

Inertia in an ombrotrophic bog ecosystem in response to 9 years' realistic perturbation by wet deposition of nitrogen, separated by form

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Abstract

Wet deposition of nitrogen (N) occurs in oxidized (nitrate) and reduced (ammonium) forms. Whether one form drives vegetation change more than the other is widely debated, as field evidence has been lacking. We are manipulating N form in wet deposition to an ombrotrophic bog, Whim (Scottish Borders), and here report nine years of results. Ammonium and nitrate were provided in rainwater spray as NH_4Cl or NaNO_3 at 8, 24 or 56 $\text{kg N ha}^{-1} \text{ yr}^{-1}$, plus a rainwater only control, via an automated system coupled to site meteorology. Detrimental N effects were observed in sensitive nonvascular plant species, with higher cumulative N loads leading to more damage at lower annual doses. Cover responses to N addition, both in relation to form and dose, were species specific and mostly dependent on N dose. Some species were generally indifferent to N form and dose, while others were dose sensitive. *Calluna vulgaris* showed a preference for higher N doses as ammonium N and *Hypnum jutlandicum* for nitrate N. However, after 9 years, the magnitude of change from wet deposited N on overall species cover is small, indicating only a slow decline in key species. Nitrogen treatment effects on soil N availability were likewise small and rarely correlated with species cover. Ammonium caused most N accumulation and damage to sensitive species at lower N loads, but toxic effects also occurred with nitrate. However, because different species respond differently to N form, setting of ecosystem level critical loads by N form is challenging. We recommend implementing the lowest value of the critical load range where communities include sensitive nonvascular plants and where ammonium dominates wet deposition chemistry. In the context of parallel assessment at the same site, N treatments for wet deposition showed overall much smaller effects than corresponding inputs of dry deposition as ammonia.

Keywords: ammonium, cover, *Calluna*, chronology, critical loads, growth, nitrate, peatland, *Sphagnum capillifolium*

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Introduction

Globally, evidence exists of detrimental effects of enhanced reactive nitrogen (N) deposition on plant species composition (Clark & Tilman, 2008; Bobbink *et al.*, 2010). Although reactive N is a key plant nutrient (Marschner, 1995), its availability varies across different habitats. Plants have evolved different, specialized strategies and traits appropriate to their natural N environments (Raven *et al.*, 1992). In the presence of increased N availability species that have evolved traits or strategies to exploit an enriched N supply can

replace N conservative N users (Aerts, 1990; Steubing & Fangmeier, 1991). Such species replacements can lead to loss of specialized communities and ecosystems, e.g. heathland transformed into grassland in the Netherlands (Bobbink & Heil, 1993). However, reactive N deposition is not simply N, but comprises two chemically different forms, oxidized and reduced N.

Enhanced reactive nitrogen deposition is a consequence of rising global emissions of the nitrogen oxides NO and NO_2 , (NO_x) from fossil fuel combustion (Dignon & Hameed, 1989), which together with nitric acid and nitrates (NO_3^-), produced through atmospheric reactions, constitute oxidized nitrogen (NO_y). These combine with increasing global emissions of

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ammonia (NH₃) from agriculture to meet the growing demand for energy and food, which reacts to form ammonium (NH₄⁺) in particulate matter and precipitation. Together with smaller contributions from organic nitrogen compounds, these components constitute the largest part of atmospheric reduced N (NH_x). The particulate and aqueous NO₃⁻ and NH₄⁺ salts or aerosols of acidic gases have a relatively long atmospheric residence time, 4 to 15 days, and when removed by precipitation contribute to N deposition in remote ecosystems, after long-range transport (Asman *et al.*, 1998). Proportions of NH₄⁺ and NO₃⁻ ions in precipitation, like the ecosystems they deposit to, vary spatially reflecting the non-uniform distribution of their sources and topography (RoTAP, 2011).

Evidence of the respective effects of reduced and oxidized nitrogen to (semi) natural habitats is urgently needed to support targeted emission control in either fields of agriculture and energy sectors or both (Bobbink & Hettelingh, 2011; RoTAP, 2011). Similarly, there is a pressing need to establish whether N critical loads (CL_N) should be separated by form, as we lack comparable evidence on which to judge whether one or other form is more damaging and under what circumstances (Bobbink & Hettelingh, 2011; RoTAP, 2011). Very few studies have addressed the importance of the form of reactive N in wet deposition (e.g. Paulissen *et al.*, 2004; Van den Berg *et al.*, 2008) and none have studied these effects in the field at low background N deposition and for an extended (>5 year) period.

Due to the fact that many species show unimodal response curves to nutrient additions, it is necessary for field N-manipulation studies to: (i) start from a clearly defined point on the response curve, (ii) be maintained long enough to fully capture the N response and (iii) avoid sites with background N loads that may have already pushed the ecosystems onto the falling side of the curve (Sverdrup *et al.*, 2013).

Semi-natural ecosystems that rely on atmospheric inputs for most of their nutrient supply e.g. ombrotrophic bogs and peatlands are considered to be particularly at risk from enhanced N deposition (Bobbink & Hettelingh, 2011). Much of the component vegetation, e.g. mosses, *Sphagnum* species and lichens are described as nitrophobes (N sensitive) and their presence has formed the basis for setting CL_N in many ecosystems (Bobbink & Hettelingh, 2011). There is a significant literature (Berendse *et al.*, 2001; Limpens & Berendse, 2003; Bragazza *et al.*, 2004; Gunnarsson *et al.*, 2004; Paulissen *et al.*, 2004; Gunnarsson & Rydin, 2000; Bobbink & Hettelingh, 2011) detailing adverse effects of enhanced N deposition on *Sphagnum* species, the main peat forming moss (Van Breeman, 1995) which can change the nature and function of bogs. There is a

pressing need to establish the extent of N-driven changes in species cover on peatlands because of their importance for the global C balance, the link between vegetation and greenhouse gas emissions and the implications for up-scaling (Joabsson *et al.*, 1999; Gray *et al.*, 2013). The experiment described here is an ongoing study of the effects of N form on species cover that has been running for more than a decade at a relatively N clean site where we have realistically manipulated wet N deposition to an ombrotrophic bog.

Our experiment addressed some key concerns on field N-manipulation experiments, namely: (i) background N deposition (ca. 8 kg N ha⁻¹ yr⁻¹) at the Scottish site is relatively low by European standards (apart from northern latitudes); (ii) the simulation of wet deposition is highly realistic (Phoenix *et al.*, 2012), being coupled to meteorology; (iii) the duration of the experiment has been sufficient to characterize N response curves over a 10 year period. We expected to find rapidly initiated, contrasting effects of enhanced wet N deposition on the cover of key species, providing corroboratory evidence on the importance of N form together with a long-term assessment of N effects on the valuable peatland ecosystem.

Aims

- To demonstrate the long-term consequences of different N forms in wet deposition on the cover of key components of an ombrotrophic bog, peatland.
- To establish whether changes in the proportions of different species can be related to cumulative (averaged over the most recent assessment period) treatment effects on soil pH and N availability, soil water nitrate and ammonium.
- To address the significance of N form in wet N deposition (NO₃⁻ vs. NH₄⁺) and the implications for separating CL_N by form.

Methods

Site

Whim bog is located in the Scottish Borders, at 282 m a.s.l., 3°16' W, 55°46' N, on 3–6 m of deep peat. No active management for at least 70 years has left a very uneven aged *Calluna vulgaris* stand (hereafter shortened to *Calluna*) with plants ranging from mature to degenerate (Gimingham, 1972). The peat is very acid, with a pH ca. 3.4 range 3.27–3.91 (H₂O), 10% base saturation and low in available P and K (Table S1). The vegetation conforms best to NVC M19 (UK national vegetation classification, mire) and repeats in heterogeneous mosaics, with hummocks dominated by *Calluna* and *Sphagnum capillifolium*, hollows with *S. fallax* and *S. papillosum* and cyperaceous graminoid species of *Eriophorum* (Mizunuma, 2008). The most

common species on this bog, *Calluna*, *E. vaginatum*, *S. capillifolium*, *Hyppium jutlandicum*, *Pleurozium schreberi* and *Cladonia portentosa* occur widely on similar habitats through the northern hemisphere (Gore, 1983).

Treatments

The treatments, replicated in four 12.8 m² plots, are supplied at three doses of N: 8, 24 and 56 kg N ha⁻¹ yr⁻¹, in addition to the total ambient deposition of ca. 8 kg N ha⁻¹ yr⁻¹, as either oxidized N in NaNO₃ or reduced N in NH₄Cl, referred to as NoxY and NredY respectively, where Y represents the annual dose applied excluding ambient deposition, e.g. Nox56. Treatments provide 10% additional rainwater, collected on site (Sheppard *et al.*, 2004a, 2008b), which dilutes the N concentrate to 0.57, 1.71 or 4.0 mM corresponding to 8, 24 and 56 kg N ha⁻¹ yr⁻¹. The treatment is applied automatically, via a spinning disc sprayer in the centre of each plot, when weather conditions permit: sufficient rainfall, air temperature >0 °C and wind speed <5 m s⁻¹, coupling application to real world conditions with a realistic frequency, ca. 120 applications yr⁻¹. Treatment commenced in June 2002 and is provided all year round, when temperatures exceed those likely to cause freezing.

Meteorological variables are measured at 1 or 15 min frequencies (Leith *et al.*, 2004; Sheppard *et al.*, 2004a). Annual wet-only N deposition at this site varies with rainfall amount in the range 4.6–6.2 kg N ha⁻¹ yr⁻¹, with dry deposited NH₃ <3 kg N ha⁻¹ yr⁻¹. Rainfall, temperature and water table data (Table S2) are presented for the periods, 2002–2004, 2004–2007, 2007–2009, 2009–2011, representing the periods between species cover assessments.

Cover assessments

Three permanent quadrats of 0.25 m² were established in each plot to include the most common species and, where possible, most species in the plot, although often in different proportions. Per cent cover data for these three quadrats were averaged to provide a plot value. The same two recorders made all the assessments. In most cases, the sum of the cover exceeded 100%, due to the multi-layering of the vegetation. *Calluna* was separated into green shoot cover (*Calluna* green), dead shoot cover and fallen litter. In 2011, cover was also estimated for all species in the whole plot (12.8 m²) by the same two recorders.

Growth, N concentrations and vitality assessments

Shoot growth of *Calluna* was measured in 2011, postgrowing season, for up to 5 years, retrospectively, on 24 shoots per plot, removed at three positions on eight compass points. Ten capitula of *S. capillifolium*, per plot, were removed in both October 2006 and November 2011, cleaned, dried and weighed for comparison. Extension growth of *H. jutlandicum* and *P. schreberi* was measured between March 2008 and March 2009. Three rectangles of plastic mesh were placed over areas of the respective mosses and anchored down. These were removed after one year by cutting below the mesh. The area

covered by the moss was estimated and 10 moss stems were measured, dried and weighed. The remaining moss was dried and weighed. N was measured on the dried samples using a CN analyser. Length and weight per cm in 2009 are presented. N was also measured in cleaned, dried, ground samples of current year *Calluna* and *E. tetralix* shoots, apices of *C. portentosa* and green leaves of *E. vaginatum* harvested at the end of the summer in 2009. Visible damage to nonvascular plants was assessed subjectively (Sheppard *et al.*, 2011) in all plots. Winter damage to *Calluna* following the cold winter and hot dry spring in 2009/2010 was scored in October 2010 as the percentage of dead grey shoots per plot (Sheppard *et al.*, 2011).

Soil pH and soil water nitrate and ammonium

In each plot, pH was measured in a 2/1 (v/v) mixture of deionized water and peat originating from one 0–10 cm core taken below a shallow layer of pleurocarpous moss. Measurements started in 2006 and between 7 and 16 measurements were made during the interval between quadrat assessments: 2006–2007, 2007–2009, 2009–2011. Soil pore water nutrients (NH₄⁺ and NO₃⁻) were sampled using rhizon suction samplers (Eijkelkamp agrisearch equipment, Gelderland, The Netherlands), comprising a 6 cm reinforced porous tube, incorporating a 2 µm filter, inserted 0–10 cm below pleurocarpous moss. NO₃⁻ and NH₄⁺ concentrations were measured by ion chromatography, with a detection limit of 0.018 mg N L⁻¹.

Data analysis

Cover and growth data were tested for normality (Bartlett's test and residual plots) and appropriate transformations (log₁₀, arcsin, square-root) were made if required. A two-way generalized model was used to separate the effects of N dose (both forms combined) and form (all doses combined), with *post hoc* tests (Tukey) to separate the interactions (N form × dose) where justified. Separate comparisons were made for individual years. All analyses were undertaken with Genstat (GenStat Release 12.1, ©2009; VSN International Ltd, Harpenden, UK). Significance is indicated when p-values were <0.05. Statistically significant chronological changes are summarized in Table S4. The data are represented as % cover with trend lines fitted, linear or polynomial ($R^2 > 0.7$) (Fig. S1) and difference in % cover from the start of the experiment (Fig. S2).

The temporal response in species cover of the four replicate plots was analysed using the fitting models of Huisman, Olf and Fresco (referred to here as HOF models) (Huisman *et al.*, 1993). These were fitted to express the shape of the species cover response over nine treatment years. Our expectation was that, where they occurred, responses would be either monotonic changes in cover (gradual increase or decrease) or curvilinear, with initial increase followed by decrease. In this way, the different fitted response models were applied as follows: I, a horizontal line; II, gradual increase or decrease; III, rapid initial increase, tending to a new stable value; IV, initial increase, then decrease. The parameter description of these fitting models is provided in Huisman *et al.* (1993). Each of

these model fits were tested using the R package 'gravy' (Ok-sanen, 2004, 2005), with justification of the best model fit described in Table S5. We applied the HOF model fitting to the untransformed cover data for each species and each N treatment (4 replicates) including controls.

Quantifying the effects of N treatment on soil chemistry and species cover change

Nitrogen treatment could drive change in species cover either through 'direct effects' from foliar uptake (e.g. mosses and ericaceous shrubs, Bates, 2002; Bobbink & Heil, 1993) or by 'indirect effects' from changes in soil chemistry, altering the favourability of conditions for each species. 'Path analysis' (statistical method for testing cause and effect, directed dependencies among the variables) was used to separate such direct and indirect effects (Cohen, 1988; Grace, 2006).

A path analysis model (Fig. 1) specifying direct and indirect relationships on individual species (those with sufficient observations) cover was tested separately for each N form, using the same control data. The path analyses were applied to two response variables: species cover in 2011, and the linear slope of change in cover over 9 years. For the purpose of the path analysis, the 2009–2011 average pH and soil solution N chemistry data were used to represent the cumulative effect of N load. Slope coefficients for the path analysis were based on arcsin square root transformed cover data, then centred to zero and standardized to unit standard deviation within each treatment across years. Cover data in 2011 were also centred to zero mean and unit standard deviation across all treatments and plots. The resulting standardized regression coefficients and path coefficients can be interpreted as effect sizes of the magnitude of change in terms of standard deviation following Cohen (1988), where values >0.2 and <0.8 are medium effects and >0.8 are considered large effects.

The path analysis model was written in the Bayesian analytical software OpenBUGS software version 3.2.1. (<http://www.openbugs.info/w/>).

A Bayesian treatment was adopted as this allowed us to estimate all regression parameters and indirect effects in one analysis, while also coping with relatively small sample sizes. Two data values were missing and the Bayesian approach allows these to be estimated by drawing from the posterior predictive distribution of each covariate in the model (Gajewski *et al.*, 2006).

As is normal in path analysis, indirect effects (in this case through soil chemistry) were computed as the product of the standardized regression coefficients for each component path (Grace, 2006). Hence if the standardized regression coefficient expressing the effect of N load on soil pH is designated as beta1 and the coefficient for the effect of soil pH on species cover is designated as beta 2, then the indirect effect of N load on species cover is the product of beta 1 and beta 2. The significance of this term can be readily tested by repeatedly sampling its value from the posterior distribution and counting how many times the estimate is greater or less than zero. The mean of the repeated draws of these binary values gives the required exact probability value for the data. Significance tests of regression parameters were performed in the same manner (Table S6).

Results

Changes in species cover

In control plots species cover changed by up to 100% over the 9 years, increasing: *Calluna*, *H. jutlandicum*, declining: *P. schreberi*, *S. capillifolium*, *E. vaginatum*, *C. portentosa* or both: *E. tetralix*. The scale of change fluctuated over time, both with and without N (Figs S1 and S2). Significant changes in green *Calluna* cover only occurred with Nred in 2007, but N-driven increases in dead *Calluna* foliage and litter were significant from 2004 (Nred56) (Fig. S1). N56 treatments mostly caused

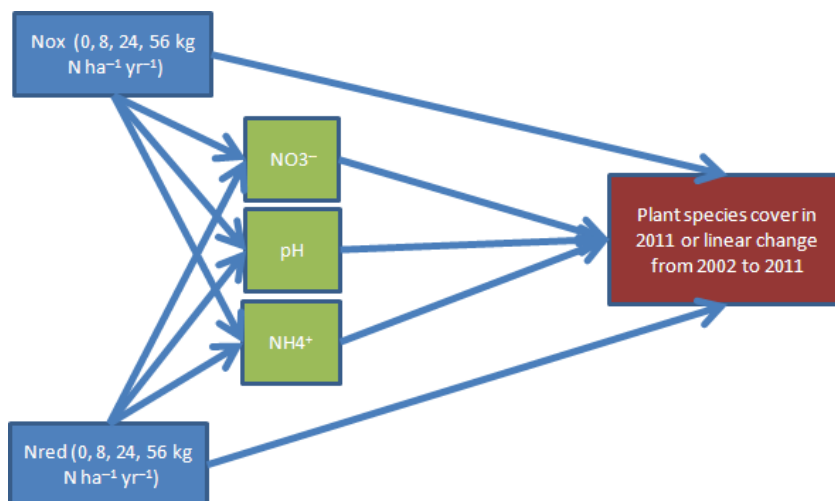


Fig. 1 Path diagram illustrating possible causal/correlative relationships between experimental treatments, soil chemistry and species abundance at Whim Bog.

changes in species cover, whereas N doses below N24 did not produce clear responses. Nonvascular species cover, particularly *S. capillifolium* did increase with a small amount of N, responding positively though not significantly to the N8 dose. This species showed the clearest dose response, with effects accumulating over time (Fig. S1).

Vaccinium oxycoccus, *V. myrtillus* and *Empetrum nigrum* occurred in too few plots for statistical analysis, but trends in cover with N56 were positive (Fig. S1). *E. tetralix* which represented <1% to 10% cover, showed a short-term positive N response, most noticeable with Nred, 3–4 fold increases significant in 2007 (Fig. S1). *E. vaginatum*, green leaves, which comprised ca. 25% of the cover, was relatively unresponsive to N form and dose although cover tended to decline, the opposite of *Calluna* (Fig. S1). Dead and green leaves of *E. vaginatum* together with *Calluna* dominated vascular plant cover (40–68%).

Within two years of treatment, there was a positive Nox effect for *H. jutlandicum* cover (Fig. S2). By 2011, increases in cover with Nox8, 24 and 56 respectively (92%, 61%, 189%) exceeded those for Nred (35%, 21%, 103%). *P. schreberi* appeared to be N sensitive, especially to Nred: within 2 years cover in the Nred56 plots had declined compared with 9 years for Nox56 (Fig. S2). Effects of ≤ 24 kg Nred were not significant, despite the 9 year cumulative dose for Nred24 exceeding the 2 year Nred56 dose. The distribution of *C. portENTOSA* among the plots was too random for statistical analysis e.g. extensive in plots with drier hummocks but sparse in wetter plots and absent from three sets of control quadrats, (Figs S1 & S2).

Species cover contributing to functional groups at the plot scale, indicated ericoid cover, excluding *E. tetralix*, was significantly lower with N56 and showed no form effect (Fig. 2). Neither N dose nor form affected graminoid cover. Moss cover responded positively to a small amount of N and Nox (Fig. 2), the latter reflecting *H. jutlandicum*. Cover of healthy *Sphagnum* (all species) and *S. capillifolium* was smaller, although not significantly, the higher the N dose.

Quantifying the shape of the species' cover response curves – HOF model

Treatments generally induced few significant changes in species cover or deviation from control plots (Fig. 3). Hence, the best fitting model in 20 of 41 possible responses was an intercept-only horizontal line (Fig. 3; model I; Table S5). In the case of *P. schreberi*, because of the extent of the N damage, there were too few positive nonzero values to fit a model to and results summarize those already described: Nred56 rapid decline and

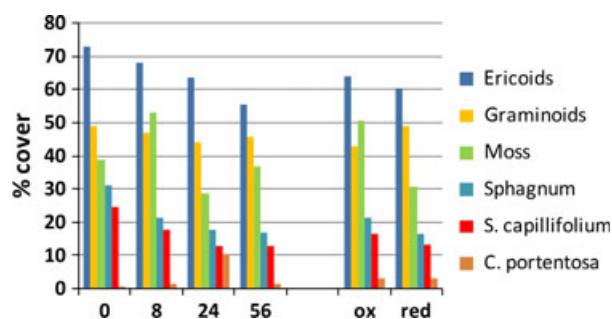


Fig. 2 Per cent cover of functional groups: ericoids, graminoids, moss excluding *Sphagnum*, *Sphagnum* species, *S. capillifolium* and *C. portENTOSA* in response to N dose 8, 24 or 56 kg N ha⁻¹ yr⁻¹ or control (0 kg N ha⁻¹ yr⁻¹) and form: oxidized N, nitrate (NaNO₃) or reduced N, ammonium (NH₄Cl) in whole plots assessed in September 2011. Dose was significant for ericoids, moss and *C. portENTOSA*, $P = 0.042$, 0.014 and 0.008 respectively; form was significant only for moss $P = 0.009$.

eradication with consistent declines in Nred24 and Nred8, and for Nox, a large decline but only for Nox56 (Fig. 3). *E. vaginatum* also declined over time in all Nred treatments and in the Nox8 treatment. *H. jutlandicum* increased significantly over time with Nox8 and Nox56 but not significantly with Nred (Fig. 3). *E. tetralix* showed a significant but small hump, peaking ca. 2007 in all Nred treatments. *Calluna* responded positively in all treatments but the magnitude was not significantly different from the controls (Fig. 3). These HOF models confirm the relatively small magnitude of species cover changes over 9 years.

Nox vs. Nred effects on difference in cover

Effects of N form on species cover change were relatively small ($R^2 = 0.69$), (Fig. S3), although the relationship with Nred was positive, $R^2 = 0.85^*$, whereas the relationship with Nox was inverse, $R^2 = -0.50$, but not significant.

Cumulative dose effects

Where N dose significantly changed species cover, e.g. for *S. capillifolium* and *P. schreberi* (Table S2) relationships with cumulative N load were investigated (Fig. 4). Once the cumulative Nred load exceeded 200 kg N *S. capillifolium* cover decreased slowly. Nred load explained 42% of variation in cover ($P = 0.02$), but there was no relationship with cumulative Nox load. *Pleurozium schreberi* cover changes were inversely related to N dose. *P. schreberi* was more N sensitive than *S. capillifolium*, halving its cover for a smaller N load (Fig. 4). Both cumulative Nox and Nred loads explained significant amounts of the cover changes in

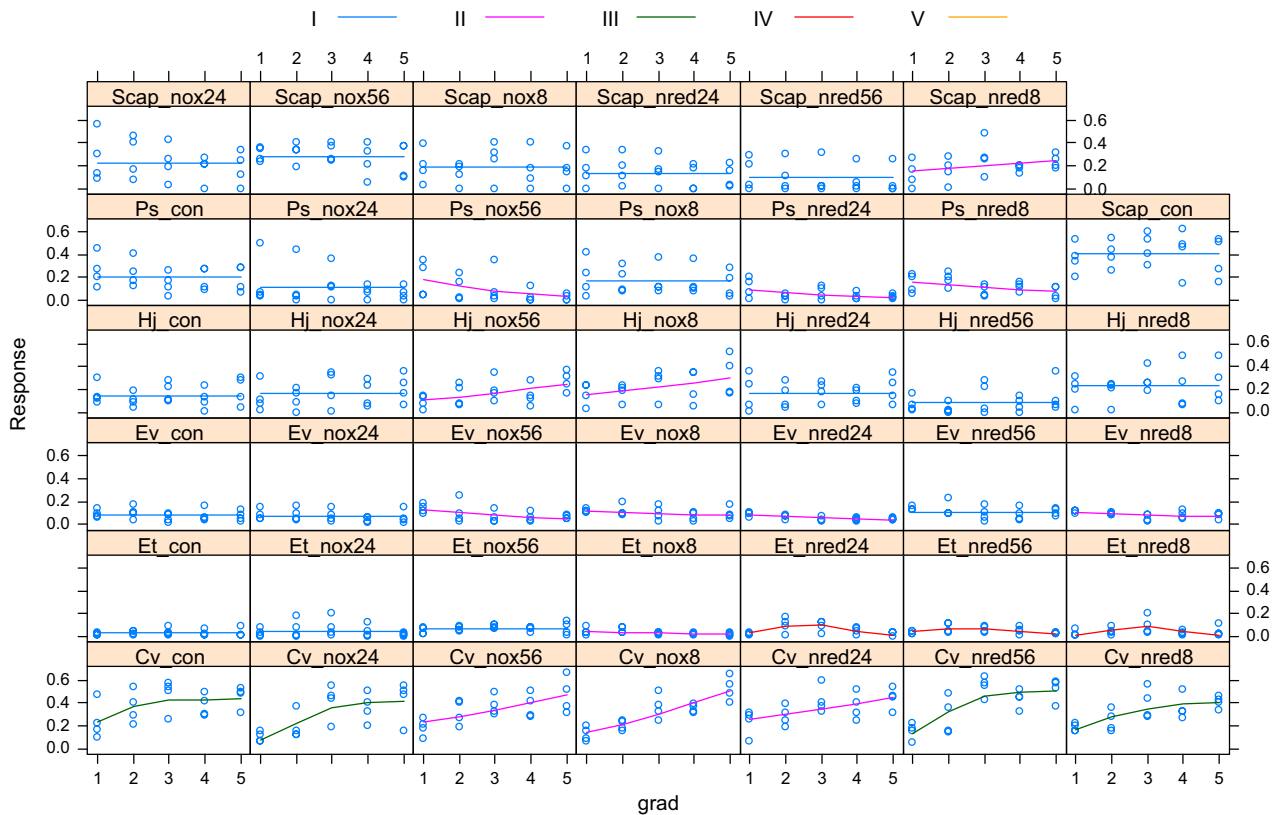


Fig. 3 Percentage cover (labelled as response proportion of cover, on the y axis) for the major species present in the experimental plots through a time gradient (grad) indicated as 1 to 5 on the x axis representing assessments in 2002 (pretreatment), 2005, 2007, 2009 and 2011 respectively. In each graph, the four points for each year show the variation between four replicate experimental plots. Species cover change over time was fitted to wet deposited oxidized nitrogen (added as NaNO_3) or reduced nitrogen (added as NH_4Cl) for 0 (control), 8, 24 or 56 $\text{kg N ha}^{-1} \text{yr}^{-1}$ = con, nox8, nox24, nox56 or nred8, nred24 or nred56 respectively. The colour of the fitted response line refers to the different empirical models I to IV of Huisman, Olf and Fresco (HOF) as outlined in the Methods. Equations (I $y = M \times 1/(1+e^x)$; II $y = M \times 1/(1+e^{a+bx})$; III $y = M \times 1/(1+e^{a+bx}) \times 1/(1+e^x)$; IV $y = M \times 1/(1+e^{a+bx}) * 1/(1+e^{-bx})$) fitted to the models in the HOF procedure; y , the species response variable; x , the gradient variable, a , b , c and d are parameters to be estimated and M is a constant representing the maximum attainable value; 1 for proportions and 100 for percentages. Cv, *Calluna*; Et, *Erica tetralix*; Ev, *Eriophorum vaginatum*; Scap, *Sphagnum capillifolium*; Hj, *Hypnum jutlandicum* and Ps, *Pleurozium schreberi*.

P. schreberi: Nox = 78%, $P = 0.0001$ and Nred = 53%, $P = 0.007$. The form effects were significantly different with Nred causing the largest cover reductions.

Shoot extension

Shoot elongation was measured to indicate whether cover changes were related to growth. In 2011, there was a significant positive dose response and effect of Nred on *Calluna* shoot extension (Table S3). Nred56 increased shoot extension significantly more than Nox56 (*post hoc* tests). There were no significant treatment effects in 2010, 2009, 2008 and 2007. Shoot extension suggests Nred56 is still stimulating *Calluna* growth. *Hypnum jutlandicum* growth data contradicted the cover changes: N dose reduced shoot length per unit weight and there

was no N form effect (Table S3). For *P. schreberi*, N dose significantly reduced both weight per unit length and shoot extension. Neither N form nor N dose significantly affected *S. capillifolium* capitulum weights in 2006 or 2011 (Table S3). In 2006, capitulum weights were larger with $\leq 24 \text{ kg N ha}^{-1} \text{yr}^{-1}$, but by 2011 only N8 maintained that positive trend.

Visible damage

In October 2010, there were more grey dead *Calluna* shoots in N56 plots (Table S3). *Pleurozium schreberi* and *H. jutlandicum* were only damaged in Nred56 plots. Some damage was seen on *S. capillifolium* but the scores exceeded 4. *Cladonia portentosa* showed most damage in the N56 plots, especially with Nred.

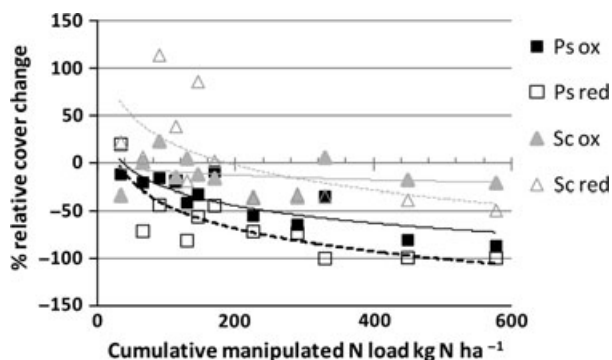


Fig. 4 Relationship between cumulative N load and relative cover for *Pleurozium schreberi* (Ps black squares) and *Sphagnum capillifolium* (Sc grey triangles). These were the only two species showing consistent negative responses to N deposition as Nox (solid symbols) and Nred (open symbols). Nred and Nox both caused large reductions in *S. capillifolium* cover (grey lines), but the effect of Nox was worse than Nred at low loads <280 kg N. At higher loads, Nred caused larger reductions. For *P. schreberi* (black lines), the effects of Nred exceeded those of Nox ($P < 0.05$).

Foliar N

The N status of all species responded positively and significantly to N dose. N concentrations were highest with Nred (Fig. 5, most points above the 1 : 1 line). The difference between % N in species treated with Nred or Nox was greater at the highest N dose. The largest increases in % N occurred among the nonvascular plants which were more N sensitive than the vascular plants.

Meteorology, soil pH and soil water nitrate and ammonium

Between 2002 and 2011, mean air temperature got colder, reflecting two very cold winters (10/11, 11/12)

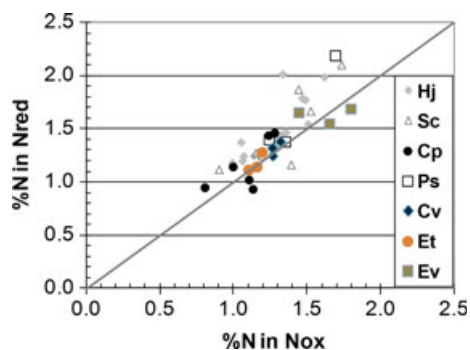


Fig. 5 Per cent N in current years' growth of lower plants, Hj, *Hypnum jutlandicum*; Sc, *Sphagnum capillifolium*; Cp, *Cladonia portentosa* and Ps, *Pleurozium schreberi* receiving N, as either Nox or Nred. % N values sitting above the one to one line indicate higher concentrations occur when the N is applied as Nred, especially at the high N dose, N56.

down to 7 °C. RH has remained relatively constant, between 88 and 91%, likewise the water table at 10 cm below the surface, once recovered from the 2003 drought, and cumulative rainfall. Pretreatment pH values measured in all plots varied by 0.2 pH units (3.45 and 3.65). By 2011, pHs in the Nox plots were up to 0.4 units higher depending on the N dose, whereas pH's in Nred remained similar to control plots but ca. 0.1 units less acid than 2002. Between 2009 and 2011, $\text{NO}_3\text{-N}$ concentrations were relatively low, <0.15 mg L⁻¹ but exceeded control values. N additions significantly increased soil water $\text{NO}_3\text{-N}$ concentrations, but there was no distinguishable trend with time and there was no effect of N form. In control plots, mean soil water $\text{NH}_4\text{-N}$ concentrations were low ca. 0.7 mg L⁻¹. Nox treatments increased soil water $\text{NH}_4\text{-N}$ concentrations, but not in relation to dose. Nred increased $\text{NH}_4\text{-N}$ concentrations in proportion to dose, and concentrations were two to threefold greater than with Nox.

Relating N treatment effects on soil chemistry and species cover using path analysis

Direct effects on species covers. Medium-sized positive and negative effects of increasing loads of Nox were detected for *H. jutlandicum* and *P. schreberi* covers in 2011 respectively (Fig. 6). Medium-sized negative effects of Nred load were seen for cover of *H. jutlandicum*, *P. schreberi* and *S. capillifolium*. Increasing Nred load had a significant positive effect on final *Calluna* cover (Fig. 6). For linear cover change between 2002 and 2011 (Fig. 7), there was only one direct effect: *E. tetralix*, positive for Nox.

Direct effects on soil chemistry with no transmitted effect on species cover. The only direct effect on soil water chemistry that was not subsequently linked to species cover was a positive relationship between increased NH_4^+ concentrations and Nred dose (Fig. 6).

Indirect effects on final cover in 2011. Nox load increased pH, positive effect, which decreased *P. schreberi* cover, large negative effect (Fig. 6). By contrast, increasing Nox had a large positive effect on final *H. jutlandicum* cover via a positive effect on soil pH (Fig. 6). Increasing Nox load also had a medium-sized negative effect on *P. schreberi* cover in 2011 via its' positive effect on soil water NH_4^+ (Fig. 6). An indirect negative effect of increased Nred on *E. vaginatum* cover was also detected via its' positive effect on NH_4^+ concentrations, but the exact Bayes p-value indicated a 90% rather than 95% chance of a credible relationship (Fig. 7).

Significant effects of soil chemistry variation on species cover were also found that were not associated

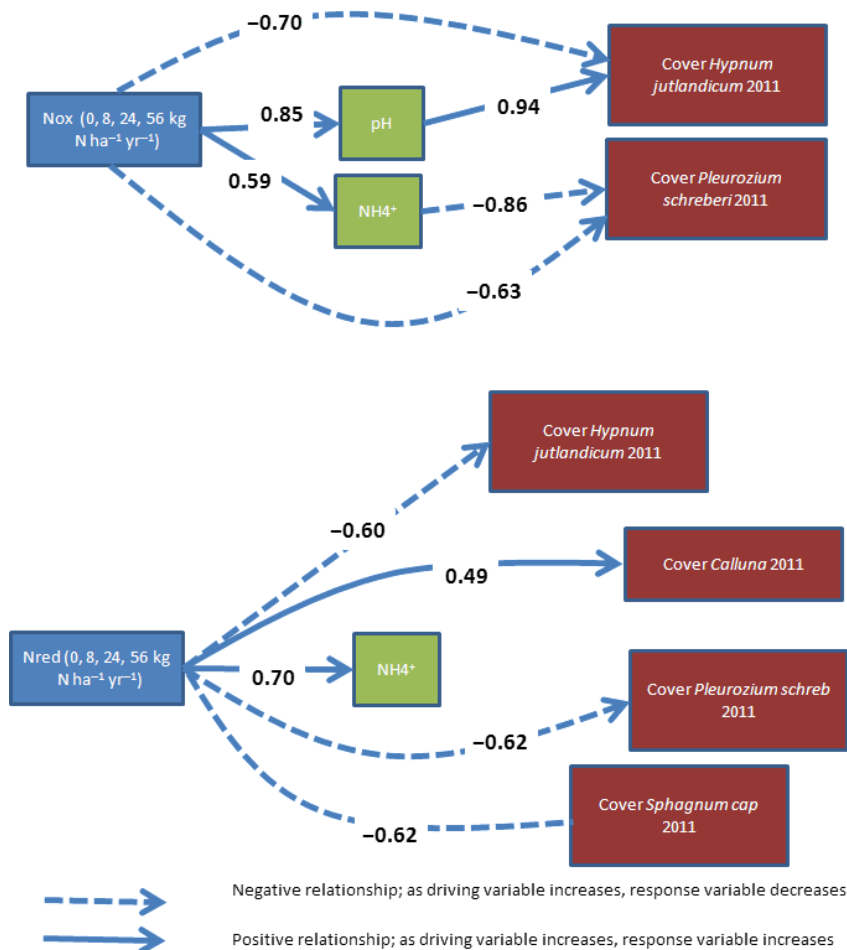


Fig. 6 Path diagram showing statistically significant relationships between Nox and Nred treatments, soil chemistry averaged over 2009–2011 and final species cover measured in 2011. Numbers on the vertices are regression coefficients based on analysis of centred and standardized data and can be interpreted as effect sizes.

with N treatment. These can either reflect lack of statistical power given the noise in the data compared to signal or, they highlight background relationships related to ecological variation in the habitat over time that are independent of the experimental treatments (Figs 6 and 7). Path analysis confirmed that significant differences in the relatively small cover changes could be attributed to changing amounts of applied N. Moreover, it demonstrated that both Nox and Nred caused detectable effects, and that most of the strong negative effects were linked to Nred.

Discussion

In the context of parallel assessment at the same site, nitrogen treatments for wet deposition showed overall smaller effects than corresponding inputs of dry deposition as ammonia (Sheppard *et al.*, 2011).

Importance of this long-term study at Whim bog: capturing a time series to detect resilience

These results highlight difficulties in demonstrating whether, in the absence of a chronology of measurements e.g. the hump back response *E. tetralix*, an N response is increasing, stagnating or declining. Combining assessments of growth and cover change with foliar N concentrations attributes change to N directly and through the growth measurements can suggest which, if any, physiological processes are being affected or whether cover changes simply reflect competition for resources. For the mosses increases in cover did not always translate into an increase in overall mass, and reduction in moss density can undermine their sustainability (Armitage *et al.*, 2012). The study has highlighted the lack of generic responses within functional groups to both N dose and form e.g. an increase in

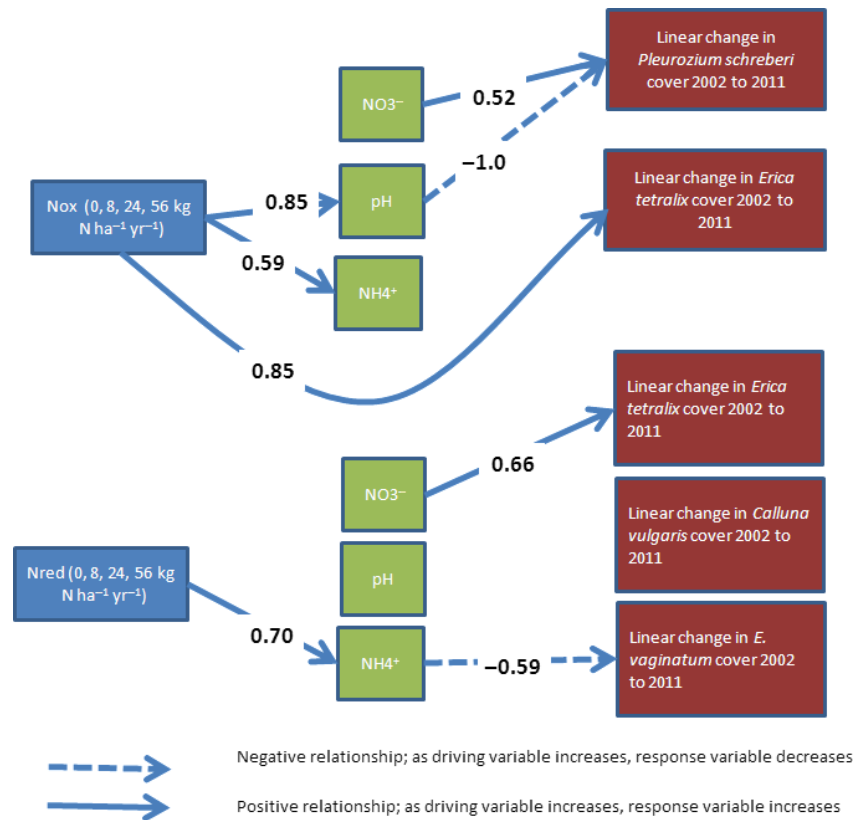


Fig. 7 Path diagram showing relationships for Nox and Nred treatments with soil chemistry (2009–2011) and slope of species cover change between 2002 and 2011. Path diagram showing statistically significant relationships between Nox and Nred treatments, soil chemistry averaged over 2009–2011 and cover change between 2002 and 2011 measured as the linear slope of cover vs. year of recording. Numbers on the vertices are regression coefficients based on the analysis of centred and standardized data and can be interpreted as effect sizes.

Hypnum moss, a large decline in *Pleurozium* but moderated decline in *Sphagnum*, the former to Nox and the latter species to both. Using path analysis, we have clearly demonstrated that N can induce change, but based on the HOF model comparisons with the controls, that the magnitude of change over 9 years is mostly small. Effects of N form were pronounced among the sensitive species, with the strongest negative effects associated with Nred and linked to greatly elevated foliar N concentrations. However, given that the keystone *Sphagnum* was also negatively impacted by Nox, both N forms would need to be controlled to protect peatlands.

A hallmark of the subtlety of realistically applied N deposition effects appears to be that impacts are played out largely via adjustment of the competitive relations of the mix of species present. N effects on *Calluna*, especially litter fall and associated moss species suggest a key role for the dominant species in responding to elevated N and in turn shaping conditions for subordinate species.

In terms of understanding the mechanisms of damage, our results caution against overinterpreting short

term, high-dose studies that elicit damage but where such impacts do not represent realistic ecosystem trajectories over realistic timescales. Some trends from the 2011 whole plot assessment supported the permanent quadrat data. Despite the halving of cover, most plots remain >10% covered in *S. capillifolium*, indicating slow rates of change and inferring this keystone species is relatively resilient to N impacts. One effect of N, demonstrated by this longer study but often overlooked, was the absence of a dose response: species were mostly impacted by the seven times ambient, 56 kg N ha⁻¹ yr⁻¹ treatment but not by inputs of 28 kg N ha⁻¹ yr⁻¹ or less. Significant form effects on change in species cover were rare except for *P. schreberi* where Nred was most detrimental.

Consequences of different N forms in wet deposition on the cover of key components of an ombrotrophic bog

Assuming this ombrotrophic bog relies on atmospheric deposition for nutrients, we had expected our manipulation of wet N deposition to change species proportions

within the vegetation. The nonvascular plant components have no cuticle, absorbing nutrient ions over their entire surface, rendering them both vulnerable to, but also effective competitors for N deposition until they saturate (Bates, 2002). Saturation of this moss filter in peat bogs (Lamers *et al.*, 2000; Curtis *et al.*, 2005) allows mineral N to leak into pore water and soil, accessible to vascular plant roots, facilitating overstorey growth, shading and moss/*Sphagnum* disappearance (Cornelissen *et al.*, 2001; Nilsson *et al.*, 2002; Bubier *et al.*, 2007). But our data show this is not the only N-induced scenario, especially in the absence of competition for light.

In an earlier study (Sheppard *et al.*, 2011), we reported highly detrimental, visible effects of elevated N deposition as dry deposited ammonia (NH₃) at equivalent N doses to those used in this study. Ammonia-N eradicated *S. capillifolium* and also the overstorey, arguably through NH₃ toxicity (Krupa, 2003). We suggested that some of the reported N sensitivity of *Sphagnum* species, such as *S. capillifolium*, could reflect exposure to elevated NH₃ concentrations, especially in areas where agriculture dominates the landscape. The data reported here support that idea indicating that, in the absence of N-induced eutrophication and ensuing competition effects, the sustainability of *S. capillifolium* is more resilient to wet N deposition than previously inferred. The curvilinear relationship with cumulative N load suggests that *S. capillifolium* can adjust to high wet N inputs. To date, the relative proportions of species in this peatland plant community, dominated by unmanaged degenerate *Calluna*, *E. vaginatum* and moss understorey have not been destabilized by realistic applications of wet N deposition separated by form.

There are characteristics of this study that could have modified the responses: (i) Method of treatment application: Unlike many N-manipulation studies (Phoenix *et al.*, 2012) N additions were automated and coupled to rainfall, facilitating frequent small N inputs at concentrations more closely resembling those in wet deposition to relatively large plots (less edge effect). This treatment scenario minimizes artefacts associated with high concentrations/doses (Pearce & van der Wal, 2008), (ii) Manipulation studies mostly apply Nox and Nred combined as NH₄NO₃, which may cancel out or restrict some effects we have seen since they affect pH differently, both within the plant and the soil (Raven, 1988), and have no associated counterions to influence the impact (Evans *et al.*, 2008). But, on balance, one might expect NH₄NO₃ deposition studies to be less, not more damaging, (iii) our study has run three times longer than most and has thus been impacted by the potential for climate interactions e.g. 2003 drought (Carfrae *et al.*, 2007) which can exacerbate N effects (Carroll *et al.*, 1999; Sheppard *et al.*, 2008a,b). Again,

this would be expected to reduce, not increase resilience. (iv) The vegetation composition at Whim is typical of NVC M19 (Rodwell, 1991), but the *Calluna* is old and unmanaged, and may not be capable of significantly growing its' overstorey and depleting light to the understorey sufficiently to exclude photosynthesis, as happened with Labrador tea (*Ledum groenlandicum*) at Mer Bleue (Bubier *et al.*, 2007; Juutinen *et al.*, 2010). Also, *Calluna* only covered 40–60% of the plots so even the significant increase in fallen litter may not have impacted all areas occupied by *Sphagnum* and the bottom 30 cm or so of stems from >60% of *Calluna* plants were devoid of greenery.

Implications

This long-term study has demonstrated that an intuitively N sensitive ecosystem can resist destabilizing changes from significant N inputs in wet deposition, such that the cumulative impacts of N deposition have yet to be fully played out. The flattening of the response of *S. capillifolium* to cumulative N load implies some sensitivity but much more tolerance than has been implied from surveys (Smart *et al.*, 2010). Our results suggest that once the initial effects of Nox have manifested themselves additional N makes very little difference, whereas for Nred increasing the N load perpetuates the slow decline. The results challenge the value of short-term manipulations for predicting N effects but suggest soil chemical changes may be the fore runner of further changes in community structure.

Were effects driven by changes in soil chemistry?

Biomass assessments, together with nutrient concentrations in 2009, inferred most N should be in the soil (Sheppard *et al.*, 2013a,b). Thus, we used soil pore water chemistry as the sensitive indicator (Sheppard *et al.*, 2008a,b) for the path analysis. Both soil water N chemistry and soil pH varied spatially and temporally, representing the difference between deposition and the biological activity of the various sink strengths: mosaics of nonidentical plant assemblages. Interestingly, although NO₃⁻ concentrations were dose sensitive in the Nox treatments, the concentrations varied too little to influence the path analysis, unlike NH₄⁺ concentrations in the Nred treatments. Overall, changes in pore water N chemistry appear to have been too small to strongly affect cover: intuitively, we would expect direct effects on vascular plant cover only, but no relationships were identified for *Calluna*, or *E. tetralix*. There was a positive effect of Nred on NH₄⁺ concentrations that appeared to influence the decline in *E. vaginatum*. However, we suggest this reflects enhanced

competition from *Calluna* stimulated by Nred (Marschner, 1995; Nordin *et al.*, 2006). Pretreatment cover of green *E. vaginatum* was negatively related ($r^2 = -0.86^*$) to *Calluna* cover. Counter-intuitively nonvascular plant cover did relate to soil chemistry: 2011 cover of *H. jutlandicum* and *P. schreberi* in Nox plots with soil pH (+ve) or soil water NH_4^+ (-ve) respectively. Ammonium was not added in the Nox treatment: suggesting N transformations (ammonification, nitrification) are occurring within or on the vegetation, producing ions in proportions that mirror those in soil water? N fixation and methanotroph activity certainly occur within this layer (DeLuca *et al.*, 2002; Raghoebarsing *et al.*, 2005; Larmola *et al.*, 2010). *Pleurozium schreberi* is sensitive to NH_4^+ (Solga *et al.*, 2005). However, mosses have no roots and these mosses occupy niches above the water table excluding direct contact with soil water N concentrations. More likely, the linkage reflects a proxy, working through competition effects which we did not explicitly test for. *Calluna* cover and litter increased with N addition, likely lowering PAR below that needed for *P. schreberi* to survive under *Calluna* (Scandrett & Gimingham, 1989), the moss was often buried under litter and 'hidden' from view, particularly in Nred plots. However, *P. schreberi* decline was shown clearly in the HOF model for Nox56 with path analysis indicating medium negative treatment effects not mediated directly via soil water chemistry, leaving the mechanism unclear.

Manninen *et al.* (2011) reported effects of the wet N treatments on *S. capillifolium* capitulum pH at Whim, and we have similar (unpublished data) for *H. jutlandicum* and *P. schreberi*, replicating those reported for soil, *i.e.* higher pH with Nox. Given pH controls many physiological processes (Raven, 1988; Marschner, 1995) we suggest that the positive relationship with pH for *H. jutlandicum* reflects a more alkaline physiological optimum.

The absence of large increases in soil N availability is consistent with the absence of destabilizing changes in proportions of the key component species and contrasts the large changes with ammonia (Sheppard *et al.*, 2011; 2013a,b).

Sensitivity to N form in nonvascular plants

Nordin *et al.* (2006) concluded, from manipulating N form in a boreal forest, that Nred decreased moss cover as a result of N accumulation, which did not occur with Nox. Tissue N data (see also Solga *et al.*, 2005) suggest *P. schreberi* cannot tolerate large (> 30%) increases in its N status; in the Nox56, Nred24 and Nred56 plots, per cent N increased by 55, 49 and 109% over the control respectively. Taking into account growth and leaf N

concentrations, and the response to the cumulative N load, our results show *P. schreberi* is damaged by both N forms, but via different mechanisms: Nox via a combination of increased pH and N accumulation and Nred through greater N accumulation. Nred56 caused damage earlier and for a lower cumulative dose than Nox56, but for long-term protection of N-sensitive bryophytes both N forms should be controlled.

Lichens take up and use both N forms equally efficiently (Crittenden, 1989, 1998). Visible damage was most conspicuous in *C. portentosa* with Nred56, which consistently elevated N concentrations (Hogan *et al.*, 2010) and eliminated it from 3 of the 4 Nred56 plots. Such damage fits the classic phytotoxicity chain response: reduced growth, NH_4^+ accumulation and ensuing cellular injury *e.g.* leaky membranes (Krupa, 2003). However, Nox was also toxic (Hauck, 2010). NO_3^- has to be converted to NO_2^- then NH_4^+ , both potentially toxic ions, in order to be assimilated (Glime, 2007). If there is insufficient energy, C skeletons or enzyme activity available, these ions can be toxic (Hauck, 2010). Bleaching commensurate with usnic acid breakdown and loss of protection from light damage (Munzi *et al.*, 2009) was recorded in both Nred and Nox plots, together with lower Fv/Fm values indicating reduced photosystem II activity (Sheppard *et al.*, 2004b). In addition, prior to bleaching, algal colonization was observed, reducing light to the phycobiont impairing C assimilation (James, 1973; Hauck, 2010).

S. capillifolium prefers acid bogs (Brown & Bates, 1990), where the NH_4^+ which dominates pore water (Hemond, 1983) can be used more effectively than NO_3^- for growth (Touffet, 1971). Both N forms, above $24 \text{ kg N ha}^{-1} \text{ yr}^{-1}$, decreased *S. capillifolium* cover, and were associated with significant increases in amino acids in 2006 (I. de Lange, unpublished data), indicating excess NH_4^+ and thus diversion of C away from growth (Ohlson *et al.*, 1995; Nordin & Gunnarsson, 2000). Twenhöven (1992) also reported that low NH_4^+ concentrations stimulated *S. fallax* growth, whereas NO_3^- decreased the abundance of both *S. fallax* and *S. magellanicum*. Nox56 may also have compromised the acid base balance, increasing *S. capillifolium* capitulum pH by 0.5 units (4.57 control, 5.04 Nox56, 4.51 Nred56) (Kivimaki, 2011; Manninen *et al.*, 2011). Not all negative effects of Nox on *S. capillifolium* were direct: stimulation of *H. jutlandicum* cover (+50%) caused some *S. capillifolium* to be overgrown and shaded. For this peatland community, the increase in plant pH associated with Nox appears to be quite deleterious. In 2011, the negative effect of Nox on *S. capillifolium* was almost significant ($P = 0.096$) corresponding to a medium size effect (-0.4 compared with -0.62 for Nred).

N form and CL_N setting

Nitrophobic nonvascular plants were most sensitive to Nred. However, 9 years of increasing N deposition, as NH₄⁺ or NO₃⁻, to the canopy of this acid peatland vegetation caused few significant changes in species cover over and above the evolving changes in control plots. *Calluna* and *S. capillifolium* preferred Nred and *H. jutlandicum* Nox, but other species were insensitive to N form. Thus, the main components of this single ecosystem differed in their responses to N form however, CL_N's are applied at the ecosystem level, making it difficult to apply form based CL_N's.

Knowledge of potential eutrophication responses of species growing at a given site to either Nox or Nred could indicate an additional risk from N deposition reflecting the dominant N form. N form could thus be used as a moderator for establishing which end of the CL_N range is appropriate for a site. It would be prudent to set the CL_N at the low end of the published range of N deposition when a site contains sensitive nonvascular plants, the input is predominantly in the damaging form, or where ecosystems contain vascular plants with nitrophilic characteristics and Nox dominates deposition.

A further aspect of the N form CL debate, not to be overlooked, concerns the interaction between N form and soil chemistry. Post deposition, these N forms are interchangeable, both in the plant and in the soil through enzyme-mediated conversions that depend heavily on pH (Stevens *et al.*, 2011). The significance of these transformations will depend on the ecosystem and the ratio of reduced to oxidized N in precipitation but requires further evaluation (Stevens *et al.*, 2011).

Other drivers influence species cover

Species cover in the control plots did not remain static, implying other factors *e.g.* the environment or just the age dynamics and competitive balance, homeostasis, within the community are actively shaping vegetation cover at Whim, unmanaged for at least 70 years. However, quantifying the extent to which these factors have exacerbated or suppressed treatment responses is confounded, because N supply is coupled to rainfall.

In 2009, the cover of most species irrespective of treatment dipped, coinciding with 25% higher rainfall the preceding year. The higher rainfall may have reduced CO₂ diffusion, and PAR was ca. 15% lower than in other years. By 2011, cover had recovered. In 2003, Whim bog, like most in Europe (Gerdol *et al.*, 2008), experienced an exceptional, prolonged drought,

which caused short-lived adverse effects in *S. capillifolium* (Carfrae *et al.*, 2007), reversed by the following wet autumn/winter, and undetectable in 2004 cover. *P. schreberi* too appears to prefer damp conditions judging from 2004 cover values, post 2003 drought, which were lower than expected, but recovered as rainfall increased through the course of the study. Climatic driven changes appear to be more transient, than those associated with N deposition.

Conservation Implications: indicator species

Pleurozium schreberi and *C. portentosa* appear to be strongly nitrophobic and unlikely to be present, or of very limited occurrence, at sites where ambient inputs exceed 24 kg N ha⁻¹ yr⁻¹. Thus, their absence from environmentally suitable sites could indicate high N deposition levels, although, water table height also determines species presence and absence in peatlands (Lindsay, 2010). Such environmental factors will also need to be accommodated within N deposition indicators. Non-responding species, tolerant of or indifferent to wet N deposition and its effects without long-term or significant cover changes were also identified. Some ericoids fell into this category *e.g.* *E. tetralix*, *V. oxycoccus* and *E. nigrum*. None of the species in the wet deposition plots, from either the graminoids or ericoids can be described as true nitrophiles. However, we saw (Sheppard *et al.*, 2011) that once competition from *Calluna* was removed *E. vaginatum* displayed its nitrophilic characteristics (Redbo-Torstensson, 1994; Wiedermann *et al.*, 2007). Many grasses, even though they have low Ellenberg values, behave as nitrophiles and up regulate their growth capability in eutrophicated environments at the expense of small forbs with similar Ellenberg values *e.g.* *Molinia caerulea* (L.J.L. van den Berg, unpublished data). *Molinia caerulea* readily capitalizes on increased NH₄⁺ *e.g.* the Netherlands during the 1990s, where it replaced *E. tetralix*, transforming wet *Ericion tetralicis* heathland into grassland (Bobbink *et al.*, 1998; Aerts & Bobbink, 1999). However, though present nearby, *M. caerulea* has not moved into plots at Whim. Although graminoids exist naturally as low N index plants, a significant cover of such species should be seen as cause for concern given their capacity to respond to N eutrophication.

Nine years of wet N deposition below 24 kg N ha⁻¹ yr⁻¹ have caused few significant changes in species cover and even twice this dose has not thus far destabilized the community. By contrast, N deposition as dry deposited ammonia has been devastating for this bog (Sheppard *et al.*, 2011). These results suggest that dry deposited NH₃ dominates changes driven by N deposition.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Soil and meteorological characteristics at Whim.

Table S2. Mean climatic variables at Whim.

Table S3. Effects of wet nitrogen deposition on growth and vitality responses.

Table S4. Summary of responses of common plant species growing on Whim bog to wet N deposition, and the year of response, ns = $P > 0.05$, $*=P < 0.05$, where Nox, nitrate and Nred, ammonium.

Table S5. Model parameters derived from the Huisman, Olff and Fresco (HOF) curve fitting procedure using the R package 'gravity'. The parameters a-d of these models were explained under the section data analysis.

Table S6. Descriptive statistics of the path model parameters (see data analysis), based on summarizing 20000 values from the posterior distributions of each.

Figure S1. Per cent cover responses of key species in permanent quadrats since 2002 (pretreatment) up to 2011, growing on Whim bog and treated with wet deposited nitrate (NaNO_3) or ammonium (NH_4Cl) at 8, 24 or 56 $\text{kg N ha}^{-1} \text{yr}^{-1}$ or wet only (control) = Nox8, Nox24, Nox56 or Nred8, Nred24 or Nred56 respectively. (Cv gr, *Calluna* green shoots; Cv dea, *Calluna* dead brown shoots; *Calluna* lit, *Calluna* litter; En, *Empetrum nigrum*; Vo, *Vaccinium oxycoccus*; Et, *Erica tetralix*; Vm, *V. myrtillus*; Sc, *Sphagnum capillifolium*; Hj, *Hypnum jutlandicum*; Ps, *Pleurozium schreberi*; Ev, *Eriophorum vaginatum*; Cp, *Cladonia portentosa*). Linear or polynomial (unimodal) trend lines describe the response.

Figure S2. Difference in mean cover, +/- standard error, from mean plot quadrats pretreatment cover (2002), after 2 year (2004), 5 year (2007), 7 year (2009), 9 year (2011) of *Calluna*, *Erica tetralix*, *Eriophorum vaginatum*, *Sphagnum capillifolium*, *Hypnum jutlandicum*, *Pleurozium schreberi* and *Cladonia portentosa* in response to wet deposited oxidized N, nitrate (NaNO_3) or reduced N, ammonium (NH_4Cl) at 8, 24 or 56 $\text{kg N ha}^{-1} \text{yr}^{-1}$ or wet only (control) = Nox8, Nox24, Nox56 or Nred8, Nred24 or Nred56 respectively.

Figure S3. Difference in cover from the start (2002) for the most common species growing on Whim bog receiving oxidized N (Nox) or reduced N (Nred) plotted against each other. The closer points are to the 1:1 solid line the more similar the response to reduced and oxidized N.