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Effects of airborne ammonium and nitrate pollution strongly differ in peat bogs, but symbiotic nitrogen fixation remains unaffected

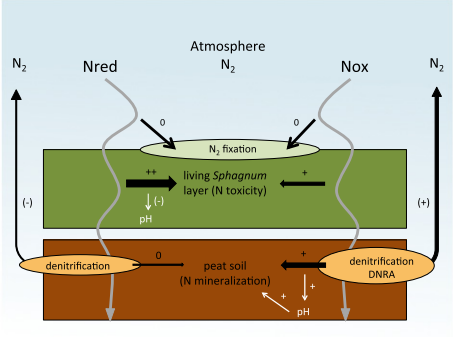
Eva van den Elzen [a,*], Leon J.L. van den Berg [b], Bas van der Weijden [a], Christian Fritz [a,d], Lucy J. Sheppard [c], Leon P.M. Lamers [a]

[a] Department of Aquatic Ecology & Environmental Biology, Institute for Water and Wetland Research, Radboud University, Heyendaalseweg 135, 6525 AJ Nijmegen, The Netherlands
[b] Bosgroep Zuid-Nederland, Huisvenseweg 14, 5591 VD Heeze, The Netherlands
[c] Centre for Ecology & Hydrology Edinburgh, Bush Estate, Penicuik EH26 0QB, UK
[d] Centre for Energy and Environmental Studies, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

HIGHLIGHTS

- N_2 fixation of moss symbionts is not down-regulated by increased N deposition.
- Ammonium N deposition leads to N stress response in keystone spp. of bogs.
- Nitrate N deposition, in contrast, leads to increased peat N mineralization.
- Differential N effects on bog ecosystem functioning should be taken into account.

GRAPHICAL ABSTRACT



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ABSTRACT

Pristine bogs, peatlands in which vegetation is exclusively fed by rainwater (ombrotrophic), typically have a low atmospheric deposition of reactive nitrogen (N) (<0.5 kg ha^{-1} y^{-1}). An important additional N source is N_2 fixation by symbiotic microorganisms (diazotrophs) in peat and mosses. Although the effects of increased total airborne N by anthropogenic emissions on bog vegetation are well documented, the important question remains how different N forms (ammonium, NH_4^+, versus nitrate, NO_3^-) affect N cycling, as their relative contribution to the total load strongly varies among regions globally.

Here, we studied the effects of 11 years of experimentally increased deposition (32 versus 8 kg N ha^{-1} y^{-1}) of either NH_4^+ or NO_3^- on N accumulation in three moss and one lichen species (*Sphagnum capillifolium*, *S. papillosum*, *Pleurozium schreberi* and *Cladonia portentosa*), N_2 fixation rates of their symbionts, and potential N losses to peat soil and atmosphere, in a bog in Scotland.

Increased input of both N forms led to 15–90% increase in N content for all moss species, without affecting their cover. The keystone species *S. capillifolium* showed 4 times higher N allocation into free amino acids, indicating N stress, but only in response to increased NH_4^+. In contrast, NO_3^- addition resulted in enhanced peat N mineralization linked to microbial NO_3^- reduction, increasing soil pH, N concentrations and N losses via denitrification. Unexpectedly, increased deposition from 8 to 32 kg ha^{-1} y^{-1} in both N forms did not affect N_2 fixation rates for any of the moss species and corresponded to an additional input of 5 kg N ha^{-1} y^{-1} with a 100% *S. capillifolium* cover.

* Corresponding author.
E-mail address: e.vandenelzen@science.ru.nl (E. van den Elzen).

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Since both N forms clearly show differential effects on living *Sphagnum* and biogeochemical processes in the underlying peat, N form should be included in the assessment of the effects of N pollution on peatlands.

1. Introduction

Nitrogen (N) is a key limiting nutrient for primary production in terrestrial ecosystems (LeBauer and Treseder, 2008), and the availability of this nutrient affects plant competition and biodiversity (Bobbink et al., 1998; Porter et al., 2013). Ombrotrophic bogs typically develop under very nutrient poor conditions, and their keystone genus, *Sphagnum* (peat moss), is highly adapted, showing a high N uptake and N use efficiency (Aerts et al., 1999; Fritz et al., 2014). By covering the peat soil, peat mosses function as a filter that efficiently absorbs N from rainwater, preventing it from leaching to the rhizosphere of vascular plants, which makes *Sphagnum* an effective competitor for nutrients (Bragazza et al., 2004; Fritz et al., 2014; Lamers et al., 2000). However, increasing anthropogenic N emissions of the last century have led to much higher N deposition loads (Dentener et al., 2006; Vitousek et al., 1997), presenting a severe threat to bogs (Bragazza et al., 2006; Tomassen et al., 2003). Reactive N in atmospheric deposition consists of two major forms: reduced N as ammonia (NH_3) and ammonium (NH_4^+), and oxidized N as nitrate (NO_3^-). Highly detrimental effects of NH_3 deposition on vegetation have been demonstrated for various ecosystems including peatlands (Fangmeier et al., 1994; Krupa, 2003; Sheppard et al., 2011), but potential differences between effects of NH_4^+ deposition (here referred to as Nred) and NO_3^- deposition (referred to as Nox) remain largely obscure. The ratio between these two forms of N deposition varies worldwide and is important in explaining changes in species composition and ecosystem functioning (Stevens et al., 2011).

Both N forms can be rapidly taken up by *Sphagnum* (Fritz et al., 2014; Rudolph et al., 1993) and other mosses (Li and Vitt, 1997) and assimilated in their tissue. In particular NH_4^+ can enter *Sphagnum* by binding to cation-exchange sites, leading to subsequent translocation to the cytoplasm (Clymo and Hayward, 1982) and can in this way be taken up 10 times faster than NO_3^- (Fritz et al., 2014; Liu et al., 2013), which must be reduced to NH_4^+ before it can be assimilated. At high loads, N deposition can have a direct detrimental effect on *Sphagnum*, as internal accumulation of NH_4^+ can become toxic (Baxter et al., 1992; Limpens and Berendse, 2003; Nordin and Gunnarsson, 2000; Tomassen et al., 2003; Wiedermann et al., 2009). Excess N is stored in N rich free amino acids in *Sphagnum*, functioning as an internal N detoxification mechanism. However, the imbalance between N uptake and N assimilation leads to N stress in *Sphagnum* that can lead to *Sphagnum* decline and gradual ecosystem change (Tomassen et al., 2003; van der Heijden et al., 2000). The amino acid content of *Sphagnum* therefore represents a sensitive indicator of *Sphagnum* N stress (Tomassen et al., 2003).

In addition, increased availability of N can lead to indirect negative effects on *Sphagnum*, since excess N is not assimilated or immobilized and becomes available in the rhizosphere of fast growing vascular plants that may subsequently outcompete *Sphagnum* for light. It is assumed that N leaches through the *Sphagnum* filter at deposition rates above 20 kg ha^{-1} y^{-1} (Harmens et al., 2014; Lamers et al., 2000). Deposition above this load may lead to ecosystem changes, from *Sphagnum* covered bogs to bogs that are more vascular plant dominated (Bubier et al., 2007; Heijmans et al., 2002; Lamers et al., 2000; Tomassen et al., 2003). Besides, N leaching to deeper anoxic peat layers may become available to the denitrifying microbial community that can quickly convert it to N_2O and subsequently to N_2. This loss of N to the atmosphere potentially represents an important pathway of N removal from peatlands (Silvan et al., 2002). However, denitrification rates reported are low, attributed to the low pH and N availability in peatlands (Aerts, 1997; Hayden and Ross, 2005).

Next to atmospheric deposition, N input to pristine ecosystems results to a large extend from N_2 fixation by microorganisms associated with peat soil and vegetation (Vitousek et al., 2013). In peatlands, the symbiosis between *Sphagnum* spp. and associated N_2 fixing microorganisms (diazotrophs) is considered a very effective mechanism to obtain sufficient N for growth (Santi et al., 2013). *Sphagnum* spp. have hyaline cells that are colonized by a diverse microbial community (Bragina et al., 2012; Opelt et al., 2007) containing several species of diazotrophs (Bragina et al., 2013). In a pristine boreal bog, this community was even found to fix 85–96% of the total bog N-input (Vile et al., 2014). In boreal forests, the bryophyte *Pleurozium* sp. (a feather moss) also grows in symbiosis with N_2 fixing cyanobacteria, supplying up to 50% of its total N input (Rousk et al., 2013). In pristine peatlands, associations between mosses and diazotrophs therefore represent an important contribution to the total N pool (Rousk et al., 2013), boosting peat accumulation through their stimulation of primary production (Vile et al., 2014). In addition to mosses, lichens such as *Cladonia portentosa* are also known to have similar symbioses (Grube et al., 2009). Although increased N availability can be expected to lower N_2 fixation rates, given the energy consuming nature of the reaction, inconsistent results have been reported on the effect of increased N deposition (0.1–2 kg N ha^{-1} y^{-1} background deposition compared to 12.5, 40 or 50 kg N ha^{-1} y^{-1}) on N_2 fixation for both feather mosses and *Sphagnum* spp. (Ackermann et al., 2012; Gundale et al., 2013; Kox et al., 2016; Leppänen et al., 2013). In addition, little is known about the relative contributions of the diazotrophic microbiomes of different species to the total N input in bogs where both bryophytes and lichens are present.

In this paper, we report on the effects of long-term (11 years) experimental addition of N deposition of 24 kg ha^{-1} y^{-1} as NO_3 versus NH_4 on the biogeochemical cycling of N in *Sphagnum* and peat soil with respect to N_2 fixation, denitrification and N loss to deeper peat. In the real-time watering experiment in Whim bog in Scotland we tested our hypotheses that: 1) increased N deposition reduces N_2 fixation of moss and lichen symbionts; 2) *Sphagnum* accumulates N rich amino acids, especially with NH_4^+; 3) N deposition, especially NO_3^-, leaches through the *Sphagnum* N filter to deeper peat layers, affecting biogeochemical processes in the soil including denitrification.

2. Methods

2.1. Study site

Whim bog is situated in the Scottish Borders, close to Edinburgh (3° 16′ W, 55° 46′ N) and represents a transition between a lowland raised bog and a blanket bog. It has a peat soil of 3–6 m deep that is relatively wet and acidic, with a pH of around 4.2. The mean annual air temperature and annual precipitation between 2003 and 2013 were 7.9 °C and 1124 mm respectively, and the ambient N deposition rate was around 8 kg N ha^{-1} y^{-1}, with similar contributions of ~3 kg of each wet N deposition form, i.e. NO_3^-, NH_4^+ and ~2 kg of dry deposition (NH_3) (Leith et al., 2004; Sheppard et al., 2004; Sheppard et al., 2014). The vegetation is classified as a *Calluna vulgaris-Eriophorum vaginatum* community (UK NVC M19) (Rodwell, 1991) with hummocks of *Sphagnum capillifolium* and hollows containing mostly *S. papillosum*. Other common species are *Calluna vulgaris*, *Eriophorum vaginatum*, *Erica tetralix*, the mosses

Pleurozium schreberi, *Hypnum jutlandicum* and the lichen *Cladonia portentosa*.

In June 2002 a long-term deposition experiment was set up in this bog, where N treatments were continuously being added by real time watering in different doses and different forms to circular plots of 12.8 m^2. The treatments, replicated in four plots, were supplied to each plot from a central spinning disc generating fine rain droplets when activated by rainfall. In this study, we focus on the N treatments of annual addition of 24 kg N ha^{-1} y^{-1} of 2 different forms of wet deposition: oxidized N or NO_3^- (Nox) applied as $NaNO_3$ and reduced N or NH_4^+ (Nred) applied as NH_4Cl. No increased concentration of Na^+ was found in the pore water or bound to soil with Nox, nor an increase in concentration of Cl^- in pore water with Nred (results not shown). Including the background N deposition of 8 kg ha^{-1} y^{-1} this translates to total N loads of 4 times ambient deposition: 32 kg N ha^{-1} y^{-1}. The solution concentration of the additional raindrops was 1.71 mM and a rainwater only control was also applied, all providing an additional 10% of treatment or control solution to rainwater (Sheppard et al., 2014). Samples were taken from each of the four replicate plots treated with control solution, 24 kg N-NH_4 and 24 kg N-NO_3.

2.2. Plant analyses

Sampling of moss and lichen tissue took place in September 2013, after a week with average rainfall (total 25.6 mm). Of each species (*Sphagnum capillifolium*, *S. papillosum*, *Pleurozium schreberi* and *Cladonia portentosa*) two 21 $cm^2 \times 2$ cm deep cores of biomass were taken, of which one was put in a zip lock bag to serve as a background isotopic signature sample. The other samples of each plot were placed in transparent, airtight 1 L glass jars. Through a septum, 100 mL of headspace was removed using a syringe with injection needle and replaced by $^{15}N_2$ gas (98 at% ^{15}N, Sigma-Aldrich, Germany) leading to a 10% $^{15}N_2$ labeling. Samples were incubated for 24 h. in the field, transferred to zip lock bags, and together with the background samples transported to Nijmegen, the Netherlands. All samples were dried at 70 °C for 48 h. and ground using a mixer mill (MM301, Retsch, Germany) for 2 min at 30 rotations s^{-1}. Total N concentrations and isotopic ratios were determined using an elemental analyzer (Type NA 1500 Carlo Erba, Thermo Fisher Scientific Inc., USA) coupled online via an interface (Finnigan Conflo III) to a mass-spectrometer (Thermo Finnigan DeltaPlus, USA). Background isotopic composition was determined for each species under each treatment separately, which is especially important given that the different N treatments (NH_4^+ versus NO_3^-) change the background isotopic signature of the mosses. This background isotopic signal was then used to correct the ^{15}N labeled signal, leading to accurate rates of N_2 fixation and high sensitivity of the method. The N_2 fixation rates measured (nmol gDW^{-1} h^{-1}) were also converted to rates per unit moss area using the average bulk density of the cores of each species per treatment. Fixation rates per hectare of the species were transformed to a yearly rate assuming N_2 fixation activity throughout the growing season (Rousk et al., 2015), which lasts around 250 days in peatlands in the Northern hemisphere with mild winters (Helfter et al., 2015; Zhu et al., 2012) and has an average temperature of 11 °C, calculated with a Q10 of 3.7 (Kravchenko and Doroshenko, 2003). Data on species cover in plots was used to calculate the average N_2 fixation rates per area of each species and these were summed to get a representation of total N_2 fixation per bog area.

An additional sample of *S. capillifolium* was collected from each plot, stored dark on ice and transported to Nijmegen. Each sample was ground in liquid nitrogen, and for amino acid analysis 3 subsamples of 1 g were transferred into 50 mL Erlenmeyer flasks with 10 mL of extraction fluid (538 mL ethanol, 262 mL demineralized water, 32 mL 25% thiodiglycol and 560 mg citric acid monohydrate), kept on ice. 200 μL of norvaline was added as an internal standard, and flasks were shaken at 100 rpm for 60 min and then centrifuged at 1000 rpm. The precipitate was diluted in 20 mL of cooled chloroform, rigorously shaken for 2 min and left to set overnight at 4 °C. Supernatant was freeze-dried, diluted in 2 mL 0.01 M HCL and filtered (Dynagard filter, 0.2 μm). Nine free amino acids were analyzed with automated precolumn derivatization and HPLC with Varian 920-LC (Varian INC., Melbourne, Australia): Alanine, Arginine, Asparagine, Aspartic acid, Glutamic acid, Glutamine, Glycine, Serine and Threonine. Their concentration was calculated based on their relative concentration compared to the known concentration of norvaline in the samples and expressed on a dry weight basis using the moisture content. The concentrations of all 9 amino acids were multiplied by the amount and weight of their N atoms and summed to get total N in free amino acids (aaN). Of the ground *Sphagnum* samples, 200 mg was digested in 4 mL HNO_3 (65%) and 1 mL H_2O_2 (30%), using an Ethos D microwave labstation (Milestone srl, Sorisole, Italy). Digestates were diluted in demineralized water and phosphorus (P) and potassium (K) concentrations were determined by inductively coupled plasma emission spectrometry (ICP-OES iCAP 6000, Thermo Fisher Scientific, Waltham, USA).

2.3. Pore water and soil sampling

Pore water samples were taken at two depths in each plot using ceramic cups (Eijkelkamp Agrisearch equipment, Wageningen, the Netherlands) at 50 cm depth and small rhizon samplers (Eijkelkamp Agrisearch equipment, Wageningen, the Netherlands) at 10 cm depth, attached to vacuumed 60 mL plastic syringes. Both devices were placed at a representative open spot with mosses, and a second rhizon was placed in a *S. capillifolium* patch at 5 cm under the capitula. Pore water for all three depths was collected overnight on three different days at two-week intervals in April and May 2014. From each sample, pH was measured (Mettler Toledo MP220 pH meter); one 20 mL vial was kept at 4 °C and one was kept frozen. In Nijmegen, the chilled samples were analyzed for dissolved organic carbon (DOC) and total nitrogen (TN) concentrations by combustion (Shimadzu, Duisburg, Germany), and the frozen samples were analyzed colorimetrically for phosphate (PO_4^{3-}), NH_4^+ and NO_3^- with an Auto Analyzer system (Bran & Luebbe, Norderstedt, Germany) using ammonium molybdate (Henriksen, 1965), hydrazine sulphate (Kamphake et al., 1967) and salicylate (Grasshoff and Johannsen, 1972). The three sampling dates were averaged to one more representative value for each plot. Dissolved organic nitrogen (DON) concentrations were calculated by subtracting the sum of NO_3^- and NH_4^+ concentrations from TN concentrations.

To determine cation exchange capacity, one soil core was collected from each plot with a metal auger (diameter 2.5 cm, length of 50 cm). Each core was separated in an upper (0–10 cm depth) and lower part (40–50 cm depth), each part was placed in a closed ziplock bag and transported to the lab in Nijmegen. In the lab, a strontium-chloride extraction and a water extraction were performed with 14 g of fresh weight (equivalent to 2.5 g dry weight) by adding 200 mL of 0.2 M $SrCl_2$ or 100 mL of N_2 bubbled demineralized water and shaking for 1 h at 105 rpm. Each sample was filtered, the extraction fluid pH was measured and analyzed for Al, Ca, Fe, S, Mg, Mn, Na, Si and K by inductively-coupled plasma emission spectrometry (IRIS Intrepid II, Thermo Electron corporation, Franklin, USA), and for NH_4 with an Auto Analyzer system (see above). Cation Exchange Capacity (CEC) was calculated by subtracting the concentrations of Ca, Mg and K extractable by $SrCl_2$ with their water-extractable concentration. All values were corrected for their charge and summed.

2.4. Denitrification measurements

Two additional cores from the upper 10 cm of soil were taken from each plot with a small plastic tube (diameter 3 cm, length 10 cm) in May 2014, closed airtight on both sides with parafilm, and transported to Nijmegen. Both cores were weighed and mixed anaerobically to one homogenized sample of which 10 mg of fresh weight was diluted in 25 mL of demineralized water and vigorously shaken by hand for

1 min. From these slurries three subsamples were put in 13 mL-vials to which 80 μL of 3 different treatment solutions was added to determine potential N loss rates to the atmosphere: A) KCl (control); B) 500 μM $K^{15}NO_3$ and 500 μM NH_4Cl; C) 500 μM $^{15}NH_4Cl$ and 500 μM KNO_3. Each vial was sealed with a plastic cap with a rubber gas sampling septum, flushed with Argon for 2 min to get anaerobic conditions and vigorously shaken (20 s) (t = 0). The vials were incubated in the dark on a rotary shaker (90 rpm) and headspace samples were taken at t = 4, 8 and 20 h and directly analyzed for isotopic composition of N gasses using gas chromatography (Agilent 6890 equipped with a Porapak Q column at 80 °C and a TCD detector at 300 °C, Agilent Technologies, Santa Clara, CA, USA) combined with mass spectrometry (Agilent 5975c quadruple inert MS, Agilent technologies, CA, USA). From these concentrations over time, slopes were calculated for increase in N_2 and N_2O in the headspace and in the slurry. Solubility ratios of 0.016 for N_2 (Weiss, 1970) and 0.6 for N_2O (Tiedje, 1982) were used, based on the Bunsen absorption coefficient, taking the dissolved fraction of gas into account. Background denitrification was then calculated from the $^{28}N_2$ signal and potential denitrification by adding up the $^{30}N_2$ and $^{30}N_2O$ production from the incubations with labeled NO_3^-. Incubations with labeled NH_4^+ enabled us to determine possible anaerobic ammonium oxidation (anammox) in production of $^{29}N_2$.

2.5. Statistics

Values displayed in bar graphs are means ± standard error (SEM) (N = 4). To test for the effect of N addition, one-way analyses of variance (ANOVAs) were conducted, using N treatment as an independent variable (fixed factor) with three categorical groups: control, Nred and Nox, and two-way ANOVAs when species data were available with species as an additional fixed factor. All dependent variables were quantitative and at a continuous scale, i.e. N_2 fixation rate, N content, amino-acid concentration, potential denitrification rate and pore water and soil nutrient concentration. Normality was tested with a Shapiro-Wilk test and data that were not normally distributed were log-transformed prior to analysis to meet conditions of parametric tests. Homogeneity of the data was checked with Levene's test of equality of variances; when variances were not equal, a nonparametric test was used. When significant differences were found, Bonferroni post hoc tests were conducted to compare groups. No interaction effects were found for any of the parameters and significance was accepted at a confidence level of $P < 0.05$. Statistical tests were performed using IBM SPSS Statistics 21.0 (IBM Corporation, released 2012, New York, USA).

3. Results

The results will be displayed in order of compartments affected by N deposition: storage in moss and lichen tissue and pore water, leaching to peat soil and its pore water, and output to and input from the atmosphere by microbial communities.

3.1. N accumulation in mosses and lichen

In the pore water of the *Sphagnum* vegetation layer concentrations of NO_3^- and NH_4^+ did not change by Nox or Nred treatments compared to the control, but there was a trend of a reduced pH with Nred, from 4.9 to 4.2 (F = 4.635; P = 0.07) (Table 1).

In moss tissue, addition of both Nox and Nred led to a 15–90% increase of N concentration for all species (F = 17.339; $P < 0.001$), with no differences between N forms. The upper 2 cm of *Sphagnum* species and *Pleurozium* had similar control N concentrations, but the lichen *Cladonia*, which had a significantly lower control N concentration (F = 15.757; $P < 0.001$) showed a very strong increase of 90% with N addition (Fig. 1). In *S. capillifolium* N concentration was examined in more detail by measuring N concentrations in amino acids. For this species, a 4-fold increase in N concentration in free amino acids (aaN) was found with Nred (F = 5.184; $P < 0.05$), corresponding to an increase in fraction of aaN per total N concentration from 7.6% to 13.8% (F = 8.890; $P < 0.01$). In contrast, no increase in amino acids was found with Nox (Fig. 2). P and K concentrations in *S. capillifolium* did not differ between treatments, but N: P ratios increased with both N additions from 1:28 to 1:46 (F = 9.340; $P < 0.01$) and N: K ratios with Nred only from 1:3.5 to 1:5.5 (F = 5.803; $P < 0.05$) as a result of increased N concentrations (Table 1).

3.2. N leaching to deeper peat

At 10 cm depth the bulk density of the peat did not differ between treatments and for both forms of N treatment the ratio between dissolved organic carbon (DOC) to total N (TN) decreased (F = 6.752, $P < 0.05$). DOC was increased with Nox compared to Nred (nonparametric tests; $P < 0.05$). The concentration of NH_4^+ in peat pore water was found to increase 2 to 3 fold for both N treatments (F = 6.210; $P < 0.05$), whereas dissolved organic N (DON) concentration was doubled by Nox, compared to Nred only (F = 4.975; $P < 0.05$), almost doubling the TN concentration from 65 μmol L^{-1} in control plots to 118 μmol L^{-1} in Nox plots (F = 4.918; $P < 0.05$) (Fig. 2a and 3). PO_4^{3-} and NO_3^- concentrations were negligible at both 10 cm and 50 cm soil depth, and unaffected by N deposition. The cation exchange capacity (CEC) of the soil and the concentration of CEC-bound NH_4^+ did not differ with treatments.

Nox also increased pore water pH from 4.2 to 4.7 at 10 cm depth (F = 13.115, $P < 0.01$) and by 0.2 pH units at 50 cm depth (F = 8.393, $P < 0.05$) (Table 2a). At 50 cm depth, NH_4^+ concentration and TN were no longer affected by either of the N deposition treatments.

Table 1
pH values (average ± SEM) of pore water within the *Sphagnum* layer and nutrient ratios of *Sphagnum capillifolium* tissue, for the control and both N addition treatments. The last column (stats) gives F values of the ANOVAs.

Sphagnum	Treatments			Stats
	Control	Nred	Nox	
pH	4.85 (±0.27)	4.14 (±0.18)	4.94 (±0.14)	F = 4.635
N:P ratio	28.23 (±3.76)	46.11* (±1.12)	45.83* (±4.28)	F = 9.340
N:K ratio	3.49 (±0.28)	5.67* (±0.38)	5.45 (±0.72)	F = 5.803

* Indicates significant difference from the control at the 0.05 confidence level.

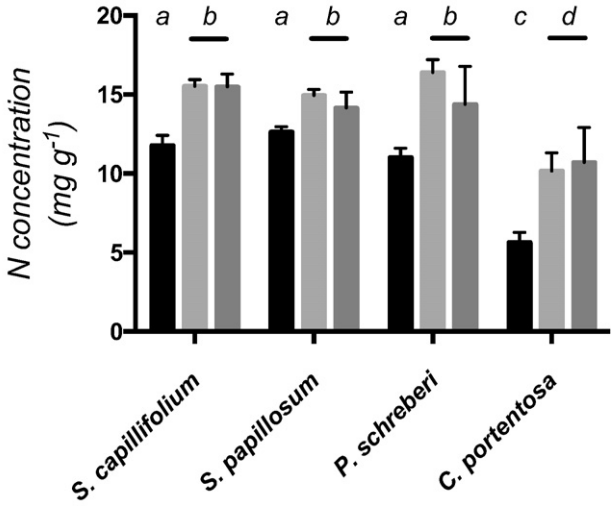

Fig. 1. N concentrations of moss species and the lichen for the control (black bars) and two N deposition treatments, Nred (light grey bars) and Nox (dark grey bars). Given are the means with their standard errors (N = 4); significant differences between groups are indicated by different letters: a–d.

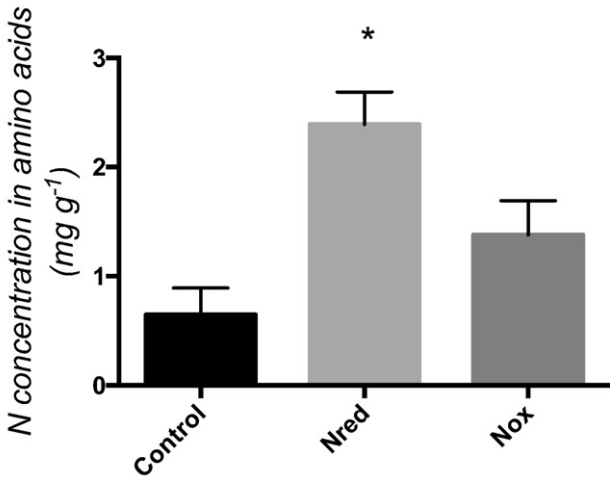

Fig. 2. Total N stored in free amino acids of *Sphagnum capillifolium*, for the control and with addition of Nox and Nred. Given are the means with their standard errors (N = 4); * indicate significant differences compared to the control.

3.3. N loss to the atmosphere

Background N_2 emission rates based on denitrification assays were 227.7 nmol N gDW^{-1} h^{-1}, increasing ~2 times with Nox, but lowered by 60% with Nred (significant difference between Nox and Nred: F = 7.649; $P < 0.05$; Table 2b). Potential denitrification, with addition of an excess of NO_3NH_4 increased emissions with 175–200 nmol N gDW^{-1} h^{-1} additional nitrogen gasses, which consisted for over 90% of N_2O for all treatments. With addition of labeled NO_3^- or NH_4^+, no increase in $^{29}N_2$ signal on top of the natural background was found, indicating that N losses by anammox in the samples taken were insignificant.

3.4. Nitrogen fixation by moss and lichen symbionts

Eleven years of increased deposition of either NH_4 or NO_3 did not affect N_2 fixation rates in any of the moss or lichen species (Fig. 4). N_2 fixation rates did differ between species and their symbiont communities (F = 48.131; $P < 0.001$). In *S. capillifolium* the highest N_2 fixation rates were found, translating to 5 kg ha^{-1} y^{-1} based on a 100% cover of this species. In *S. papillosum* rates based on dry biomass were 40% lower. *Pleurozium schreberi* and *Cladonia portentosa* show respectively two and four times lower N_2 fixation rates than *S. capillifolium* per gram dry biomass.

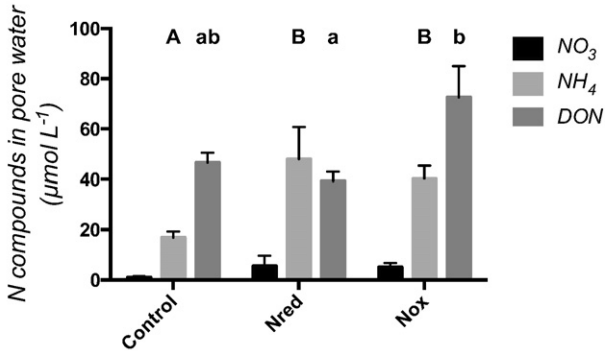

Fig. 3. Concentrations of N compounds NO_3^-, NH_4^+ and dissolved organic nitrogen (DON) in µmol L^{-1} in pore water of the peat soil at 10 cm depth for the control and N deposition treatments Nred and Nox. Given are the means (N = 4) with their standard errors; letters indicate significant differences between treatments (lower case for DON and capitalized for NH_4^+).

Table 2
a. pH and concentrations of dissolved organic carbon (DOC) and total nitrogen (TN) (mean ± SEM) of pore water in the peat soil at 10 cm depth for control and both N addition treatments. Concentrations of DOC and TN are in µmol L^{-1}. b. Incubations of peat soil of 10 cm depth without (background) and with addition of NO_3^- (potential). N_2 and N_2O losses are in nmol N gDW^{-1} h^{-1}.

	Treatments		
	Control	Nred	Nox
a. Peat soil pore water			
DOC	3304.6 (±247.6)	2682.2 (±160.5)	4568.7* (±909.5)
TN	64.61 (±3.68)	92.76 (±16.40)	117.80* (±12.22)
pH	4.25 (±0.04)	4.16 (±0.08)	4.66* (±0.09)
b. Peat soil incubations			
background N_2 losses	227.71 (±78.85)	82.27 (±42.14)	441.80* (±57.80)
potential N_2 losses	11.75 (±5.32)	11.62 (±4.71)	11.00 (±4.92)
potential N_2O losses	189.50 (±7.76)	164.19 (±51.96)	183.50 (±76.60)

* Indicates significant difference of Nox (compared to control: pH and TN; or compared to Nred: DOC, background N_2 losses) at the 0.05 confidence level.

4. Discussion

4.1. Effects of increased N deposition on N_2 fixation rates and moss N concentration

Eleven years of increased N deposition resulted in increased tissue N concentrations for all bryophytes and the lichen, in accordance with previous studies (Granath et al., 2012; Nordin et al., 1998; Remke et

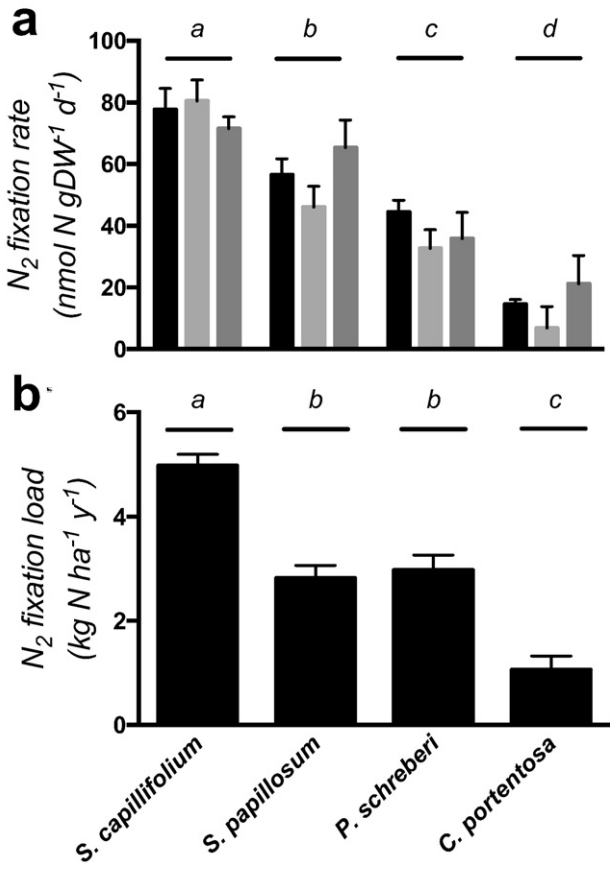

Fig. 4. a) N_2 fixation rates in 3 moss species and the lichen for controls (black bars), and for addition of Nred (light grey bars) or Nox (dark grey bars) nitrogen. Given are the means with their standard error (N = 4). Both N deposition forms did not significantly change N_2 fixation rates compared to the controls. b) Average N loads by N_2 fixation for each moss species, for patches with 100% cover by the species. In both graphs significant differences between species are indicated by different letter codes (a–d).

al., 2009), but no differences were found between different deposition forms (Fig. 1). Especially *Cladonia portentosa*, often used as biomonitor for N pollution (Remke et al., 2009), but also *Pleurozium schreberi* were found to be sensitive to N deposition with a tissue N increase of respectively 90 and 40%. Interestingly, these species were also found to show the lowest rates of N_2 fixation by their microbial communities (Fig. 4). For all species, higher N_2 fixation rates of symbionts coincide with higher N concentrations in host species at background N deposition. *Sphagnum capillifolium* received over 30% higher N_2 fixation rates compared to the other species, both based on dry weight and on unit area (Fig. 4), showing that host species are important in explaining the activity of their microbial community. This variation in diazotrophic communities between species may, however, also be driven by differences in the abiotic conditions of the habitats that different host species provide, for example humidity, light and elements like phosphorus (P) or molybdenum can determine composition and thus activity of their microbial communities (Vitousek et al., 2002; Warren et al., 2017).

Surprisingly, given the theoretical suppression of N_2 fixation by high N availability, we found no effect of the N deposition load of 32 kg ha^{-1} y^{-1} in either form in any of the moss species. Similar results were found in short-term N addition experiments for *Sphagnum magellanicum* from a low N deposition (<0.5 kg N ha^{-1} y^{-1}) site with addition of 200 kg N ha^{-1} y^{-1} (Kox et al., 2016) and in *Pleurozium schreberi* with N additions <10 kg ha^{-1} y^{-1} (Rousk et al., 2014). Long-term field studies in boreal forests showed no difference in N_2 fixation rates of *Pleurozium schreberi* for N deposition loads between 3 and 12 kg ha^{-1} y^{-1} (Gundale et al., 2011), but higher N_2 fixation rates were found at N deposition loads between 0 and 3 kg ha^{-1} y^{-1} (Gundale et al., 2011; Leppänen et al., 2013). In our study, the additional N deposition of 24 kg ha^{-1} y^{-1} on top of the background deposition of ~8 kg ha^{-1} y^{-1} in both reduced and oxidized form does not affect the activity of the diazotrophs of any of the moss and lichen species tested. This strongly suggests that the background deposition at this research site already exceeds the critical load of N deposition beyond which N_2 fixation rates are affected, in agreement with literature. Evidently, N_2 fixation activity of the microbial communities is not completely down-regulated by high N deposition, probably because of fast uptake or leaching of N by host species and N_2 fixation rates are appreciable compared to the background N deposition (Fig. 4b). Adding up loads from N_2 fixation in all assessed species based on their coverage gives a rate of 1.8 kg N ha^{-1} y^{-1} of the system, which is within the range of earlier estimates of N_2 fixation rates in bogs: 0.7–10 kg N ha^{-1} y^{-1} (Granhall and Selander, 1973; Larmola et al., 2014; Waughman and Bellamy, 1980) and represents an ecologically significant load of ~23% of total background deposition in this bog.

S. capillifolium, the keystone species, received highest N input from N_2 fixation rates (Fig. 4), which, based on the species bog cover add up to 1.2 kg N ha^{-1} y^{-1}. In this species, P, an important regulating factor of N_2 fixation (van den Elzen et al., 2017; Vitousek et al., 2002) was found to remain unchanged with N treatments. This unchanged P availability for the microbial community can explain why N_2 fixation rates are unaffected by N addition. The ratio's of N:P and N:K in *Sphagnum* (Table 1), however, were found to increase from slight N limitation in the control to strong P and K limitation (Bragazza et al., 2004) as a result of the long-term N treatments. So, over the course of 11 years of N treatments, N limitation for growth was relieved resulting in N accumulation to concentrations of over 15 mg g^{-1}, which was postulated as the threshold above which peat moss growth is hampered (Fritz et al., 2012; van der Heijden et al., 2000).

4.2. Sphagnum physiology affected by reduced N deposition

The concentration of N in amino acids (aaN) was increased with Nred only, to over 2 mg g^{-1} (Fig. 2), the given threshold for decrease in *Sphagnum* growth (Nordin and Gunnarsson, 2000). This indicates a stress response to the increased uptake of NH_4^+ by *S. capillifolium* that is consistent with the slow decline in cover of this species in response to cumulative Nred loads, and not to Nox loads, as was found by Sheppard et al. (2014) at this experimental site. This detrimental effect of Nred by *Sphagnum* spp. taking up more N than they can detoxify as amino acids was found before (Paulissen et al., 2016) and the fraction of total N in amino acids with Nred that we found compare to aaN fractions in *Sphagnum* spp. with addition of high N loads (20 kg ha^{-1} y^{-1}) accumulating over several years (Tomassen et al., 2003). We also found this N accumulation in amino acids to increase over time and with increasing N deposition load (results not shown). These results clearly indicate that in peatlands Nred is more detrimental to the *Sphagnum* vegetation than Nox, which was found for heathlands and mesotrophic peatlands too (Van Den Berg et al., 2005; Verhoeven et al., 2011).

Although more N is stored in N-rich free amino acids with Nred, tissue N concentrations do not differ between both forms of N deposition (Fig. 1). Since *Sphagnum* is very effective at the reallocation of N from old to new tissue (Aldous, 2002), the total tissue N concentration represents a balance between N acquired from reabsorption, N deposition, N_2 fixation and growth. It is likely that less N is reabsorbed from older tissue to compensate the excess uptake of N with Nred, and that in the capitula with Nox relative to Nred more N was used in important cell structures and proteins. This shows that the assessment of N allocation to N-rich amino acids provides a better indicator of ecosystem status in response to increased N deposition than tissue N concentrations alone.

4.3. Differential effects of Nred and Nox on peat biogeochemistry

In earlier studies on this unique long-term N application experiment in a peat bog, it was shown that high loads of 64 kg ha^{-1} y^{-1} of wet deposition, particularly as NH_4^+, compromise the *Sphagnum* filter function (Chiwa et al., 2016; Sheppard et al., 2013). The low dissolved N concentrations in *Sphagnum* pore water suggested that the filter was still functioning at 32 kg ha^{-1} y^{-1}. However, this dose of N deposition well exceeds the suggested threshold of 20 kg ha^{-1} y^{-1} (Harmens et al., 2014; Lamers et al., 2000), and we indeed found 3 times higher NH_4^+ concentrations in pore water in peat soil (upper 10 cm) with both N deposition forms (Fig. 3). The low concentrations of inorganic N in *Sphagnum* pore water rather reflect the rapid uptake of N by *S. capillifolium* than unaffected functioning of the *Sphagnum* filter. The effective (passive) uptake of NH_4^+ (Fritz et al., 2014) in exchange for H^+ ions lowered the pH in the *Sphagnum* vegetation layer, and excess NH_4^+ was still leaching rapidly through the *Sphagnum* vegetation. At 50 cm depth all additional NH_4^+ seemed to be denitrified and/or taken up by vascular plant roots.

Nox did not change the concentration of NO_3^- of the pore water in peat soil, indicating that all NO_3^- was rapidly converted to different N forms. In the upper 10 cm layer of the peat Nox led to increased NH_4^+ concentrations, probably by increased peat N mineralization. NO_3^-, which is a strong electron acceptor, is quickly denitrified, speeding up N mineralization, and this may lead to increased concentrations of NH_4^+ and also of DON (Fig. 3). DOC was 1.5 times increased with Nox compared to Nred, suggesting increased decomposition. Alternatively, leaching NO_3^- could be converted to NH_4^+, as a result of dissimilatory NO_3^- reduction to NH_4^+ (DNRA), a process that can be expected in anoxic soils with high organic matter content (Rutting et al., 2011). Both DNRA and organotrophic denitrification lead to an increase in pH of the soil (Simek and Cooper, 2002), which again speeds up decomposition rates (Lamers et al., 1999; Smolders et al., 2002). Increased concentrations of total N in the peat soil by Nox and Nred leaching deeper in the peat soil seem to be taken up to a certain extent by deeper rooting vascular plants. Indeed, vascular plants, especially *Calluna vulgaris*, were shown to profit from increased N loads of Nred and Nox at this experimental site (Sheppard et al., 2014).

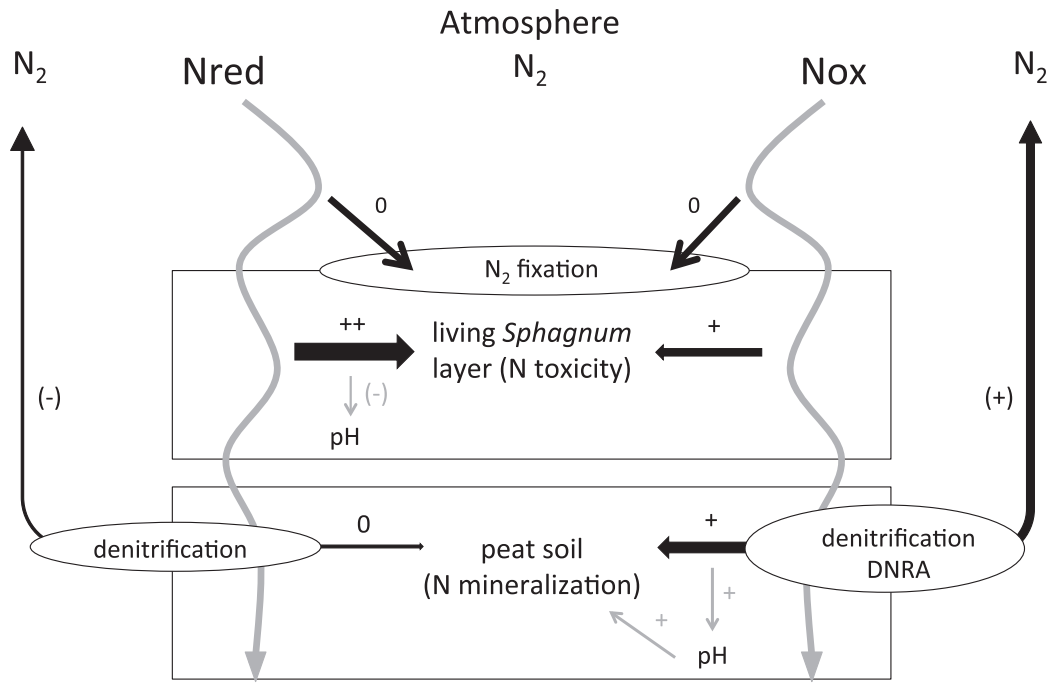

Fig. 5. Conceptual model for the differential effects of Nred (NH_4^+ deposition) and Nox (NO_3^- deposition) in a bog, relative to background deposition. Rectangles represent compartments of bog system (living *Sphagnum* layer and peat soil); microbial processes converting N compounds are displayed in circles. Grey arrows indicate the N deposition flow for Nred and Nox, black arrows represent effects of deposition on different departments and N conversions of microbial communities. Effect magnitude relative to the control is represented by the width of the arrow and by: +: doubling/2 times increase; ++: <2 times increase; 0: no effect and (+) or (−) refer to an increasing or decreasing trend. Side effects of pH are displayed by grey arrows with grey + (increase) or − (decrease).

4.4. N losses to the atmosphere

Background N_2 emissions of the controls translated to 364 μmol N m^{-2} h^{-1} (for a soil depth of 1 cm) and were high compared to a range of denitrification rates of different types of wetlands of 20–260 μmol N m^{-2} h^{-1} (Seitzinger, 1994). We found a 2.5 times higher denitrification rate with Nox compared to Nred (Table 2b), comparable with the trend of N_2O emissions found by Sheppard et al. (2013) in this bog. Increased N_2 emissions with Nox can be expected as NO_3^- can directly be used for denitrification, while NH_4^+ from Nred first has to be oxidized to NO_3^- and nitrification rates can be expected to be low in acidic bogs (Bayley et al., 2005). In addition, the increased N_2 emissions may also be induced by the increased pH (Francez et al., 2011; Seitzinger, 1994) or by the higher availability of DON in these soils (Hill et al., 2016). No indications for anammox were found in this bog, probably as a result of the fast conversion of NO_3^- by denitrification and/or DNRA. Potential denitrification consisted mostly of N_2O (Table 2b), consistent with the relatively low pH of the peat soil (Simek and Cooper, 2002; Van den Heuvel et al., 2011) and this corresponds with the finding that Nox increases gaseous N emissions, especially of N_2O (Lozanovska et al., 2016; Roobroeck et al., 2010). This is important because N_2O is the third most important contributor to global warming (Forster et al., 2007).

5. Conclusion

Although the tissue N concentration strongly increased in moss species in this raised bog as an effect of increased oxidized and reduced N deposition, N_2 fixation rates of their symbiotic microbiomes were, surprisingly, not affected by high loads of N deposition in either form. Apparently, the keystone species, *Sphagnum* spp., that evolved in N limited environments, are adapted to effectively assimilate N, but are not capable of down-regulating N uptake at high N inputs. Their N_2 fixing symbionts are not actively inhibited, leading to appreciable additional N inputs of around 1.2 kg ha^{-1} y^{-1} to the system, based on *S. capillifolium* bog coverage. In addition, Nred affects moss vitality more than Nox, given the fact that the amino acid N content increased to the threshold in concert with a decline in cover (Sheppard et al., 2014). Where Nred has negative effects on *Sphagnum* physiology and cover in the bog, Nox in contrast leads to stronger leaching of N to the peat soil, where it results in higher N reduction rates and increasing pH, speeding up N mineralization rates, and leading to higher N_2O emissions. A synthesis of our results is shown in fig. 5.

Our results show the need to consider both N forms in atmospheric deposition (NH_4^+ and NO_3^-) separately when assessing the effects of increased N deposition on bog ecosystem functioning. Moreover, this study adds to the scientific evidence that elevated N input targets the weak spot of a living bog, impacting all parts of the N cycle.

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