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**Biomonitoring methods for assessing  
the impacts of nitrogen pollution:  
refinement and testing**

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## Executive Summary

- The conservation agencies are required to identify, monitor and protect sites designated for nature conservation under UK and European legislation. The ability to determine the impacts of enhanced N concentrations and N deposition is important for assessing effects on site condition and integrity. Currently, assessment of atmospheric pollution effects on these sites is not part of the common standards monitoring. The use of biomonitoring methods is one approach, which could provide an early warning of sites at risk from N deposition.
- This report describes a two-part field study, which applied bioindicator methods in parallel. Firstly methods were applied to 4 key 'intensive' UK sites with contrasting habitats and atmospheric N concentrations and deposition. Then secondly, bioindicator methods identified from the intensive study were applied at the UK scale (extensive study) at 32 sites with a range of habitat types, NH<sub>3</sub> concentrations and N deposition.
- The bioindicator methods tested at the intensive sites were a) chemical (foliar N content and soluble foliar ammonium concentration using pleurocarpous mosses), b) standardised grass bioindicator (*Deschampsia flexuosa*): effects on biomass and foliar N concentrations, c) Ellenberg N index and d) epiphytic lichen frequency and species composition, including associated measurement of bark pH. The methods selected for application at the UK scale were the chemical analysis of mosses (a) and epiphytic macrolichen frequency and community composition (d).
- The use of conservation and environment agencies' staff to record epiphytic macrolichen frequency and to sample lichens and pleurocarpous mosses for the UK extensive study was successful. The quality of sampling and recording was high and their participation enabled a much more comprehensive study to be undertaken, as well as dissemination of the methodology.

### Ellenberg N Index

- Ellenberg N index was shown to be a useful bioindicator method at the intensive sites for assessing the N status along a known gradient of NH<sub>3</sub> concentration, NO<sub>2</sub> concentration and N deposition.
- At the sites dominated by long-range wet N deposition, the use of Ellenberg N index did not detect significant change in vegetation due to N deposition.
- The application of an acidophyte-nitrophyte index for vascular plants and bryophytes was tested at the intensive sites and could provide a more sensitive measure of N deposition and eutrophication impacts to a target habitat.

### Standardised grass transplants

- The grass, *Deschampsia flexuosa* was found to be a robust bioindicator of NH<sub>3</sub>, NO<sub>2</sub> concentrations and N deposition at sites with a large gradient of atmospheric N with the nitrogen impacting strongly on above ground biomass, foliar N contents and soluble ammonium concentrations. However, *Deschampsia flexuosa* exposed for 3 months at

the sites dominated by wet N deposition did not show any changes in biomass or foliar N concentrations in relation to amount of N deposition.

### **Chemical methods (foliar N content and soluble ammonium concentration)**

- Both foliar N content and soluble ammonium concentration proved to be robust bioindicator methods for the detection of N impacts when using *Deschampsia flexuosa* and the pleurocarpous mosses at sites with a strong gradient in NH<sub>3</sub>, NO<sub>2</sub> concentration and N deposition.
- Overall, the UK extensive study showed a positive but weak correlation between moss foliar N content, soluble ammonium concentrations and NH<sub>3</sub> concentration and N deposition. The low overall relationship indicates that (for the amount of sample replication used here) for sites with diffuse sources of N deposition over a modest range, the foliar N concentration and soluble ammonium concentration of bryophytes may not provide a sufficient signal for spatial comparisons at one time. Increased replication of samples from wider search areas is therefore recommended for studies at such low N deposition levels.
- There was strong interspecies variation in the sampled mosses in response to NH<sub>3</sub> and N deposition. In general the response to NH<sub>3</sub> concentration was greater than that found for N deposition. This could be due to a number of habitat and climatic factors including interactions with regional precipitation differences.

### **Lichens diversity**

- Frequency of lichen indicator species was found to be a robust bioindicator method at both the 4 key intensive sites and at the UK scale. The main restriction on the method is the requirement for the presence of deciduous trees at the sampling site. Comparison of lichens growing on twigs and trunks showed that those on twigs were more sensitive to NH<sub>3</sub> concentration. This is associated with the higher bark pH of twigs.
- Macrolichen frequency was recorded in the UK extensive study, which found nitrophyte lichen species increased on twigs and trunks with increasing atmospheric NH<sub>3</sub> concentration. The loss of acidophyte lichen species was found to occur at lower ammonia concentrations than the subsequent increase in nitrophyte species.
- Comparison of expert and non-expert lichenologists sampling and field identification of epiphytic lichens showed that a simplified recording system using frequency of macrolichens could be used to detect change in site condition resulting from N deposition impacts. At the UK scale, the lichens were found to respond most closely to the NH<sub>3</sub> concentration.

### **Application of the results**

- The results are used to indicate the potential for significant adverse effects at the different UK intensive sites and extensive sites. In many cases the results of different biomonitoring methods confirm a wider picture of effects or no effects at the sites. Hence increased robustness in the application of nitrogen bioindicators and biomonitoring may be obtained by using several approaches simultaneously.
- The concept of robustness may be extended by considering different nitrogen indicators in a “biomonitoring chain” from source to conservation relevant impact: emission, air

concentration, deposition, N accumulation, physiological response, injury, growth response, species composition change (most sensitive species), species composition change (designated species for conservation).

- While it is difficult to measure all of these stages, selecting the easier methods from along the range of this biomonitoring chain, both increases robustness (multiple methods), and makes the link between source attribution (methods closest to emission) and adverse effect (methods closest to impact on designated species).

## Technical Summary

### Background and structure of the report

1. The conservation agencies are responsible for the identification and protection of designated sites of nature conservation (such as Sites of Special Scientific Interest (SSSIs), Special Areas of Conservation (SACs) and Special Protection Areas (SPAs). An assessment of these designated sites is carried out on a 6-year cycle to monitor the condition of the designated interest feature/features for that specific site, with key attributes being identified and targets set for each feature.
2. Although there is concern about the potential impacts of atmospheric nitrogen (N) deposition on conservation areas with N sensitive plant species, an assessment of N impacts is currently not explicitly included in the Common Standards Monitoring (CSM) of designated sites. An assessment of air pollution impacts, including N, is also required as part of the permitting process for the Pollution Prevention and Control Regulations and to fulfil the obligations placed on competent authorities, such as Scottish Environmental Protection Agency (SEPA), by the Habitats Regulations.
3. A comprehensive review of existing biomonitoring methods for determining the impacts of N deposition on plant species and habitats was produced by Sutton *et al.* (2004a) on behalf of the Joint Nature Conservation Committee (JNCC, Report No: 356). That report reviewed N biomonitoring approaches and identified robust N bioindicator methods, which could be applied by the conservation agencies to designated nature conservation areas to assess potential N impacts. The study also included a field component at an agricultural NH<sub>3</sub> point source, where several N biomonitor techniques were examined in parallel.
4. This report describes the work commissioned by JNCC and Scotland and Northern Ireland Forum for Environmental Research (SNIFFER) to assess rigorously a short-list of specific N biomonitoring methods identified by Sutton *et al.* (2004a) for wider application by the conservation agencies and SEPA and EHS at designated nature conservation sites for pollution impact assessment.
5. The present report is structured in six parts:
  - a) Intensive site study.
    - This study assesses in detail the simplified biomonitoring methods (Ellenberg N index, Lichen diversity and the chemical methods: foliar N and soluble ammonium concentration in mosses) tested in parallel at 4 key sites with contrasting N sources and habitat types.

- b) Extensive UK study.
  - This study evaluates the selected simplified N bioindicator methods identified in the intensive site study at the UK scale. The methods applied were the chemical methods (foliar N content and soluble ammonium concentration) and the lichen diversity (with associated measurement of bark pH). The study also compares expert and non-expert epiphytic lichen diversity identification and sampling quality at a small number of UK extensive study sites.
- c) Synthesis of the tested biomonitoring methods.
  - Synthesis of the intensive and UK scale extensive site biomonitoring method results.
  - Evaluation of the robustness, applicability of the bioindicator methods for use by the conservation and environment agencies.
- d) Appendix I summarizes the development of an improved methodology for the chemical biomonitor method, soluble plant  $\text{NH}_4\text{-N}$  concentration.
- e) Appendix II summarizes a pilot study comparing the effects of  $\text{NH}_3$  along a concentration gradient on the above and below ground biomass and foliar N concentrations of the standardised grass biomonitor (*Lolium multiflorum*).
- f) Appendix III details the lichen diversity sampling protocols and site description data sheets used by the conservation and environment agencies' staff in sampling for the extensive UK study.

#### **Method development and testing at four key intensively measured sites.**

6. The sites were selected for a contrasting range of habitat and atmospheric N deposition and N form.
  - a) **A lowland mixed deciduous woodland.** N source: agricultural  $\text{NH}_3$  point source (poultry farm adjacent to Piddles Wood SSSI, Dorset).
  - b) **A lowland mixed deciduous and conifer woodland.** N source: vehicle emissions ( $\text{NO}_x$ ) from the M74 motorway at a site near Happendon, Lanarkshire, Scotland.
  - c) **An upland and lowland moorland.** N source: wet deposition in precipitation ( $\text{NH}_x$  and  $\text{NO}_y$ ) comparing Auchencorth Moss and Bowbeat Hill, sites in the southern uplands of Scotland.
  - d) **An N manipulation study on blanket bog vegetation.** N sources: dry  $\text{NH}_3$  and the two main N species in precipitation ( $\text{NO}_3^-$  and  $\text{NH}_4^+$ ) at the CEH Edinburgh Whim Moss experimental facility.

#### **Application of the Ellenberg indicator approach at the four intensive sites**

7. Ellenberg devised a comprehensive indicator system for vascular plants of central Europe (Ellenberg 1979; Ellenberg *et al.* 1992) to describe the response of individual species to a range of ecological conditions (light, temperature, continentality, moisture, pH and N). The Ellenberg N index is a robust indicator of enhanced N deposition, which has been used extensively in Europe to indicate vegetation change due to increased atmospheric N deposition.

8. The Ellenberg N index method relies on the preclassification of different species preferences to N availability, which were modified by Hill *et al.* (1999) for British conditions. Although the method is relatively simple, it requires a sound botanical knowledge to identify accurately a wide range of species.
9. In the current study, the Ellenberg N Index was tested at all the intensive study sites, but excluded (due to resource availability) from the N manipulation study at Whim Moss.
10. Ellenberg N Index was particularly useful in assessing the N status along known gradients in N deposition, confirming the strength of the method in indicating enhanced N deposition, and providing an important standard for comparison between sites, and within sites on a spatial and temporal scale. The determination of the Ellenberg N index along a gradient of NH<sub>3</sub> concentration and N deposition at Piddles Wood, and a gradient of NO<sub>2</sub> concentrations and N deposition at Happendon Wood showed changes in vegetation composition, which could be attributed to N deposition.
11. Ellenberg N Index appears to be a weaker predictor of the relative N status of sites dominated by wet deposited N. At the two moorland sites both of naturally low N status, Auchencorth Moss and Bowbeat Hill, but with different atmospheric N inputs, mean Ellenberg N Index did not indicate any N driven change in species composition. At such sites, the presence of mainly stress-tolerant, low N value species and the absence of propagules of high Ellenberg Index plants may restrict changes in the mean Ellenberg N Index.
12. The first attempt to apply an index based on the selection of acidophyte and nitrophyte species (vascular + bryophytes) provided useful information on the eutrophication of the sites. In this approach, previously applied only for lichens, the assessment is based not on the full species list at a site, but only on those species known to favour nitrogen-rich or nitrogen-poor N conditions.
13. The acidophyte-nitrophyte approach for higher plants, bryophytes and lichens has the potential to provide a more sensitive measure of N deposition induced changes, than the Ellenberg approach, since it focuses on the species changes most relevant for each habitat. For example, at Piddles Wood the flora was shown to be dominated by nitrophyte species at NH<sub>3</sub> concentrations greater than 3 µg m<sup>-3</sup>. There is now a need to develop the database of key species of acidophytes and nitrophytes for major habitats, followed by site evaluation in the UK to develop the robustness and scope of this approach.
14. The application of both the Ellenberg and acidophyte-nitrophyte methods by conservation and environment agencies staff requires training in botanical identification. However, while a full botanical survey is necessary to obtain an accurate Ellenberg Index, the simpler index based on the acidophyte/nitrophyte balance may require less training (as fewer species need to be identified). Development of key acidophyte and nitrophyte species for habitat types should facilitate application by conservation officers.

#### **Application of *D. flexuosa* as a standard biomonitor at the intensive sites.**

15. *Lolium multiflorum* has been used extensively as a standardised grass biomonitor in the past and was tested here in relation to N deposition. It can be used to assess N deposition along an exposure gradient with short exposure periods of 20-50 days.

However, in areas with diffuse N inputs from long-range transport (i.e. no strong individual local source), and low N deposition the exposure period required is much longer (60 + days), mainly due to the episodicity of precipitation and wet deposition. The fast growing *Lolium* spp. is therefore unsuitable and a slower growing grass species is required. This study investigated whether *Deschampsia flexuosa* could be used as an alternative to the faster growing *Lolium* spp.

16. The standardised grass transplant system with a reservoir of water worked well in all the different habitats and environmental conditions. The method was relatively cheap to use and does not require specialist equipment (the equipment cost per six plant tray was ~ £25). There would be an analytical cost of ~ £20 per sample for tissue N content and soluble NH<sub>4</sub>-N concentration. The grass N biomonitors only required minimal management over the three months exposure period with a site visit every 10-14 days.
17. The measurements show that *D. flexuosa* could be used effectively as a grass N biomonitor especially at sites with a local point source, such as intensive agricultural livestock units. However, the application of standardised grass N biomonitors at sites with diffuse wet N inputs from long-range pollutant transport and lower N deposition values is not an effective method to monitor N deposition over a short period (0-3 months comparison between Bowbeat Hill and Auchencorth Moss). The germination and propagation of the *D. flexuosa* seed was not as straight forward as for *Lolium* spp. Therefore, further work to improve the germination rate/propagation techniques for this species is required.
18. Significant increases in above ground biomass, tissue N content and soluble NH<sub>4</sub>-N concentration were found with increasing NH<sub>3</sub> concentration and N deposition in *D. flexuosa* plants after 3 months exposure at the intensive agricultural site at Piddles Wood.
19. Contrary to the results adjacent to the poultry unit, the biomass of the *D. flexuosa* grass biomonitors decreased with increasing NO<sub>2</sub>, NH<sub>3</sub> concentrations and N deposition at Happendon Wood, the site adjacent to the M74 motorway. This decrease in biomass suggests that factors other than simple N supply (which would have a positive effect) associated with vehicle emissions have a negative impact on *D. flexuosa* growth (e.g. NO<sub>2</sub> toxicity, particle emissions, road salt etc). There were strong linear (negative) relationships with log NO<sub>2</sub> concentration and log N deposition and tissue N content but not with soluble NH<sub>4</sub>-N concentrations. This would indicate that for sites with high NO<sub>2</sub> concentrations the soluble NH<sub>4</sub>-N concentration method is less influenced by the NO<sub>2</sub> and other transport related pollutants.
20. No effects on grass bioindicator plant biomass and foliar chemistry were found for the sites with contrasting wet N deposition, Auchencorth Moss and Bowbeat Hill. This indicates that an exposure period of 3 months at sites with atmospheric N deposition derived principally from NO<sub>3</sub><sup>-</sup> and NH<sub>4</sub><sup>+</sup> in precipitation is insufficient to detect impacts on grass biomonitors. The interacting effects of altitude, temperature and low N deposition appear to be responsible for the lack of response found. Further work is required to test grass biomonitors at long-range sites over a longer exposure period (6-12 months) to determine if N deposition can be detected in the grass foliage.
21. At the experimental manipulation site (Whim Moss) comparing wet and dry N deposition the mean tissue N content and soluble NH<sub>4</sub>-N concentration were higher but not significantly in the NH<sub>4</sub>-N treatments compared to the NO<sub>3</sub>-N treatments. However, the biomass was greater in the NO<sub>3</sub>-N treatments at both 32 and 64 kg N ha<sup>-1</sup> y<sup>-1</sup> and this is reflected in the higher N inventory. These different results point to the potential for

the grass biomonitor approach to distinguish the impacts of oxidised versus reduced N deposition. All N treatments significantly increased foliar N inventory compared to the control.

22. Comparison of wet and dry  $\text{NH}_x$  treatments (3 month exposure period at Whim Moss) show that at  $10 \text{ kg N ha}^{-1} \text{ y}^{-1}$  inputs (estimated N deposition for 3 month experimental period for  $64 \text{ kg N ha}^{-1} \text{ y}^{-1}$  treatments) the *D. flexuosa* biomass, foliar N concentrations and N inventory were all increased in wet treatment  $\text{NH}_4\text{-N}$  compared to the equivalent dry  $\text{NH}_3$  treatment.

### **Application of tissue and soluble nitrogen determination in mosses as bioindicators at the intensive sites.**

23. Total tissue N content is a widely recognised biomonitor of N impacts in a range of vegetation species. The use of soluble  $\text{NH}_4\text{-N}$  concentration of foliage has been measured in several previous studies, with much more recent application of this method as an N bioindicator (Sutton *et al.* 2004a). In the current study, the method has been compared directly with total tissue N and both have also been compared directly at both the intensive and UK scale extensive sites.
24. There were strong, robust relationships between tissue N content, soluble  $\text{NH}_4\text{-N}$  concentration in the mosses *Eurhynchium praelongum* and *Eurhynchium striatum* with  $\text{NH}_3$  concentration and N deposition at the agricultural  $\text{NH}_3$  source site (Piddles Wood). This indicates that both these chemical methods can be used effectively as bioindicators of N impacts at designated sites with a strong local N point source. There were differences between the two species with *Eurhynchium praelongum* appearing to be N saturated at the high  $\text{NH}_3$  concentrations/N deposition. As both species were found within 5 m of the poultry house this would indicate that the two *Eurhynchium* species have a high tolerance to N.
25. The response to N deposition was much larger for soluble  $\text{NH}_4\text{-N}$  concentration than it was for tissue N content. Overall tissue N content increased by a factor of 2.7 in both *Eurhynchium praelongum* and *Eurhynchium striatum* while the soluble  $\text{NH}_4\text{-N}$  concentration increased by a factor of 20 for *Eurhynchium praelongum* and by a factor of 40 for *Eurhynchium striatum*.
26. Overall, the N content and soluble  $\text{NH}_4\text{-N}$  concentrations in the four moss species decreased with distance from the M74 motorway site (Happendon Wood). There was a relatively poor relationship between tissue N content, soluble  $\text{NH}_4\text{-N}$  concentration and  $\text{NH}_3$  concentration ( $R^2=0.23$  and  $0.43$  respectively). As the  $\text{NH}_3$  concentrations measured were low along the gradient away from the M74 ( $1.27\text{--}0.45 \mu\text{g m}^{-3}$ ), this suggests that other factors (including  $\text{NO}_2$  concentrations) could be influencing N uptake as strong linear relationships were found between soluble  $\text{NH}_4\text{-N}$  concentration and both log  $\text{NO}_2$  concentration and log N deposition. Although, a weak relationship was found between tissue N content of the four moss species and log N deposition ( $R^2=0.26$ ) there was a much stronger relationship with log  $\text{NO}_2$  concentration ( $R^2=0.96$ ). The results show that the two chemical methods differ in their response to the atmospheric pollutants at Happendon Wood. This indicates that the application of individual chemical methods must be tailored to the pollutant at the site and also highlights the need for further research to determine what chemical method is applicable for different atmospheric N pollutants.
27. The results for the sites dominated by long-range transport of wet N deposition, Auchencorth Moss and Bowbeat Hill, suggest that the use of the foliar N bioindicator

methods would only be applicable with long-term monitoring as an ‘early warning’ of N deposition increases.

28. There were strong positive log-linear relationships (in *H. jutlandicum*) between tissue N content (% dry weight) and soluble NH<sub>4</sub>-N concentration and NH<sub>3</sub> concentration along the NH<sub>3</sub> gradient at Whim Moss. The mean monthly NH<sub>3</sub> concentration ranged from 0.5 µg m<sup>-3</sup> to 70 µg m<sup>-3</sup> along the transect. The results show that both chemical methods were able to detect differences in N in *H. jutlandicum* after 2 years exposure to NH<sub>3</sub> concentration. The magnitude of responses was broadly proportional to the log of NH<sub>3</sub> concentration.

#### **In the wet N deposition treatments at Whim Moss:**

29. Differences were found between N forms in the wet N treatments. There was a strong linear-log relationship between tissue N content (% dry weight) and soluble NH<sub>4</sub>-N concentration and wet N deposition of both N forms in *H. jutlandicum*. However, the increases in foliar N per unit N were greater in the NH<sub>4</sub>-N treatments than the NO<sub>3</sub>-N treatments.
30. The tissue N content (% dry weight) increased significantly when compared to ambient N deposition (10 kg N ha<sup>-1</sup> y<sup>-1</sup>) in both forms of N at N deposition of 32 and 64 kg N ha<sup>-1</sup> y<sup>-1</sup>.
31. In contrast, soluble NH<sub>4</sub>-N concentrations were only significantly increased in the NH<sub>4</sub>Cl, 64 kg N ha<sup>-1</sup> y<sup>-1</sup> treatment compared to the control and the other NH<sub>4</sub>-N and NO<sub>3</sub>-N treatments. This result suggests that a critical threshold wet deposition may have been exceeded in this treatment for soluble NH<sub>4</sub>-N concentrations.
32. The comparison between dry NH<sub>3</sub>, wet NO<sub>3</sub>-N and wet NH<sub>4</sub>-N inputs shows that these forms of N input do not all have the same impact on foliar N and soluble NH<sub>4</sub>-N concentrations of mosses. For tissue N the sensitivity appears to be highest for NH<sub>3</sub>, intermediate for wet NH<sub>4</sub>-N and lowest for wet NO<sub>3</sub>-N. This differentiation is similar but even stronger for soluble NH<sub>4</sub>-N in mosses, which was very sensitive to NH<sub>3</sub>, but only responded to high levels of wet N inputs.

#### **Application of lichens diversity methods as N bioindicators at the intensive sites.**

33. Transects were undertaken at Piddles Wood adjacent to a poultry unit with a NH<sub>3</sub> point source, and adjacent to the M74 motorway at Happendon Wood. At other sites (Whim Moss and Auchencorth Moss and Dunslair Hill near Bowbeat Hill) a basic comparison was made due to the limited availability of suitable trees). At these sites all lichen species were investigated on trunks and twigs, allowing “nitrophyte” and “acidophyte” species values to be calculated based on a modification of the van Herk approach. Ellenberg Index values were used as described in Wirth (1992).
34. The results showed that epiphytic lichen communities of twigs are strongly correlated with NH<sub>3</sub> concentrations. Acidophyte lichens on twigs are more sensitive to NH<sub>3</sub> than those on trunks, associated with higher bark pH of twigs than trunks. In addition lichen communities of trunks may carry relict species from previous conditions, allowing assessment of recent changes in NH<sub>3</sub> exposure based on a comparison of acidophyte and nitrophyte communities on twigs and trunks.

35. Lichen on trunks may also cover a wide tree age range and be subject to variation in environmental conditions such as shade. Where the same tree species could be used throughout the transect, as at Piddles Wood, the results showed a good correlation with ammonia concentrations. By contrast, in cases where tree species and habitat homogeneity varied, as across the M74 transect at Happendon Wood, the results were more difficult to interpret. The most consistent results were in the loss of acidophytes on acid-barked tree species at relatively low levels of  $\text{NH}_3$  in all sites suggesting that the effects of ammonia on areas of natural vegetation are more widespread than previously thought. In sites where acidophyte vegetation was naturally dominant, loss of acidophytes was more conspicuous than the appearance of nitrophytes, these being often slow to colonise.
36. The results also demonstrated that in local situations on the same tree species that bark pH was highly correlated with atmospheric  $\text{NH}_3$  concentrations.

### **Comparison of expert and non-expert lichen sampling.**

37. In its original application, the method of van Herk (1999) provided a complex sampling method recording the presence of both macrolichens (foliose and fruticose species) and crustose lichens. While this method was previously shown in the UK to give good results (Sutton *et al.* 2004a), the scoring system was labour intensive and it required advanced lichen identification skills. The results of testing in a range of conditions; parkland at Bush Estate in Midlothian, coastal woodland at Stackpole, Pembrokeshire, an upland site at Pwll Peiran, Ceredigion and an inland oak wood at Yarner Wood in S. Devon demonstrated that the simplified recording system used in this study, based on macrolichen frequency forms a reliable basis to detect responses to increased  $\text{NH}_3$  and N deposition.
38. The increase in nitrophytes on twigs at lower  $\text{NH}_3$  concentrations than their appearance on trunks is consistent with the higher pH on twigs and the results of the extensive survey. There was also less difference in the results from macrolichen and total species sampling on twigs than on trunks where crustose species may be dominant. Following the testing of epiphytic macrolichen indicator species against pollution and environmental data the use of a standardised method and illustrated guide to indicator species would permit their widespread use in the UK.

### **Synthesis of bioindicator and biomonitoring results from the four intensive sites.**

39. The comparison of the methods at Piddles Wood (agricultural  $\text{NH}_3$  point source) show that all the simplified biomonitoring methods tested were found to be robust and could be applied by the conservation agencies at sites where there is a defined point source and a strong gradient of N deposition/concentrations.
40. The results for the other intensive site with a defined source, the M74 motorway at Happendon Wood, showed that Ellenberg index was suitable, the soluble  $\text{NH}_4\text{-N}$  concentration method was robust for mosses and *D. flexuosa*, but the other methods were considered to be suitable but only under specific conditions i.e. larger N concentration gradients.
41. None of the biomonitoring methods tested at the diffuse N source wet N dominated sites (Auchencorth Moss or Bowbeat Hill) were sufficient to show a statistically significant increase with N deposition. While soluble foliar ammonium of bryophytes increased as expected, tissue N actually decreased, possibly due to a lower ratio of dry to wet deposition at the high deposition site (Bowbeat Hill).

42. For higher plants and bryophytes, the Ellenberg N Index and a new modified acidophyte/nitrophyte index for these plants provide measures of current species composition and the extent to which nitrophyte species dominate a site. They confirm the current status of the site within the NVC classification and can show areas where change has already occurred. With comprehensive botanical knowledge, the Ellenberg index is straightforward to apply and has the added benefit of providing a species list for the site. The new acidophyte/nitrophyte index and future refinement of this approach using key species is simpler to apply with limited botanical training, as well as being more sensitive to the key species responses to N.
43. Foliar N chemistry measurements are a more sensitive indication of N exposure than species changes for higher plants and show the potential for change and damage to the 'health' of the habitat. These methods can act as an 'early warning' of potential N impacts to a designated site. These techniques also provide a robust approach, which can be conducted cost-effectively in one-off surveys of spatial differences or as part of long-term monitoring programmes.
44. Use of standardised grass plants as biomonitors of N deposition has the advantage that the effects may be demonstrated visually over the short-term through altered biomass, as well as in foliar N concentrations and the above ground plant N inventory. For studies on diffuse sources of N deposition, such as enhanced wet deposition, a longer time period (several months) is necessary both to integrate the atmospheric inputs and to detect a significant response.
45. The comparison shows that while the different methods give broadly the same result, some are more sensitive than others, or respond differentially to different forms of atmospheric N supply. In simple terms, robustness in biomonitoring for N may be found by using several different approaches.

#### **Testing of nitrogen bioindicator methods on the UK scale.**

46. The chemical methods (tissue N content and soluble  $\text{NH}_4\text{-N}$  concentration) in pleurocarpous mosses and the lichen diversity methods were selected for use in the extensive UK scale study (32 sites throughout the UK, selected for a range of habitat types and atmospheric pollutant inputs). The use of Ellenberg N index for higher plants and standardised grass biomonitors (intensive study) were not practical within the confines of the study for wider application at the UK scale.
47. Local conservation agency staff and SEPA, CEH and NHM staff participated in the moss sampling, the lichen diversity measurements and collection of bark samples for pH measurements on the trunks and twigs. If required, local officers were given basic training (including a short training course) in moss and lichen identification and instruction on the application of the sampling protocols. All sites were provided with a sampling pack, which included sampling protocols and a ladder quadrat for the lichen survey of the tree trunks.
48. The criteria for site selection was based on a) availability of atmospheric monitoring at the site, b) the site being of conservation interest and c) the availability of local conservation/environment agencies' staff to conduct the lichen survey and vegetation sampling. The UK sites selected were all sites dominated by diffuse N deposition, with approximately 44% dominated by dry N deposition and the 54% by wet N deposition. A

site was assessed to be dry N dominated if > 50% of the total N was as NH<sub>3</sub>-N deposition.

### **Bryophyte tissue and soluble nitrogen concentrations at the extensive UK sites.**

49. A weak but significant relationship was found for both tissue N content and soluble NH<sub>4</sub>-N concentration with atmospheric NH<sub>3</sub> concentration for the pleurocarpous mosses sampled in the UK extensive study. There was also a relationship between both indicators and total N deposition. The correlations were higher in response to NH<sub>3</sub> concentration than in response to N deposition, but overall the data showed a higher scatter, which may be due to the fact that the sites were all subject to diffuse sources of N deposition, providing a smaller N deposition range for comparison.
50. Comparing the foliar N content and soluble NH<sub>4</sub>-N concentration data for the two most frequently sampled mosses in the UK study (*R. squarrosus* and *S. purum*) shows that there are species differences in response to NH<sub>3</sub> concentrations and N deposition. *R. squarrosus* shows a similar relationship between both NH<sub>3</sub> concentration and N deposition, whereas *S. purum* shows weaker relationship for N deposition and no relationship at all with NH<sub>3</sub> concentration.
51. By contrast, there was a reasonable relationship between total N content and soluble NH<sub>4</sub>-N concentration using all the UK site data ( $R^2 = 0.484$ ). This would indicate that the measurements reflect real differences in N availability to the bryophytes, and that the weak overall responses in relation to mapped N deposition are due to the other inter-site differences noted above. This demonstrates the benefit of measuring more than one chemical bioindicator simultaneously.
52. More detailed examination of the tissue N content and NH<sub>4</sub>-N concentration values at individual sites revealed a number of unexpected values. For example, at Inverpolly/Knockan (one of the cleanest UK sites) values for *Thuidium tamariscinum* were much higher than expected. This indicates that caution may be needed in such an extensive approach, which utilizes a simple collection of sample at one time. This highlights the need for more detailed checking of such values by more intensive sampling at particular sites.
53. The high scatter in the overall relationship between the foliar parameters and N supply indicates that for sites with diffuse sources of N deposition over a modest range, the foliar N concentration and soluble NH<sub>4</sub>-N concentration of bryophytes may not on their own provide a reliable predictor for spatial comparisons at one time (according to the level of replication used in this study). By contrast, such methods may be better suited to implementing within more intensive monitoring, such as a) near a local gradient or in b) more detailed long term monitoring, including repeated sampling over a period of time. Although, the intensive studies in the present project have shown the potential near known N sources, long-term biomonitoring using these methods still needs to be tested.
54. While recognizing the significant scatter in the data from the UK sites, it is possible to compare the chemical bioindicator data to establish critical loads for the habitats. Based on previous intensive studies of the relationship between total N deposition and total foliar N content, a threshold value of 1.3% N was used as an indicator of N impacts at sites. Application of this value to the present sites showed that 20 out of the 32 sites were estimated to be affected by N deposition. This could have long-term problems for integrity of these sites.

55. By comparison, comparing habitat specific critical loads with estimated N deposition for each of the UK sites showed that 23 out of the 32 sites had N deposition above the current load for their specific habitat. While the individual sites identified were not always the same, this overall number is broadly consistent with that estimated by the bioindicator approach. The advantage of the threshold bioindicator value over the comparison of critical loads with estimated N deposition is that it assigns an actual value based on site measurement.
56. This approach may be applied for other bioindicator parameters, such as foliar ammonium. Previously, a threshold value of  $20 \mu\text{g NH}_4\text{-N g}^{-1} \text{FW}$  was identified as a threshold value for pleurocarpous mosses of woodland (Sutton *et al.* 2004a), however, the extraction methodology used for the present study gives some what smaller values, so a lower value would be more appropriate with the revised sampling protocol (e.g.  $6 \mu\text{g NH}_4\text{-N g}^{-1} \text{FW}$ ).

### **UK extensive lichen diversity survey**

57. The UK extensive survey was restricted to macrophytes on trunks and twigs of a range of available tree species in sites where ammonia is monitored across the UK. Macrophytes were classified as acidophytes and nitrophytes and others. Indices for acidophyte and nitrophyte frequency were estimated for all sites. In addition the Ellenberg nitrophyte scores were used for all species where available.
58. The results showed that there was a strong correlation of lower acidophyte values and increasing nitrophyte values on trunks and twigs with increasing  $\text{NH}_3$ , and that loss of acidophytes is occurring at lower concentrations of  $\text{NH}_3$  than an increase in nitrophytes. The combined index of AV-NV is strongly correlated with increasing  $\text{NH}_3$ . In areas of high  $\text{NH}_3$  deposition nitrophyte values were higher on twigs than on trunks and associated with higher bark pH. The results suggest that a comparison of macrolichens on twigs and trunks allows an evaluation of changes occurring with time.
59. The results of the UK scale lichen assessment support the previous result of Sutton *et al.* (2004a) that the critical level for  $\text{NH}_3$  is set too high. In the present UK dataset for twig lichens, nitrophyte (NV) lichen species tend to dominate over acidophyte lichen species (AV) ( $\text{AV-NV} < 0$ ) at  $\text{NH}_3$  concentrations of above  $1\text{-}2 \mu\text{g m}^{-3}$ . Above similar levels of  $\text{NH}_3$  exposure trunk values of AV-NV tend to be typically reduced to  $< 5$ . The data point to the need to revise the annual critical level for  $\text{NH}_3$  effects on lichens to  $\sim 1.5 \mu\text{g m}^{-3}$ , which is roughly a factor of 5 less than the current value adopted by the UNECE ( $8 \mu\text{g m}^{-3}$  annual average).

### **Interpretation of the nitrogen bioindicator results and relationship to site condition.**

60. Using the  $\text{NH}_3$  concentration, N deposition and sulphur (S) deposition data and the results from the lichen indicator value survey and moss sampling, a generalised impact assessment of the UK sites was carried out. Using the critical load for each of the selected habitats and the estimated N deposition ( $\text{kg N ha}^{-1} \text{y}^{-1}$ ) critical load exceedance was determined for each site.
61. The 32 UK sites were grouped, where possible, into four general habitat types (upland, mixed broadleaved woodland, Atlantic oak woodland and lowland wetland). Using the grouped data for total N content and soluble  $\text{NH}_4\text{-N}$  concentration, a mean N content was determined for each habitat. This mean value was used to estimate whether the individual sites were potentially being impacted by N deposition.

### **Implications for impacts of N from the sampling at the UK sites.**

62. The application of simplified biomonitoring on a UK scale using local field officers to carry out lichen surveys, collect moss, twig and bark samples worked well. Using the field officers allowed a greater number and range of habitats to be sampled. The standard of moss identification and the quality of the sampled material collected was high.
63. It needs to be emphasized that very clear guidance is necessary to ensure agency staff make the best sampling decisions in the field. Sampling decisions, which may appear obvious to an expert, are often difficult for non-experts. A key need is to make clear which are the priority species for sampling (due to better established relationships) and to underline the need for calibration sampling to be undertaken immediately (<20-50 m) adjacent to air monitoring locations due local variability in atmospheric NH<sub>3</sub> levels.
64. It was found that annual rainfall appears to influence the N content of pleurocarpous mosses with increased precipitation reducing the foliar N content in the UK extensive study mosses. This provides a complicating factor to the interpretation of tissue N content response to N deposition. Further work is required to determine the influence of precipitation volume, episodicity of rain events, and precipitation frequency on foliar N concentrations on a UK scale.
65. Using the mean foliar N content and soluble NH<sub>4</sub>-N concentrations derived for four habitat types it is possible to determine a mean concentration for the different habitat types using the moss data collected as part of the UK extensive study.
66. The upland moorland and Atlantic oak woodland had the lowest foliar total N content, followed by the lowland wetlands at 1.30% N and finally the mature woodland at 1.45% N. A similar pattern was found for the habitats soluble NH<sub>4</sub>-N concentrations, but there was virtually no difference between the lowland wetland and the mature woodland habitats at 7.4 and 7.7 μg g<sup>-1</sup> FW respectively.
67. Twenty-three out of the 32 sites exceeded the critical load for their habitat type, and a similar fraction was identified on the basis of exceeding a critical threshold of total N content of bryophytes of 1.3% N.

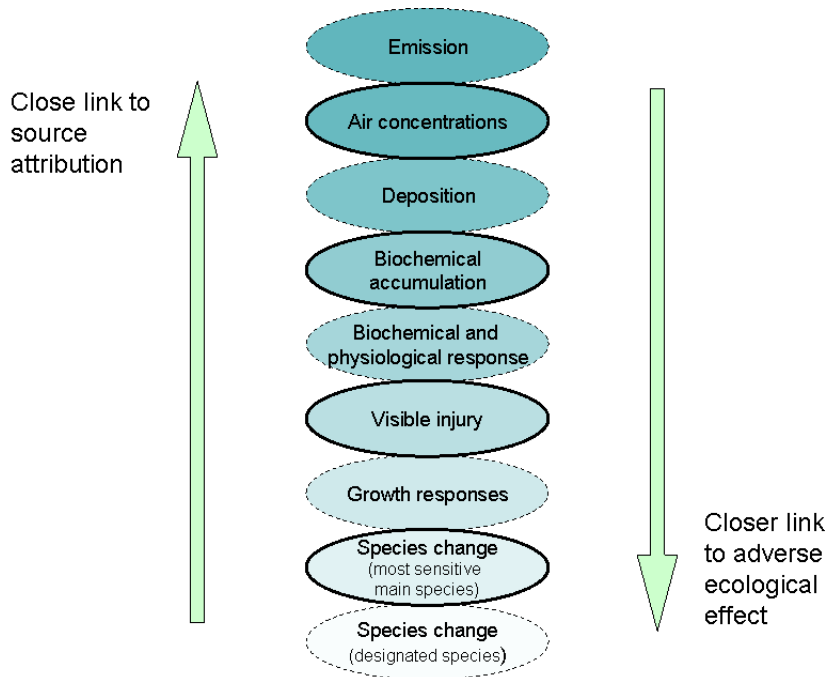
### **Implications for impacts of nitrogen deposition on condition and integrity for four detailed case studies.**

68. The results from a) the simplified biomonitoring methods from both the intensive and the UK extensive studies and b) the N and S deposition data were used to assess the likely impacts of N & S deposition on the condition and integrity of four contrasting SSSI sites. If N biomonitors are to be incorporated as part of site assessment and/or monitoring they must be shown to give added value compared to modelling and critical load assessment.
69. The sites selected were 1) a lowland wood with an agricultural NH<sub>3</sub> source (Piddles Wood SSSI), 2) a lowland raised bog (Caldanagh bog ASSI), 3) an Atlantic oak woodland (Ariundle SSSI) and a mixed broadleaved and yew woodland with neutral and calcareous grasslands (Llanymynech and Llyncllys Hills SSSI). At each site, the specific attributes defined under CSM for the site interest feature were assessed in relation to the applicability of biomonitoring methods and the atmospheric pollutant inputs. In general, it was difficult to relate directly the biomonitoring method to the site interest feature.

The interest features were not normally specific N or S sensitive plant/lichen species making direct application difficult, especially for those sites with diffuse N sources.

### **Developing robustness in biomonitoring and the biomonitoring chain.**

70. It is possible to envisage different methods as a “biomonitoring chain” from source to ultimate impact of conservation concern: emission, air concentration, deposition, accumulation, biochemical response, visible injury, growth response, species composition change (most sensitive species), species change (designated species). Bearing in mind the interest to obtain information that links source attribution and ultimate effect, the most robust biomonitoring program can therefore be envisaged as one which uses several methods well distributed along the biomonitoring chain.
71. At its simplest, robustness of biomonitoring may be enhanced by the use of several different methods. However, the difficulty of linking nitrogen biomonitoring directly to interest features demonstrates the need for a cross cutting approach to biomonitoring that links changes in biological features with the source of the pollution. Such a linkage can be conceptualized in the “biomonitoring chain” (Figure 1), which notes that monitoring tools available are distributed across the pathway from source to final effect.
72. The different positions of monitoring along the biomonitoring chain (Figure 1) may be envisaged as: 1) emissions, 2) air concentrations, 3) deposition, 4) biochemical accumulation, 5) biochemical response, 6) visible injury 7) growth responses, 8) species composition change of main species within the habitat, 9) species composition change of designated species. It should be noted that not all stages of the chain may occur or be relevant in all contexts. However, a robust program of monitoring, that is able to link species effects to pollution, would include measurements that are well distributed across this chain.



**Figure 1.** The “biomonitoring chain” demonstrating how use of complementary monitoring methods can help establish the link between source attribution and adverse ecological effect on designated species. Approaches shown with a bold border are in general easier to measure, while those with a dashed border are harder to measure. Not all links in the chain apply in all circumstances.

73. It should be noted that some measurements are easier than others and that these are fortuitously, well distributed along the biomonitoring chain. In particular, a practical program of easier indicator measurements may focus on: 2) air concentrations, 4) biochemical accumulation, 6) visible injury and 8) species composition change of main species within the habitat (the even numbered stages). By contrast, measurement of the odd numbered stages in the chain requires much more resources.
74. The robustness of the biomonitoring chain depends on the logical and observed connections between source and effects. It is obviously most robust to measure all the stages, however, measurement of the even numbered stages should in most cases be sufficient to examine the link between cause and effect.
75. The biomonitoring chain concept provides a helpful tool to guide the practical application of biomonitoring for statutory conservation and environment agencies, as well as helping to identify the challenges. Foremost among the challenges is the extent to which stage 8) and 9) may be linked if it is not feasible to monitor 9) directly. It can be argued that if some species respond to N, then unmeasured designated species may also be at risk.
76. The extent of risk will depend on improving our understanding of the relative sensitivity of the main different species groups and for particular designated species. For example, the success of acidophyte lichens may be relatively independent of the success of different woodland ground flora communities at the same location, and may differ in sensitivity to nitrogen. In such examples, use of different biomonitoring approaches to indicate impacts on the habitat interest features relies on improving the calibration of responses between the indicator and the interest features and N exposure (as air

concentration or N deposition). For this purpose, further refinement is required in “benchmarking” of critical values of the indicators appropriate for different habitats.

77. An example may serve to demonstrate the application of these principles. At Fressingfield in Suffolk, the  $\text{NH}_3$  air concentration was measured as  $5.3 \mu\text{g m}^{-3}$ , and the measures of N accumulation in *Eurynchium praelongum*: tissue N content and soluble ammonium concentration were 2.87 total N and  $60.4 \mu\text{g NH}_4\text{-N } \mu\text{g g}^{-1}$  FW, respectively. Visible injury for N effects was not assessed. On oak trunks acidophyte (AV) lichens scored 0.7 while nitrophyte (NV) lichen species scored 20.7. On oak twigs, the AV score was 0 and the NV score was 10.4. Overall this provided scores of AV-NV of -20 for trunks and -10.4 for twigs. Hence both the trunk and lichens indicate a nitrophyte-dominated site. The high values of tissue N content and soluble  $\text{NH}_4\text{-N}$  of bryophytes are somewhat above critical thresholds, indicating a significant amount of N accumulation at this site (although not with the highest values). This is supported by the clear dominance of nitrophyte lichens at this site as compared with acidophyte lichens. The biological measurements therefore point to this site being under threat from atmospheric N, in particular  $\text{NH}_3$  (as indicated by the lichen scores). These values are consistent with the N deposition to the site of  $41 \text{ kg N ha}^{-1} \text{ y}^{-1}$ , (based on the measured  $\text{NH}_3$  concentration and mapped values for other terms), which is larger than the critical load for this habitat (critical load is  $20\text{-}30 \text{ kg N ha}^{-1} \text{ y}^{-1}$ ). Although the selected sampling conducted did not directly demonstrate a loss of favourable condition, the results point to a site under significant threat of N deposition to the integrity of the site with the main source being  $\text{NH}_3$  emissions.
78. The example above demonstrates how selected measurements may be used to provide a screening assessment of a site. Where potential problems are identified, there is therefore a need for more intensive measurements, for example using a wider range of approaches along the biomonitoring chain, including more detailed monitoring of the designated species and habitat elements most sensitive to elevated N.
79. Finally, it should be noted that the present short study has necessarily focused on bioindicator methods applied for single sampling periods. Each of the methods increase their robustness when applied repeatedly over time in a planned program of biomonitoring. The two main timescales of biomonitoring for nitrogen may be envisaged as a) short term monitoring (months—a few years) following a local change in conditions (e.g. the impact of emissions from a recent development) and b) long term monitoring following the impact of regional air pollution policies (e.g. several years to decades).

## Appendices

### Refinement of the foliar ammonium concentration bioindicator method.

80. The development of a simplified extraction procedure for the chemical biomonitoring method, soluble foliar ammonium ( $\text{NH}_4\text{-N}$ ) concentration was an integral part of the study. A range of extraction methods and times were tested using: de-ionised water, liquid nitrogen, autoclaving (in water and in sulphuric acid) and ultra-sonic bath.
81. After testing of different extraction solutions and methods using moss tissue, it was established that an extraction time in water of 4 hours, produced levels of  $\text{NH}_4\text{-N}$  at measurable concentrations. This was before significant alternation of the sample with additional ammonium as a product of biological activity took place.

82. The four-hour extraction in water was found to be the quickest, simplest and most cost effective method and produced results, which were comparable with other methods. This extraction procedure was therefore adopted for all soluble  $\text{NH}_4\text{-N}$  concentration measurements of standardised grass and pleurocarpous moss samples from both the intensive and extensive UK studies.
83. The study compared soluble ammonium and nitrate concentrations in moss tissue and established that soluble ammonium concentrations in moss tissue was a better chemical bioindicator than nitrate, which had extremely low measurable concentrations close to the limit of detection.

**Application of *Lolium multiflorum* as a standard biomonitor at an intensive site, Whim Moss.**

84. The potential application of below ground (roots) biomass and foliar N content as a bioindicator of dry N deposition (along a  $\text{NH}_3$  concentration gradient over mire vegetation) was examined using standardised grass biomonitor plants of *Lolium multiflorum*. The above and below-ground biomass and N contents were compared after 2 months of  $\text{NH}_3$  exposure ( $\text{NH}_3$  concentration range 2-200  $\mu\text{g m}^{-3}$ ) at the Whim Moss manipulation facility.
85. *Lolium multiflorum* was found to be a suitable species for use as a standardised grass bioindicator with a defined  $\text{NH}_3$  point source under experimental field conditions. The *Lolium multiflorum* above ground and below ground biomass increased significantly with increasing  $\text{NH}_3$  concentrations.
86. Although there was an increase in tissue N content in both the above and below ground biomass, the N uptake was greater in the above ground biomass (76%) compared to the root biomass (24%). For the total plant inventory of N, this was dominated by the above ground biomass, accounting for 93% of the overall response.
87. Inorganic clay granules (Agsorb) were tested as a growing medium for *Lolium multiflorum* as compared with normal peat/soil substrate. Use of Agsorb speeded up the extraction/cleaning time of the roots compared to soil/peat based composts although such root extraction is still very time consuming. Each root system took on average 30 minutes to clean.
88. Given the extraction time involved in the determination of root biomass and N content, and the fact that the plant response to N was dominated by the above ground plant material, the use of roots as an N bioindicator is considered not suitable for large scale studies. This simplifies the approach, as in practice it is only necessary to measure the above ground plant parts.