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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

• N₂ 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

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 N₂ 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, NH₄⁺, versus nitrate, NO₃⁻) 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 NH₄⁺ or NO₃⁻ addition resulted in enhanced peat N mineralization linked to microbial NO₃⁻ 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 N₂ 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.

Keywords:
Sphagnum capillifolium
Nitrogen deposition
Biogeochemical processes
Diazotrophs
Amino acids
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., 1998; Porter et al., 2013). Ombrotrophic bogs typically develop under conditions where N leaches through the peat moss, which makes Sphagnum spp. (a feather moss) also grows in symbiosis with N-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 peak accumulation through their stimulation of primary production (Vile et al., 2014).

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$_2$O 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).


denitrification by microorganisms associated with peat soil and vegetation (Vitousek et al., 2013).

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--Ericophorum vaginatum community (UK NVC M9) (Rodwell, 1991) with hummocks of Sphagnum capillifolium and hollows containing mostly S. papillosum. Other common species are Calluna vulgaris, Ericophorum vaginatum, Erica tetralix; the mosses

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.

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**Pleurozium schreberi, Hymen 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 m². 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 NO₃⁻ (Nox) applied as NaNO₃ and reduced N or NH₄⁺ (NRed) applied as NH₄Cl. 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, 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-NH₄ and 24 kg N-NO₃.

### 2.2. Plant analyses

Sampling of moss and lichen tissue took place in September 2013, with a week after average rainfall (total 25.6 mm). Of each species (Sphagnum capillifolium, S. papillosum, Pleurozium schreberi and Cladonia portentosa) two 21 cm² × 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 which one was put in a zip lock bag to serve as a background isotopic signature. Each core was separated in an upper (0–10 cm depth), a middle (10–50 cm depth), each part was placed in a closed ziplock bag and transported to the lab in Nijmegen, the Netherlands. All samples were dried at 100 °C 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 HNO₃ (65%) and 1 mL H₂O₂ (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 rhizion 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 (PO₄³⁻), NH₄⁺ and NO₃⁻ with an Auto Analyzer system (Bran & Luebbe, Norderstedt, Germany) using ammonium molybdate (Henriksen, 1965), hydrazine sulphate (Kampfke 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 NO₃⁻ and NH₄⁺ 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 SrCl₂ or 100 mL of N₂ 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, Mg, Mn, Na, Si and K by inductively-coupled plasma emission spectrometry (IRIS Intrepid II, Thermo Electron corporation, Franklin, USA), and for NH₄ with an Auto Analyzer system (see above). Cation Exchange Capacity (CEC) was calculated by subtracting the concentrations of Ca, Mg and K extractable by SrCl₂ 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 slurrys three subsamples were put in 13 mL-vials to which 80 μL of three different treatment solutions was added to determine potential N loss rates to the atmosphere: A) KCl (control); B) 500 μM K\(^{15}\text{NO}_3\) and 500 μM NH\(_4\)Cl; C) 500 μM \(^{15}\text{NH}_4\)Cl 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 gases 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). From these concentrations over time, slopes were calculated for increase in N\(_2\) and N\(_2\)O in the headspace and in the slurry. Solubility ratios of 0.016 for N\(_2\) (Weiss, 1970) and 0.6 for N\(_2\)O (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 \(^{29}\text{N}_2\) signal and potential denitrification by adding up the \(^{28}\text{N}_2\) and \(^{29}\text{N}_2\)O 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 \(^{29}\text{N}_2\).

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 (ANOVA)s were conducted, using N treatment as an independent variable (fixed factor) with three categorical groups: control, Nred and Nox, and two-way ANOVA}s 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 homogeneity of the variances was checked with Levene’s test of equality of variances; when variances were not equal, a nonparametric test was used. When 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

<table>
<thead>
<tr>
<th>Sphagnum Species</th>
<th>Treatments</th>
<th>Stats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Nred</td>
</tr>
<tr>
<td>pH</td>
<td>4.85 (±0.27)</td>
<td>4.14 (±0.18)</td>
</tr>
<tr>
<td>N:P ratio</td>
<td>28.23 (±3.76)</td>
<td>46.17 (±1.12)</td>
</tr>
<tr>
<td>N:K ratio</td>
<td>3.40 (±0.28)</td>
<td>5.67 (±0.38)</td>
</tr>
</tbody>
</table>

* Indicates significant difference from the control at the 0.05 confidence level.
3.3. N loss to the atmosphere

Background N₂ emission rates based on denitrification assays were 227.7 nmol N g⁻¹ DW⁻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 NO₃⁻NH₄⁺ increased emissions with 175–200 nmol N g⁻¹ DW⁻¹ h⁻¹ additional nitrogen gasses, which consisted for over 90% of N₂O for all treatments. With addition of labeled NO₃⁻ or NH₄⁺, no increase in ²⁹N₂ 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 NH₄⁺ or NO₃⁻ did not affect N₂ fixation rates in any of the moss or lichen species (Fig. 4). N₂ fixation rates did differ between species and their symbiont communities (F = 48.131; P < 0.001). In S. capillifolium the highest N₂ 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 N₂ fixation rates than S. capillifolium per gram dry biomass.

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### Table 2

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Control</th>
<th>Nred</th>
<th>Nox</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. Peat soil pore water</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DOC</td>
<td>3304.6 (±247.6)</td>
<td>2682.2 (±160.5)</td>
<td>4588.7 (±909.5)</td>
</tr>
<tr>
<td>TN</td>
<td>64.61 (±3.68)</td>
<td>92.76 (±16.40)</td>
<td>117.80 (±12.22)</td>
</tr>
<tr>
<td>pH</td>
<td>4.25 (±0.04)</td>
<td>4.16 (±0.08)</td>
<td>4.66* (±0.09)</td>
</tr>
<tr>
<td>b. Peat soil incubations</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>background N₂ losses</td>
<td>227.71 (±78.85)</td>
<td>82.27 (±42.14)</td>
<td>441.80* (±57.80)</td>
</tr>
<tr>
<td>potential N₂ losses</td>
<td>11.75 (±5.32)</td>
<td>11.62 (±4.71)</td>
<td>11.00 (±4.92)</td>
</tr>
<tr>
<td>potential N₂O losses</td>
<td>189.50 (±7.76)</td>
<td>164.19 (±51.96)</td>
<td>183.50 (±76.60)</td>
</tr>
</tbody>
</table>

* Indicates significant difference of Nox (compared to control: pH and TN; or compared to Nred: DOC, background N₂ losses) at the 0.05 confidence level.

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4. Discussion

#### 4.1. Effects of increased N deposition on N₂ 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 al., 2001).
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 N₂ fixation by their microbial communities (Fig. 4). For all species, higher N₂ fixation rates of symbionts coincide with higher N concentrations in host species at background N deposition. Sphagnum capillifolium received over 30% higher N₂ 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 N₂ 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 N₂ fixation rates of Pleurozium schreberi for N deposition loads between 3 and 12 kg ha⁻¹ y⁻¹ (Gundale et al., 2011), but higher N₂ 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 N₂ fixation rates are affected, in agreement with literature. Evidently, N₂ 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 N₂ fixation rates are appreciable compared to the background N deposition (Fig. 4b). Adding up loads from N₂ 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 N₂ 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 N₂ 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 N₂ 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 N₂ fixation rates are unaffected by N addition. The ratio 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 NH₄⁺ 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, N₂ 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 capilula 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 NH₄+, 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 NH₄⁺ concentrations in pore water in peat soil (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 NH₄⁺ (Fritz et al., 2014) in exchange for H⁺ ions lowered the pH in the Sphagnum vegetation layer, and excess NH₄⁺ was still leaching rapidly through the Sphagnum vegetation. At 50 cm depth all additional NH₄⁺ seemed to be denitrified and/or taken up by vascular plant roots.

Nox did not change the concentration of NO₃⁻ of the pore water in peat soil, indicating that all NO₃⁻ was rapidly converted to different N forms. In the upper 10 cm layer of the peat Nox led to increased NH₄⁺ concentrations, probably by increased N mineralization. NO₃⁻, which is a strong electron acceptor, is quickly denitrified, speeding up N mineralization, and this may lead to increased concentrations of NH₄⁺ and also of DON (Fig. 3). DOC was 1.5 times increased with Nox compared to Nred, suggesting increased decomposition. Alternatively, leaching NO₃⁻ could be converted to NH₄⁺, as a result of dissimilatory NO₃⁻ reduction to NH₄⁺ (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).
4.4. N losses to the atmosphere

Background N₂ 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 N₂O emissions found by Sheppard et al. (2013) in this bog. Increased N₂ emissions with Nox can be expected as NO₃⁻ can directly be used for denitrification, while NH₄⁺ from Nred first has to be oxidized to NO₃⁻ and nitrification rates can be expected to be low in acidic bogs (Bayley et al., 2005). In addition, the increased N₂ 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 NO₃⁻ by denitrification and/or DNRA. Potential denitrification consisted mostly of N₂O (Table 2b), consistent with the relatively low pH of the peat soil (Simk and Cooper, 2002; Van den Heuvel et al., 2011) and this corresponds with the finding that Nox increases gaseous N emissions, especially of N₂O (Lozanovská et al., 2012; Rönbrok et al., 2010). This is important because N₂O 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, N₂ fixation rates of their symbiotic microorganisms 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 N₂ 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 N₂O emissions. A synthesis of our results is shown in Fig. 5.

Our results show the need to consider both N forms in atmospheric deposition (NH₄⁺ and NO₃⁻) 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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