impact of N. However, the method is not well suited to monitoring N impacts, as diversity is influenced by the presence of both nitrophyte and acidophyte species.
Sensitivity to other factors	There are many changes that are occurring in urban areas following the fall in SO ₂ levels over the past two decades, and the data provided from frequency of selected species does not allow multivariate analysis to provide a basis for assessing ongoing environmental changes.
Time constant	Lichen diversity on tree trunks represents the accumulated effect of pollution climate over the past few decades. Under conditions of improving air quality, it may take many years for recovery. Lichen diversity measurements on twigs represent changes over a shorter period.
Limitations to applicability	Only applicable where sufficient number of trees fulfilling sampling criteria.
Expertise in field	Requires staff trained to recognise a few selected lichen species.
Expertise in laboratory	Need for specialist processing of samples or data, in order to calculate results. Trained personnel. Trained personnel are not required as equation applied to quantified results.

Cost	<p><i>Cost per unit of sampling (e.g. one SAC sampled at the edge, centre and an intermediate location at one time). Number of person days of required (Conservation agency + External specialists) in field and laboratory. Indicative cost as of 2003-2004.</i></p> <p>Conservation agency staff may be trained to conduct the basic level of identification necessary. Locating sites in the vicinity needs 1 day, and sampling of 10 trees per site needs 1.5 days. The total cost also needs to account for travel and data analysis time.</p>
References	<p>VDI (1995) Messung von Immissionswirkungen: Ermittlung und Beurteilung photoxischer Wirkungen von Immission mit Flechten – Flechtenkartierung zur Ermittlung des Luftgütwertes (LGW), <i>VDI – Richtlinie 3799</i>, Blatt 1, Berlin.</p> <p>Dilg, C. (1998) Epiphytische Moose und Flechten als Bioindikatoren der Luftqualität im Stadtgebiet von Bonn, <i>Limprichtia - Zeitschrift zur Moosforschung in Deutschland</i> 11, 1-94.</p> <p>Kirschbaum, U. (1995) Immissionsbezogene Flechtenkartierung von Hessen nach der neuen Richtlinie VDI 3799 Blatt 1, <i>Staub - Reinhaltung der Luft</i> 55, 305-309.</p> <p>Kirschbaum, U. and Hanewald, K. (1998) Immissionsbezogene Flechtenkartierung in hessischen Dauerbeobachtungsflächen, <i>Journal of Applied Botany</i> 72, 212-227.</p> <p>Kirschbaum, U. and Windisch, U. (1995) Beurteilung der lufthygienischen Situation Hessens mittels epiphytischer Flechten, <i>Schriftenreihe der Hessischen Landesanstalt für Umwelt</i>, Wiesbaden.</p> <p>Kirschbaum, U., Marx, A., and Schiek, J.E. (1996) Beurteilung der lufthygienischen Situation Giessens und Wetzlars mittels epiphytischer Flechten (1995), <i>Angewandte Botanik</i> 70, 78-96.</p> <p>Nobel, W., Worm, R., Schritz, C., Beron, J., and Nitschke, C. (1999) Flechtenkartierung nach VDI 3799: Methodische Modifikationen und Grundlage für eine ökologische Stadtplanung, <i>Verh. Ges. Ökologie</i> 29: 571-577.</p> <p>Werner, A. (1993) <i>Aktives Biomonitoring mit der Flechte Hypogymnia physodes zur Ermittlung der Luftqualität in Hannover</i>, J. Cramer, Berlin, Stuttgart.</p> <p>Windisch, U. (1999) <i>Evaluierung der Bioindikationsverfahren mit Flechten zur Bestimmung der Luftgüte anhand landesweiter Erhebungen in Hessen und Bayern</i>. J. Cramer, Lehre.</p> <p>Loppi, S/ De Dominicis, V 1996: Effects of agriculture on epiphytic lichen vegetation in central Italy. - <i>Israel Journal of Plant Sciences</i> 44: 297-307.</p> <p>Loppi, S. & Pirintsos, S. (2000) Effect of dust ion epiphytic lichen vegetation in the Mediterranean area (Italy and Greece) <i>Israel Journal of Plant Sciences</i>. 48: 91-95</p>

Method	Lichen Diversity /Lichens on twigs method
Basis of the approach	<p>Twigs provide a new substrate every year and colonising lichen communities are strongly affected by existing climatic and atmospheric conditions as well as by availability of propagules. Data on lichen diversity from a standard sampling procedure along woodland and or hedgerow margins allows a comparison of lichen communities in the vicinity of a range of environmental conditions, particularly those associated with agricultural conditions. The procedure is repeatable allowing an assessment of changes over time.</p> <p>Twigs (5-10) of exposed trees are randomly selected along a boundary (woodland, hedgerow or parkland) where twigs are accessible. Lichens recorded on annual increments, or on 1-5 years and 5+ years, or presence on whole twig up to c. 10 yrs. Mean diversity for each site calculated and PCO used to identify shifts in lichen communities and their association with environmental conditions (Wolseley et al, 1999, Wolseley, 2002a, 2000b). This method simplified to produce an easy to use key to lichens on twigs (Wolseley <i>et al.</i> 2002) combined with survey methodology and data collection on a website www.nhm.ac.uk/botany/lichen/twig.</p> <p>The twig method may be used with the Nitrophyte – Acidophyte species lists van Herk (1999) (see Dutch Method) to provide a robust indication of atmospheric ammonia and nitrogen deposition.</p>
Previous experience	<p>Preliminary investigation of annual increments of <i>Quercus petraea</i> twigs along boundaries of an SSSI exposed to different land management practices showed that colonising lichen species and communities varied with surrounding land management. The characteristic acidophyte communities of Usneion and Pseudevernon of woodland glades and moorland were replaced by species of the Xanthorion (Wolseley and Pryor, 1999).</p> <p>In a further project, <i>Fraxinus</i> twigs were sampled along a transect across Pembrokeshire from a point source from oil refineries. The mean frequency of lichen species on twigs at each site showed a positive correlation with distance from source and prevailing wind direction (Purvis <i>et al.</i> 1998).</p> <p>In the vicinity of point sources of ammonia in Norfolk and Devon twigs of <i>Quercus</i> were sampled at sites at known distances from the point source. In both sites twig flora showed a better correlation with distance from source than the flora of the trunks, which showed little overall change with distance from source (Wolseley and James, 2002). These results were improved by weighting nitrophyte (NIW) and acidophyte (AIW) species.</p> <p>Lichen communities on twigs of <i>Betula</i> species at Earlston and Gordon moss were sampled to assess changes with distance from source and NH₃ deposition near a poultry farm in Scotland, scoring for Nitrophyte and Acidophyte species (Appendix I). The complete disappearance of acidophyte species occurred with lower levels of NH₃ for twigs than for trunks, suggesting that the twig method may be more sensitive.</p>
Suitability to indicate atmospheric concentrations	<p>There is currently a shortage of data, however, the preliminary results suggest that a good relationship with NH₃ concentrations exists between the NIW and AIW species. With further data, this method may prove to be the most robust lichen diversity method to indicate NH₃ concentrations. There is a lack of data on the relationship to oxidized nitrogen (NO_x and HNO₃) concentrations.</p>
Suitability to indicate atmospheric deposition	<p>The close correlation with NH₃ concentration suggests a parallel response to N deposition. However, it is expected that oxidized nitrogen and wet deposited ammonium may have different effects to NH₃, since these do not increase bark pH.</p>
Suitability to indicate environmental impacts of N	<p>By definition the method reflects an ecological impact of atmospheric nitrogen. It is also highly sensitive to very levels of NH₃ concentration (e.g. 1 ug m⁻³, Appendix I).</p>
Sensitivity to other factors	<p>Variation in available tree species may affect results through variation in the natural pH of bark and bark surface structure. Environmental conditions especially shading may affect the results obtained, although no shading effect on</p>

	NIW-AIW was detected in the present study by a poultry farm in Scotland.
Time constant	Lichen biodiversity on twigs represents a much shorter timescale of pollution climate than trunks. More data are necessary, but twig diversity may represent pollution climate over periods as short as 3-5 years.
Limitations to applicability	Currently the method is only applicable in areas where native trees are a component of the landscape. Initial testing has focused on Oak, but the recent work has shown that the method can also be applied to birch and spruce. The method is less applicable for trees species with higher bark pH.
Expertise in field	Requires a specialist to collect data.
Expertise in laboratory	Some laboratory work is required in order to identify sterile or small samples.
Cost	<p><i>Cost per unit of sampling (e.g. one SAC sampled at the edge, centre and an intermediate location at one time). Number of person days of required (Conservation agency + External specialists) in field and laboratory. Indicative cost as of 2003-2004.</i></p> <p>Selection and laying out of random samples for each site and sampling of 10 twigs requires 1-2 days of specialist time depending on species diversity on twigs. The total cost also needs to account for travel and data analysis time of specialists.</p>
References	<p>Wolseley, P.A. (2002a) Using lichens on twigs to assess changes in ambient atmospheric conditions. In <i>Monitoring with Lichens – Monitoring Lichens</i>. Eds Nimis, P.L., Scheidegger, C. and Wolseley, P.A. Nato Science Series, Kluwer, Dordrecht. 291-294.</p> <p>Wolseley, P.A. and Pryor, K.V. (1999) The potential of epiphytic twig communities on <i>Quercus petraea</i> in a Welsh woodland site (Tycanol) for evaluating environmental changes. <i>Lichenologist</i> 31: 41-61.</p> <p>Purvis, O.W., Wolseley, P.A., Reed, M.E., Wilson, P.J. and James, P.W. (1998) <i>Monitoring lichen communities as indicators of air quality in Pembrokeshire</i>. Report to Texaco Ltd, Gulf Oil (Great Britain) Ltd and Elf Oil (UK).</p> <p>Wolseley, P.A., and James, P.W. (2002) Assessing the role of Biological Monitoring using lichens to monitor excessive ammonia deposition in the UK. . In: Anon. <i>Effects of NO_x and NH₃ on lichen communities and urban ecosystems</i>. Report for DEFRA by Imperial College and the Natural History Museum. Pp 1-22 pp: 50-69. http://www.airquality.co.uk/archive/reports/reports.php?action=category&section_id=10</p> <p>Wolseley, P., James, P. and Alexander, D. (2002b) A key to lichens on Twigs. Field Studies Council. Also on www.nhm.ac.uk/botany/lichen/twig</p> <p>Wolseley, James and Sutton (in prep.) Comparison of methods for detecting changes in lichen communities in areas of increased atmospheric nitrogen.</p> <p>Pitcairn C.E.R., Leith I.D., Sheppard L.J., van Dijk N., Tang Y.S., Wolseley P.A., James P.W. and Sutton M.A. (2004) Field inter-comparison of different bio-indicator methods to assess the impacts of atmospheric nitrogen deposition. Appendix I. (This volume).</p>

Method	Lichen Diversity /French method (Lallement)
Basis of the approach	<p>This method was developed in France to provide a rapid assessment of air pollution particularly nitrogen over a large geographical area using 14 easily recognisable macro-lichen species associated with well defined phytosociological communities selected from the van Haluwyn and Lerond scale (1988). Land use in each unit was used as a basis for assessing nitrogen levels.</p> <p>The area to be surveyed is plotted using a grid 0.834 km². Trees (number not specified but in van Haluwyn et al it is c. 10) with a diameter >0.3 m are selected (preferably oak) in the centre of each plot as far as possible and selected lichen species recorded on the trunk between 0.8-2 m above ground. Lichen species presence is used to define 5 pollution zones ranked from zone 1 coinciding with zone D in van Haluwyn and Lerond with presence of <i>Diploicia canescens</i> and <i>Xanthoria parietina</i> at high frequency, to zone 5 corresponding to zone F in van Haluwyn and Lerond distinguished by presence of <i>Flavoparmelia caperata</i>, <i>Melanelia glabratula</i> and absence of nitrophilous species of <i>Physcia</i>, <i>Xanthoria</i> and <i>Diploicia</i>. Zones are mapped and land management and topography recorded. Zone 1 coincides with high pigsty density and maize production and zone 5 coincides with protected areas (Giraudeau <i>et al.</i>, 1997)</p>
Previous experience	<p>This method has been used in a number of oceanic sites in France with similar lichen communities and zones mapped across urban and rural areas (Lallement <i>et al.</i>, 1999). The method was tested across a woodland in Scotland adjacent to a poultry farm (Appendix I). In this context a low resolution of the method was found, with all sites scoring either 2 or 3. The method is therefore quick, but of rather low sensitivity and resolution.</p>
Suitability to indicate atmospheric concentrations	<p>The method responds to NH₃ concentrations, but has a low sensitivity to indicate concentrations.</p>
Suitability to indicate atmospheric deposition	<p>Not known. Results have mostly so far been tested against land use rather than against N deposition.</p>
Suitability to indicate environmental impacts of N	<p>By definition the method records an impact of N, however, it is currently rather uncertain regarding its implementation in the UK (where different key species occur).</p>
Sensitivity to other factors	<p>The method has not been tested against other climatic factors.</p>
Time constant	<p>The method scores lichens on trunks and therefore reflects changes in pollution climate over the previous several decades.</p>
Limitations to applicability	<p>Surveys with this method have so far focused on areas with oceanic vegetation where trees are a component of the landscape. Only selected species used, so that the method cannot be used to assess changes in other species or in total diversity. Not useful to assess changes in sites of nature conservation interest.</p>
Expertise in field	<p>Easy to train observers to identify selected species</p>
Expertise in laboratory	<p>No laboratory work required.</p>
Cost	<p><i>Cost per unit of sampling (e.g. one SAC sampled at the edge, centre and an intermediate location at one time). Number of person days of required (Conservation agency + External specialists) in field and laboratory. Indicative cost as of 2003-2004.</i></p> <p>Preliminary identification of area and grid sampling units 2-3 days. Sampling of 10 trees per unit area for selected species – approx half a day.</p>
References	<p>Giraudeau P. and Lallement, R. (1997) Etude de l'impact des émissions ammoniacales agricoles sur la qualité atmosphériques. DDASS, Nantes Van Haluwyn, C. and Lerond, M. (1988) Lichénosociologie et qualité de l'air: protocole opératoire et limites. <i>Cryptogamie, Bryologie et Lichénologie</i> 9: 313-336. Lallement, R, Joslain H. Houssay, I. and Cyprien A-L.(1999) The use of lichens for estimating air pollution in Western France. Unpublished report, University of Nantes, France.</p>

Method	Lichen Diversity /Photographic method
Basis of the approach	Lichen growth and vitality may be influenced by environmental stress within a short time frame (e.g. within one growing season). Permanent quadrats are established using stainless steel screws to locate a quadrat frame with scale and colour reference bar. Ladders may be used to locate samples which are difficult to reach and where lichens are rare owing to stemflow / nutrient effects. Digital photographs are taken of the quadrat and species recorded in quadrat and on the whole tree. Rates of lichen growth, health and changes in assemblage composition are assessed at typically yearly intervals. Using image analysis software changes in area of thallus can be calculated and expressed as a percentage growth or loss of thallus area, which may be correlated with pollutants and other environmental parameters (Purvis <i>et al.</i> , 2002). Images can be stored for subsequent investigation.
Previous experience	<p>Between 1986-1990 monitoring of species of <i>Lobaria</i> across conservation sites in the vicinity of atmospheric recording stations across Britain showed a characteristic pattern of changes associated with pollution in those areas with high levels of acid deposition (Looney <i>et al.</i> 1990, Wolseley <i>et al.</i> 1990). However, where acid deposition was not high there was considerable variation in response of specimens to local environmental conditions (Wolseley <i>et al.</i> 2001). Growth rate responses are expected to be correlated to atmospheric N supply, and provide the basis for changes in species composition, such as measured with the Dutch and Twig lichen diversity methods for nitrogen biomonitoring. The photographic method and measurement of growth rate changes is therefore expected to be a more sensitive and faster responding parameter than the methods based on presence of characteristic species. By examining changes in time, the method provides a technique to look at changes in air pollution with time.</p> <p>The application of this method at Burnham Beeches led to analysis of changes in area cover of <i>Parmelia</i> spp. in relation to various physicochemical data in order to interpret which factors were responsible for the changing lichen floras at the site in the vicinity of London (Purvis <i>et al.</i> 2002). Subsequent analysis of specimens showed an accumulation of nitrogen over time in specimens of <i>Parmelia sulcata</i> which were colonised by alien algae and <i>Physcia</i>, a 'nitrophytic' species (Purvis <i>et al.</i> 2003).</p> <p>Long term monitoring of (saxicolous) lichens growing on rocks on the Sarsen stones at Fyfield Down in Wiltshire was set up to evaluate changes in animal stocking rates at a conservation site where stock numbers were increased. No digital analysis was undertaken, but this is possible at a later date (O'Dare & Coppins, 1994).</p>
Suitability to indicate atmospheric concentrations	The approach is expected to be highly sensitive to NH ₃ concentrations, as well as possibly NO _x , although the latter is less certain. At present, however, the quantitative relationships are not well established, due to the range of other factors affecting growth rates of individual lichens.
Suitability to indicate atmospheric deposition	An association between growth of <i>Parmelia sulcata</i> , traffic levels, regional NO ₂ levels, black smoke emissions, sticky pad readings (but not SO ₂ levels) was identified. However, the method cannot be used on its own to indicate deposition without additional studies (e.g. chemical analysis).
Suitability to indicate environmental impacts of N	By definition the method monitors an impact of N on ecosystem functioning. It provides detailed information on the dynamics of species/individuals within a permanent quadrat. Suitable for monitoring local conditions within an area of conservation interest and to compare growth and/or loss of rare species. However, the method does not monitor status of the local population unless these are quantified outside the quadrat. The method is also subject to local changes in environmental conditions independent of N deposition.
Sensitivity to other factors	Permanent quadrats are subject to tree loss and to changes other than impacts of nitrogen. Large numbers of quadrats would circumvent the loss of samples.
Time constant	The approach has the shortest time constant of the lichen diversity methods, since changes in growth rate precede changes in species composition. For fast growing species, change may in principle be detected within a year. In practice, there is no

	standard time interval between taking photographs, as this depends on the species' growth rate and habitat. Once a year is usually adequate in disturbed urban habitats and highly enriched habitats typified by a high turn over of species.
Limitations to applicability	Applicable to monitoring of lichen communities on a variety of substrata including epiphytic (tree bark growing), saxicolous (bare rock growing) and terricolous (bare soil growing) lichens. At present the limitations are in assessing nitrogen deposition in local areas as a reference to further establish the method. Such quadrat monitoring is most more applicable to local scale temporal changes and is therefore not suitable for indicating N concentrations where recording of data is on a large scale.
Expertise in field	Specialist staff are required to identify lichens and situations that should be monitored. Appropriate equipment for standardized digital photography is also required.
Expertise in laboratory	Image analysis software required to calculate growth rates.
Cost	<i>Cost per unit of sampling (e.g. one SAC sampled at the edge, centre and an intermediate location at one time). Number of person days of required (Conservation agency + External specialists) in field and laboratory. Indicative cost as of 2003-2004.</i> External specialist time needed for quadrat location and setting up: 6 man days for 21 quadrats e.g. Burnham), for quadrat recording and photography: 4 man days for 21 quadrats, and for analysis of digital images - (7 man days for 21 images). Time taken will vary with numbers of species present.
References	Purvis, O.W., Chimonides, J., Din, V. Erotokritou, L. Jeffries, T. and Jones, G.C., Louwhoff, S., Read, H. and Spiro, B. (2003) Which factors are responsible for the changing lichen floras of London? <i>Science of the Total Environment</i> 310 , 179-189. Purvis, O.W., Erotokritou, L., Wolseley, P.A., Williamson, B. and Read, H. (2002). In <i>Monitoring with Lichens – Monitoring Lichens</i> . Eds Nimis, PL, Scheidegger, C. and Wolseley, P.A. Nato Science Series, Kluwer, Dordrecht. 291-294. Purvis, O.W., Bamber, R.N., Chimonides, J., Din, V. Erotokritou, L. Jeffries, T. and Jones, G. (2001) <i>Burnham beeches Lichen Monitoring phase 2. Year 2</i> . Report for the Corporation of London by the Natural History Museum, 125 pp. Wolseley, P.A. & James, P.W. (2001) Factors affecting changes in species of <i>Lobaria</i> in sites across Britain 1986-1998. <i>Forest Snow and Landscape Research</i> 75 (3), 319-338.

Method	Lichen Diversity /Ellenberg Method
Basis of the approach	<p>Additional nitrogen supply both leads to a eutrophication of tree bark and changes in bark pH. In particular, enhanced levels of NH₃ have been shown to increase bark pH. The environmental preferences of lichens in relation to these different conditions may be classified into two extremes: “nitrophyte” lichen species prefer high supply of nitrogen and high bark pH, while “acidophyte” lichen species prefer a low supply of nitrogen and the naturally low pH of clean bark.</p> <p>In the Ellenberg approach (as applied by Wirth, 1991 for lichens), the relative preference of lichens as nitrophytes or acidophytes is incorporated in to an empirical scale of 1 to 7. The most nitrophytic species have the highest scores, while the most acidophytic species are given the lowest scores. Species with intermediate preference are scored in between. The lichen Ellenberg score for the site is calculated simply as the average of the Ellenberg values of the different species present. In more refined approaches, the result may be weighted by the relative frequency of the different species to estimate an “abundance-weighted Ellenberg score.</p> <p>The method may be applied to either tree trunks or twigs (see also twig sampling method), with sampling on a range of tree species with naturally acidic bark (e.g. oak, birch, pine, spruce).</p>
Previous experience	<p>The method has been tested in an inter-comparison of nitrogen biomonitoring methods across a mixed woodland downwind of a poultry farm in Scotland (Appendix I). The advantage of the method is that it is relatively quick and simple to score and that, as far as possible, all lichen species are assigned an Ellenberg score. This means that even in situations with very low lichen diversity, the method can still be applied. The correlation with NH₃ concentrations was better with sampling on twigs than on trunks (Appendix I).</p> <p>The method has the advantage is that it is amenable to simplification to a restricted set of species that can be identified by non-specialists, which is of use to encourage wider public appreciation of lichen monitoring for nitrogen (Appendix I, this report). Application of the simplified method at the showed that it is less sensitive to low NH₃ levels, but provided a clear signal of whether NH₃ concentrations were above or below 8 ug m⁻³, which is the currently established critical level for NH₃.</p>
Suitability to indicate atmospheric concentrations	<p>The method has shown a clear relationship to atmospheric NH₃ concentrations above 1.7 ug m⁻³, but is less sensitive than the most detailed lichen approach (Dutch method/Twig method). The simplified Ellenberg approach is again less sensitive, but more suited to application by non-experts.</p> <p>The extent of relationship to oxidized nitrogen concentrations has yet to be established.</p>
Suitability to indicate atmospheric deposition	<p>The response of the lichens to NH₃ concentrations implies a relationship also exists with nitrogen deposition. However, a differential sensitivity is expected between oxidized nitrogen deposition (HNO₃, NO₂, NO₃⁻) and the different forms of reduced nitrogen deposition (NH₃, NH₄⁺).</p>
Suitability to indicate environmental impacts of N	<p>By definition, the method represents an ecological impact of enhanced nitrogen. The simplified scheme (using only a limited set of easily identifiable species), is well suited to demonstrate effects to stakeholders, but has a lower sensitivity than a survey (by specialists) which scores all species present.</p>
Sensitivity to other factors	<p>The varying bark characteristics of different tree species affects the occurrence of nitrophyte species, so caution is needed in applying the method between tree species. The scores obtained at a site may be expected to be modified under conditions with very high SO₂ levels.</p>
Time constant	<p>The lichen flora of trunks and twigs respond to atmospheric conditions on different time scales. The time constant of response for trunks may be a decade or longer (especially under conditions of improving air quality, with a slow recolonization of previously eutrophicated bark). The time constant of response for twigs is faster and may reflect atmospheric conditions over previous periods as short as 3-5 years.</p>

Limitations to applicability	The method is currently limited to the trunks and twigs of trees with naturally acidic bark.
Expertise in field	Specialists are required to conduct a full survey identifying all lichen species. For the simplified approach, identification by conservation agency staff is possible following training.
Expertise in laboratory	The full identification of species requires some basic chemical tests by specialists. The numerical calculation of Ellenberg scores is very simple.
Cost	<p><i>Cost per unit of sampling (e.g. one SAC sampled at the edge, centre and an intermediate location at one time). Number of person days of required (conservation agency + External specialists) in field and laboratory. Indicative cost as of 2003-2004.</i></p> <p>For the full species approach, two specialist days are required for identification of species and chemical tests, plus one person day for data analysis. Time for travelling to sites needs to be added. Costs may be reduced where several sites should be assessed in parallel.</p> <p>The simple approach would require typically one day in the field by a non-specialist, but substantial further development of this approach is necessary before it can be considered ready for general use.</p>
References	<p>Pitcairn C.E.R., Leith I.D., Sheppard L.J., van Dijk N., Tang Y.S., Wolseley P.A., James P.W. and Sutton M.A. (2004) Field inter-comparison of different bio-indicator methods to assess the impacts of atmospheric nitrogen deposition. Appendix I. (This volume).</p> <p>Wirth V. (1992) Zeigerwerte von Flechten. Chapter 4 in: Zeigerwerte von Pflanzen in Mitteleuropa. Scripta Geobotanica, pp 215-237 (with English summary pp 249-258).</p>