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Effects of Reduced and Oxidised Nitrogen on Rich-Fen Mosses: a 4-Year Field Experiment

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Abstract Dutch fens, subjected to high nitrogen (N) deposition levels with reduced N (NH_y) highly dominating over oxidised N (NO_x), have since the second half of the past century seen a significant decline of *Scorpidium* and other characteristic brown moss species, while several *Sphagnum* species have increased rapidly. This promotes acidification and the transition from rich to poor fens. In line with the outcomes of previous short-term water culture experiments, we hypothesised that *Scorpidium* growth is negatively affected by NH_y due to ammonium toxicity, but not by NO_x deposition, and that *Sphagnum* grows equally well on both N forms. To test this hypothesis under field conditions, we carried out a 4-year N addition experiment (5.0 g N m^{-2} $year^{-1}$, applied either as NO_3^--N or as NH_4^+-N) on natural mixed *Scorpidium revolvens–Sphagnum contortum* stands in a rich fen with relatively low background N deposition. After 4 years, ammonium addition had significantly reduced *Scorpidium* growth, while *Sphagnum* had not significantly been affected by N additions. Increased ammonium levels were directly toxic to *Scorpidium*, while *Sphagnum* was not affected. Furthermore, N addition (in particular nitrate) also indirectly influenced moss growth through promoting vascular plants. Our study confirms that it is ecologically relevant to consider the specific form in which N enrichment occurs, i.e. the ratio of NH_y vs. NO_x. We conclude that in rich fens, the risk of rapid transition of the moss layer to dominance of poor-fen species is strongly promoted by increased deposition of reduced N.

Keywords Ammonium · Bryophytes · Fens · Nitrate · Nitrogen deposition · Succession rate

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1 Introduction

In the second half of the 20th century, atmospheric deposition of reactive nitrogen (N) has strongly increased over industrialised global regions (e.g. Lövblad and Erisman 1992; Galloway 1995; Galloway et al. 2008). In many (semi-)natural ecosystems, plant species are adapted to low N availability and can only compete successfully and survive under conditions of low N input (Aerts and Chapin 2000). Bryophytes may be very

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sensitive to increased N deposition (Mäkipää 1998; Proctor 2000), both through direct toxicity effects (e.g. Jones et al. 2002; Pearce et al. 2003; Bobbink and Hettelingh 2011) and through competition with vascular plants (e.g. Bergamini and Pauli 2001). Therefore, (semi-)natural ecosystems in which bryophytes constitute a significant vegetation component—such as fens and bogs—may be especially at risk under increased N deposition (e.g. Lamers et al. 2000; Verhoeven et al. 2011).

While it is well known that many ecosystems respond strongly to elevated nitrogen deposition, the effect of the N form (in particular oxidised N vs. reduced N) has only more recently began to draw attention and is not yet fully understood (Bobbink et al. 2003; Van den Berg et al. 2008; Stevens et al. 2011; Verhoeven et al. 2011; Dorland et al. 2013; Dias et al. 2014). Increased levels of reduced N mainly originate from modern farming systems such as intensive animal husbandry, while increased oxidised N inputs originate from the combustion of fuels (e.g., Galloway et al. 2008). In the densely populated Netherlands, maximum N deposition rates (NO_x+NH_y) of >4 g m^{-2} $year^{-1}$ were attained in the second half of the 1980s and early 1990s, with ≥70 % of this in the form of NH_y (Eerens et al. 2001). Since the mid1990s, NH_y/NO_x ratios in atmospheric deposition have generally increased in Europe (Fagerli and Aas 2008).

Plant species differ in preference of and response to N form, although ammonium is more readily taken up than nitrate—even by sensitive species—because of lower energetic costs (Stevens et al. 2011). In contrast to vascular plants, bryophytes mainly rely on direct foliar uptake from atmospheric inputs, and it has been shown that dense moss layers, e.g. in peatlands, can significantly limit N availability for vascular plants as long as the bryophyte layer is not N saturated (Lamers et al. 2000; Limpens et al. 2003). In contrast to NO_x, NH_y may be toxic to many vascular plants and bryophytes, especially at high deposition rates and low acidity sites (e.g. Lucassen et al. 2003; Pearce et al. 2003; Paulissen et al. 2004, 2005; Van den Berg et al. 2005). Three non-exclusive mechanisms have been put forward to explain ammonium toxicity in plants (Stevens et al. 2011 and references therein): (1) reduced uptake of important cations K, Ca and Mg; (2) immediate metabolism of ammonium into N-rich amides and amino acids reduces available resources for plant growth and (3) following ammonium uptake and assimilation, plants need to actively control cell homeostasis including cytosol pH, and these energetic costs may slow growth. Another hypothesis, put forward by Soares and Pearson (1997), is that ammonium build-up inhibits nitrate reductase, leading to direct toxicity of ammonium.

In this paper, we focus on the effects of contrasting N forms in wet deposition on the bryophyte layer of rich fens. Rich fens are minerotrophic peatlands, fed by precipitation as well as by naturally nutrient-poor, base-rich groundwater or surface water (Sjörs 1950). The vegetation of rich fens is typically species rich with many threatened species (Verhoeven and Bobbink 2001). Bryophytes are very important in rich fens, in terms of biomass, biodiversity and as regulators of ecosystem functioning and succession via peat production (Kooijman 1992; Vitt 2000). Brown mosses dominate the bryoflora of rich fens and include *Scorpidium*, *Calliergon* and *Campylium* species (Kooijman 1992; Paulissen et al. 2014). In Europe, many fens are listed as Natura 2000 sites, and characteristic fen species and habitat types are included in the EU Habitats Directive.

In the long term, expansion of *Sphagnum* and concomitant acidification occur in many rich and poor fens, although these may persist for centuries without significant acidification, especially under pristine conditions (O'Connell 1980; Gorham et al. 1987; Vitt 2000). In contrast, the rate of floristic change in Dutch fens has been very high (cf. Bakker et al. 1994, 1997; Van Diggelen et al. 1996). Especially since 1980, many brown moss species have shown a marked decline, whereas several *Sphagnum* and *Polytrichum* species have shown a rapid increase (Kooijman 1992; Paulissen et al. 2014). This has resulted in an accelerated transition of Dutch rich fens into floristically impoverished poor fens (Beltman et al. 1995; Schaminée et al. 1995). Several non-exclusive causes for these floristic changes have been put forward: hydrological change and P enrichment (e.g. Van Wirdum et al. 1992; Kooijman and Paulissen 2006). but also ammonium enrichment (Van Baaren et al. 1988; Paulissen et al. 2004, 2005). In Dutch fens, atmospheric N inputs are much more important than N supply via surface water or groundwater (Koerselman et al. 1990).

Water culture experiments in which typical brown mosses, *Sphagnum* and *Polytrichum* species from Dutch fens, were exposed over a 2–3-month period to nitrate, ammonium or a mixture of both N forms have shown

that brown mosses prefer nitrate to ammonium and are sensitive to ammonium levels as measured in Dutch rainwater in the 1980s and 1990s. In contrast, *Sphagnum* and *Polytrichum* are indifferent to N form and are much less sensitive to ammonium (*Sphagnum contortum* being less sensitive than brown mosses, but more sensitive than *Sphagnum squarrosum*). The efficiency of the ammonium assimilation apparatus, producing N-rich amino acids, probably plays a key role in determining the sensitivity to increased ammonium deposition (Paulissen et al. 2004, 2005). Verhoeven et al. (2011) have shown in a field experiment that ammonium addition strongly reduces species diversity and biomass of rich-fen mosses, while nitrate addition does not. However, the expected shift from a brown moss to a *Sphagnum*-dominated bryophyte layer, which marks the start of the transition into a poor fen, has so far not been demonstrated in a detailed field study.

To investigate the effects of increased NO_x or NH_y inputs on contrasting bryophyte species from the initial rich-to-poor fen transition, we carried out a 4-year nitrogen addition experiment in a relatively unpolluted site in central Ireland. We added nitrate or ammonium to naturally mixed *Scorpidium revolvens–Sphagnum contortum* stands. The rate of N addition that we applied corresponds to the higher end of the N deposition range measured in industrialised countries since the 1980s (e.g. Roelofs 1986; Bobbink et al. 2010). We hypothesised that *Scorpidium* growth is negatively affected by ammonium due to its toxicity, but not by nitrate addition and that *Sphagnum* grows equally well on both N forms (cf. Paulissen et al. 2004, 2005). We measured the response to the nutrient addition treatments of both bryophyte species in terms of mass growth and cover. In addition, we measured nutrient and free amino acid concentrations in the bryophyte tissue. Previous studies have indicated the importance of phosphomonoesterase (PMEase) and phosphodiesterase (PDEase) in the P nutrition of bryophytes and vascular plants under conditions of increased P limitation due to high external N inputs (e.g. Press and Lee 1983; Turner et al. 2001, 2003; Phoenix et al. 2003). Therefore, potential uptake rates of organically bound phosphate (phosphatase activity) were measured halfway through the experiment in both species as an indication of possible N treatment effects. Furthermore, the change in vascular plant cover was investigated, because this may also indirectly influence the cover of the mosses.

2 Materials and Methods

2.1 Site Description

The experiment was carried out at Scragh Bog in central Ireland (53° 35′ N, 7° 22′ W). This is a calcareous valley fen (EUNIS D4.1; European Environment Agency 2004) surrounded by eskers (glacial melt water deposits) that are intensively used as pastureland (O'Connell 1980, 1981). During the experiment, estimated background deposition in this region was 0.7–1.0 g N m^{-2} $year^{-1}$ (cf. Aherne and Farrell 2002), and the site was not subjected to any form of active management. Scragh Bog is supposedly fed by springs and drained by a single stream at its north end. Occasionally, the fen is flooded during the winter season, with water levels up to 50 cm above the soil surface. The fen is partially fed by runoff and shallow groundwater originating from the surrounding pastureland. This has created a marginal zone (ca. 5 m wide) containing high-productive plant species. However, the major part of the fen is characterised by typical rich-fen vegetation and oligotrophic conditions, with very low water and soil concentrations of N and P (Beltman et al. 2002).

2.2 Experimental Set-Up

From June 2000 to August 2004, we carried out a nitrogen addition experiment in the oligotrophic part of the fen, which was characterised by a mosaic of EU Habitat types 7230 (Alkaline fens) and 7140 (Transition mires and quaking bogs). The experiment had a randomised block design, with five blocks of three plots each. Treatments were not replicated within the blocks. The plots consisted of 40×70 cm plots within natural and apparently P-limited (Paulissen 2004) bryophyte gradients of *Scorpidium revolvens* (Swartz) Rubers (hereafter referred to as *Scorpidium*) to *Sphagnum contortum* Schultz (hereafter referred to as *Sphagnum*). All plots were situated within an overall area of ca. 50× 25 m. Distance between replicate blocks ranged from ca. 3–11 m and between plots within blocks from ca. 1.3–11 m. The plots were treated twice per year (in spring and in summer or early autumn) by applying an artificial rain solution. The treatments were: control (water only), 5.0 g NO_3^--N m^{-2} $year^{-1}$ (as $NaNO_3$) and 5.0 g NH_4^+-N m^{-2} $year^{-1}$ (as NH_4Cl). We observed no visual damage to the vegetation resulting from the addition of the nutrient solutions (0.5 l per plot).

2.3 Phosphatase Assays

In August 2002, shoots of *Scorpidium* and *Sphagnum* were collected from each plot of the experiment. The most recent nutrient addition event had been 1 month earlier. The shoots were transported to the lab in a cool box and stored at 4 °C. Surface PMEase and PDEase activity of the shoot tips was measured within 24 h. The assay procedure largely followed Turner et al. (2001), but deviated in the following way: prior to the assay, the shoots were washed in deionised water (cf. Christmas and Whitton 1998). Our buffered (180 mM citric acid, pH 5.5) assay medium largely reflected nutrient concentrations in the field and was composed as follows: 25 μM KCl, 1000 μM $CaCl_2$, 101 μM $MgSO_4$, 188 μM $NaHCO_3$, 4.5 μM $FeCl_3$ (plus 40 μM Na_2EDTA), 11.6 μM H_3BO_3, 0.2 μM $MnSO_4$, 0.2 μM $ZnSO_4$, 0.03 μM Na_2MoO_4, 0.08 μM $CuSO_4$ and 0.04 μM $CoCl_2$. For each species and field plot, three vials containing plant material incubated in assay medium were prepared. Two vials received analogue substrate for PMEase and PDEase, respectively (cf. Turner et al. 2001). The final concentration of the substrate in the assay mixture was 100 μM. The third vial served as a control and received assay medium instead of substrate solution. In addition, nine blank vials (three for the PMEase, PDEase and control series, respectively) were established. These vials contained no plant material but did receive substrate solution. Enzyme activity was terminated after 50–75 min, and absorbance of the assay medium was measured at 405 nm (cf. Turner et al. 2001). To calculate PMEase and PDEase activity, a calibration curve was constructed from *para*-nitrophenol (*p*NP) standards (0–40 μM).

2.4 Analysis of Cover, Biomass, Tissue Nutrient and Free Amino Acid Concentrations

Cover of *Scorpidium* and *Sphagnum* (the dominant bryophyte species) was measured at the start of the experiment (June 2000), in August 2002 and in August 2004, using the point intercept method (Jonasson 1988). This was done using a 30×60-cm frame with a permanent grid of 189 points. The frame was put in the same position in 2000, 2002 and 2004, and the level position of the grid frame was checked before the measurements started. Parallel to the bryophyte cover measurements in 2004, vascular plant cover in the experimental plots was determined by averaging two simultaneous and independent visual estimations.

Standing crop of *Scorpidium* and *Sphagnum* was determined in August 2004 by cutting one turf (∅ 7.25 cm, depth ca. 20 cm) per species from each plot. The turfs were transported to the laboratory in plastic bags (closed tops with perforations allowing air exchange) put in a cool box. After storage at 4 °C, the green upper part of the turfs was carefully cut off and, in case shoots of other moss species were found, this material was removed. Dry mass of the collected *Scorpidium* and *Sphagnum* material was determined after drying for 72 h at 70 °C.

The dried plant material was ground using a ball mill and digested according to a modified Kjeldahl method (Bremner and Mulvaney 1982). The acid digestion samples were analysed colorimetrically (N, P) and flame-photometrically (K) on a Skalar SA-40 continuous flow analyser (Skalar BV, Breda, the Netherlands).

In addition, ca. 2–3 g fresh weight of green *Scorpidium* and *Sphagnum* shoots was collected from each plot in August 2004 and transported to the lab in sealed plastic bags in frozen condition. This material was used for analysis of free amino acids by high-performance liquid chromatography (HPLC). The method largely followed Van Dijk and Roelofs (1988), with some modifications as described in Paulissen et al. (2005).

2.5 Data Analysis

Data were analysed using IBM® SPSS® Statistics version 22. For all dependent variables except vascular plant cover and tissue-free amino acid concentrations, separate ANOVAs were run for *Scorpidium* and *Sphagnum*, with nitrogen addition treatment as independent variable and percentage vascular plant cover as a covariate. Because the block effect was consistently non-significant for the species and dependent variables tested, blocks were not included as a fixed factor in the ANOVAs. The ANOVAs were followed by Tukey's HSD analysis to compare individual treatment effects. As the assumptions underlying parametric analysis of variance (notably homogeneity of variance) could not be met for all amino acids, we analysed the effect of N treatment on tissue-free amino acid concentrations using the non-parametric Kruskal–Wallis test followed by Dunn's post hoc analysis. Dunn's test is not included in SPSS 22 and was performed in MS Excel 2000 (calculation file copyright Edwin Martens, Centre for Biostatistics, Utrecht University).

3 Results

3.1 Surface Phosphatase Activities of the Mosses

Scorpidium showed higher phosphatase activity than *Sphagnum* did after 2 years of N addition. In the latter species, PDEase activity was particularly low (Fig. 1). *Scorpidium* had developed significantly higher PMEase activity in the plots to which nitrate was added than in the control (ca. 60 % higher) and ammonium plots. *Sphagnum* showed a different PMEase response pattern. We measured a significant 70 % increase in PMEase activity of this species in the ammonium treatment, compared to the control. Nitrate addition caused a non-significant intermediate response. Neither species showed significant differences in PDEase activity between the treatments (Fig. 1).

3.2 Change in Percentage Cover and Bryophyte Living Standing Crop

Neither nitrate nor ammonium addition did significantly influence the change in percentage cover of the co-dominant bryophyte species during the 4 years of the experiment. *Sphagnum* cover increased with 20–30 % in all treatments, while *Scorpidium* cover decreased with 25–40 % (Fig. 2).

Vascular plant cover tended to increase to around 80 % after 4 years of nitrate addition, compared with 60 % in the control situation, but the difference was non-significant. Ammonium addition led to a cover level intermediate between that of the control and the nitrate addition plots (Fig. 3).

Biomass production, measured as green standing crop at harvest, of *Scorpidium* responded differently to N treatment than *Sphagnum* (Table 1, Fig. 4). In the case of *Scorpidium*, both N treatment and percentage vascular plant cover significantly impacted biomass production. In *Sphagnum*, there was no significant effect of N treatment or percentage vascular plant cover on biomass production (Table 1). Nitrate addition tended to decrease biomass production in *Scorpidium*, but the difference with the control was not significant. While ammonium tended to reduce *Sphagnum* growth, the reduction of green standing crop following ammonium addition was only significant in *Scorpidium*: it became ca. 50 g m^{-2} after 4 years of ammonium addition, compared with almost 150 g m^{-2} in the control vegetation (Fig. 4).

3.3 Tissue N, P and K Concentrations

In *Scorpidium*, tissue N concentrations significantly increased to around 22 mg N g dwt^{-1} after 4 years of ammonium addition, compared with only 12.5 mg N g dwt^{-1} in the control plots. Nitrate addition caused a non-significant intermediate increase (Fig. 5). *Sphagnum* showed a different response with much smaller changes in N concentrations. In this species, nitrate addition tended to increase tissue N concentration, while ammonium addition tended to decrease *Sphagnum* tissue N concentrations. Only the difference between the nitrate and ammonium treatment was significant (Fig. 5).

Tissue P concentrations in *Scorpidium* and in *Sphagnum* were not significantly affected by N treatment. Tissue P concentrations in *Scorpidium* were higher than in *Sphagnum* (Fig. 5).

In *Scorpidium*, tissue K concentrations after 4 years of ammonium addition were more than halved compared with the control. Nitrate addition also tended to decrease tissue K levels, but the difference with either the control or the ammonium treatment was not significant. Tissue K concentrations in *Sphagnum* showed a different response to N addition. In this species, nitrate addition tended to increase tissue K concentrations, while ammonium tended to decrease the concentrations. However, only the difference between the nitrate and ammonium treatments was significant (Fig. 5).

3.4 Tissue Free Amino Acid Concentrations

N treatment generally did not lead to a significant response of tissue free N-rich amino acid concentrations in *Scorpidium* (Table 2). The exception was the arginine concentration, which showed an almost threefold increase in response to nitrate addition and an eightfold increase in response to ammonium addition. The effect of the nitrate treatment did not differ significantly from the control and the ammonium treatment.

In contrast, tissue concentrations of the free (N-rich) amino acids in *Sphagnum* showed a significant response to N treatment, notably to ammonium addition (Table 2). Alanine concentrations significantly increased by 50 % in response to both nitrate and ammonium addition. Tissue arginine, asparagine, glutamine and serine concentrations all increased significantly in response to ammonium addition, while nitrate addition led to an intermediate increase

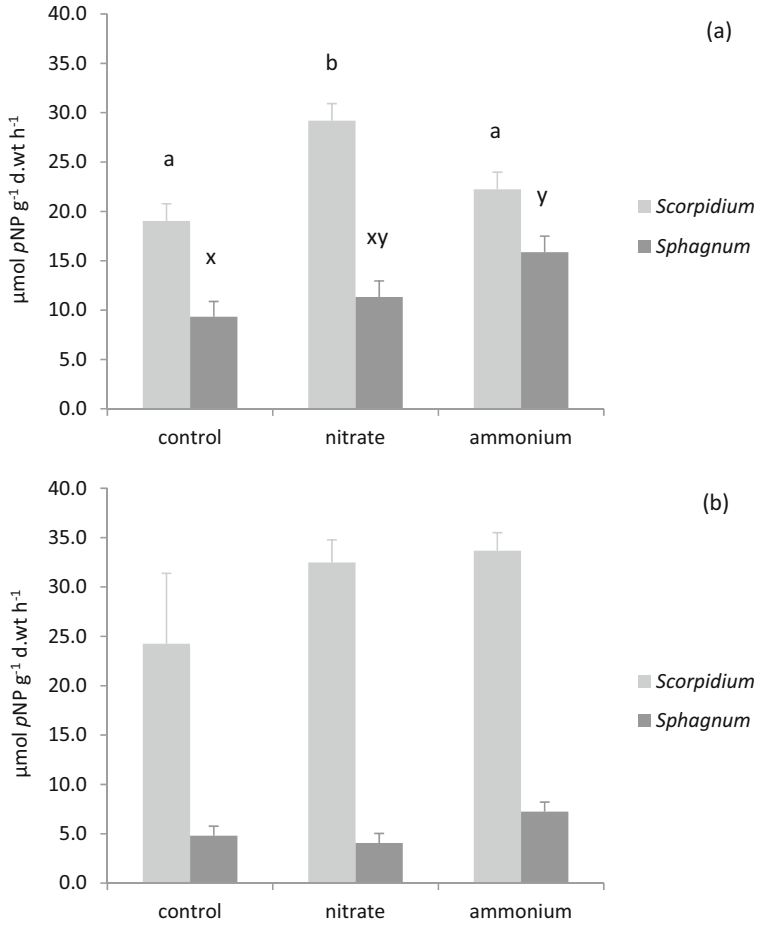

Fig. 1 PMEase (**a**) and PDEase activity (**b**) of *Scorpidium revolvens* and *Sphagnum contortum* as measured 26 months after the start of the nutrient addition treatments. *Bars* represent treatment means+1 SE; *N*=5. Within each species and enzyme type, *bars* with *different letters* are significantly different (*P*<0.05)

which did not differ significantly from the control and the ammonium treatment. Responses were strongest for arginine, asparagine and glutamine, which showed a 2 to 25-fold increase following nitrate addition and a 22 to 67-fold increase following ammonium addition (Table 2).

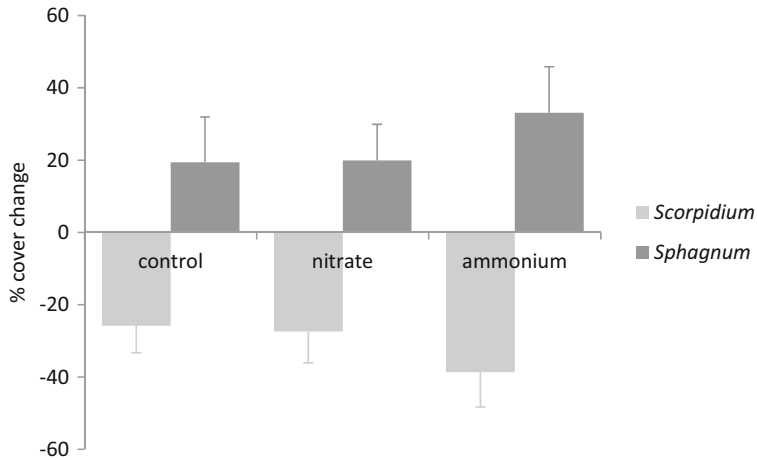

Fig. 2 Effects of nitrate or ammonium addition (4 years, 5 g N m^{-2} $year^{-1}$) on percentage cover change of *Scorpidium revolvens* and *Sphagnum contortum* in natural bryophyte gradients. *Bars* represent treatment means+1 SE, with *N*=3 for control, *N*=5 for nitrate and *N*=4 for ammonium

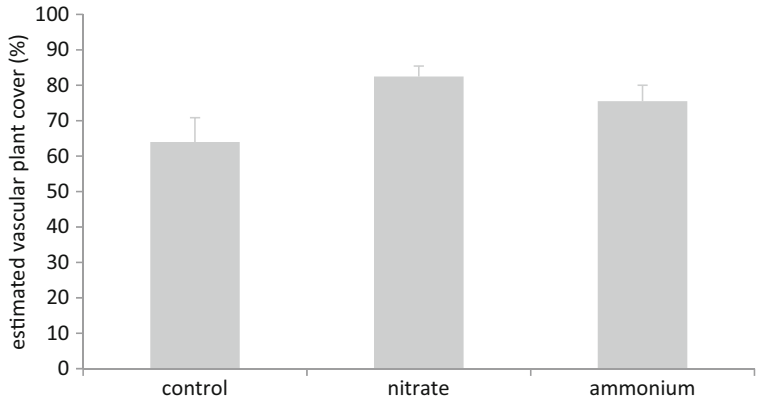

Fig. 3 Effect of 4 years of nitrate or ammonium addition (5 g N m^{-2} $year^{-1}$) on percentage cover of vascular plants present in the studied natural bryophyte gradients. *Bars* represent treatment means+1 SE; with N=5

4 Discussion

The main aim of this field study was to investigate the differential effects of increased NO_x or NH_y input on naturally mixed stands of two co-dominant rich-fen bryophytes: *Scorpidium revolvens* (a brown moss) and *Sphagnum contortum* (a peat moss). These species represent characteristic and consecutive genera in the rich-to-poor fen transition, which has accelerated in the Netherlands in the second half of the 20th century (Kooijman 1992; Paulissen et al. 2014).

The most striking result of our experiment was that after 4 years, ammonium addition significantly reduced *Scorpidium* biomass, while *Sphagnum* was not significantly affected by N addition. These results are in line with those of a previous short-term water culture experiment (Paulissen et al. 2004).

Although analysis of the main N treatment effect, with vascular plant cover as a covariate, indicated that *Scorpidium* growth was also indirectly impaired by increased shading, vascular plant growth tended to respond stronger to nitrate than to ammonium addition, confirming the toxicity effects of ammonium addition to *Scorpidium*. This was also suggested by the contrasting response of *Scorpidium* versus *Sphagnum* in several physiological characteristics (PMEase activity, tissue N and K concentrations). Similar responses have also been found in other studies for species sensitive to ammonium (e.g. De Graaf et al. 1998; Lucassen et al. 2003; Pearce et al. 2003; Paulissen et al. 2004, 2005). The most striking difference between the species in physiological response to N form was found for tissue concentrations of several N-rich free amino acids. Unlike *Scorpidium*, *Sphagnum* was able to strongly increase tissue concentrations of asparagine, arginine and glutamine, while *Scorpidium* only did so for arginine. These results agree with those of previous short-term water culture experiments in which different rich-fen and poor-fen bryophytes were exposed to increased ammonium levels (Paulissen et al. 2005). Many studies have shown that plant species, including bryophytes, can reduce or avoid ammonium toxicity by synthesising excess N into (N-rich) amino acids and amines (e.g. Nordin and Gunnarsson 2000; Limpens and Berendse 2003; Tomassen et al. 2003; Van den Berg et al. 2008). Our results show that this ammonium detoxification mechanism is relatively poorly developed in *Scorpidium*, as compared to *Sphagnum*. Other studies have also found differences in the ability of individual plant species to detoxify ammonium, with *Sphagnum* species from bogs having a relatively well-developed accumulation mechanism in response to increased N addition (Nordin and Gunnarsson 2000; Limpens and Berendse 2003; Tomassen et al. 2003).

Could the outcomes of our field experiment possibly have been influenced by the high solute concentrations in the applied treatment solutions? Although we did not observe any visual damage to the vegetation (such as chlorosis), adverse effects of ion application in high concentrations cannot fully be excluded. If in our experiment ammonium effects were exaggerated compared with the longer-term exposure at lower concentrations in the real world, the fact remains that *Sphagnum* was not sensitive, in contrast to *Scorpidium*. It seems—in addition—unlikely that any of the differential effects of nitrate and ammonium could be attributed to the different ions with which they were associated. While chloride (in our treatment bound to ammonium) is metabolically inert, it may provoke negative osmotic effects at high concentrations. Sodium, on the other

Table 1 ANOVA results table for the dependent variables tested after 2 or 4 years of nitrate or ammonium addition

Dependent variable	Source of variation	*Scorpidium*				*Sphagnum*				Vascular plants			
		df	Mean square	F statistic	Significance	df	Mean square	F statistic	Significance	df	Mean square	F statistic	Significance
PMEase activity after 2 years	N treatment	2	124.776	9.982	0.004	2	31.604	2.717	0.119				
	Vascular plant cover (covariate)	1	12.292	0.983	0.345	1	2.808	0.241	0.635				
	Error	10	12.500			9	11.630						
PDEase activity after 2 years	N treatment	2	88.757	0.787	0.481	2	19.451	1.578	0.258				
	Vascular plant cover (covariate)	1	30.578	0.271	0.614	1	69.201	5.615	0.042				
	Error	10	112.772			9	12.323						
Percentage cover change over 4 years	N treatment	2	201.599	0.547	0.599	2	296.407	0.502	0.623				
	Vascular plant cover (covariate)	1	26.905	0.073	0.794	1	180.749	0.306	0.595				
	Error	8	368.517			8	590.196						
Percentage cover after 4 years	N treatment									2	436.250	3.461	0.065
	Error									12	126.042		
Green biomass after 4 years	N treatment	2	6149.807	10.956	0.003	2	5095.760	1.050	0.389				
	Vascular plant cover (covariate)	1	3350.812	5.970	0.035	1	24,776.612	5.104	0.050				
	Error	10	561.321			9	4854.402						
Tissue N concentration after 4 years	N treatment	2	83.176	4369.000	0.047	2	20.955	3.709	0.080				
	Vascular plant cover (covariate)	1	10.981	0.577	0.467	1	0.334	0.059	0.815				
	Error	9	19.037			7	5.649						
Tissue P concentration after 4 years	N treatment	2	0.065	0.334	0.724	2	0.099	3.471	0.090				
	Vascular plant cover (covariate)	1	0.120	0.619	0.452	1	0.034	1.176	0.314				
	Error	9	0.194			7	0.029						
Tissue K concentration after 4 years	N treatment	2	34.283	6.813	0.016	2	11.655	4.001	0.069				
	Vascular plant cover (covariate)	1	7.406	1.472	0.256	1	4.408	1.513	0.258				
	Error	9	5.032			7	2.913						

No ANOVA results were available for tissue-free amino acid concentrations as these were tested using the non-parametric Kruskal–Wallis test

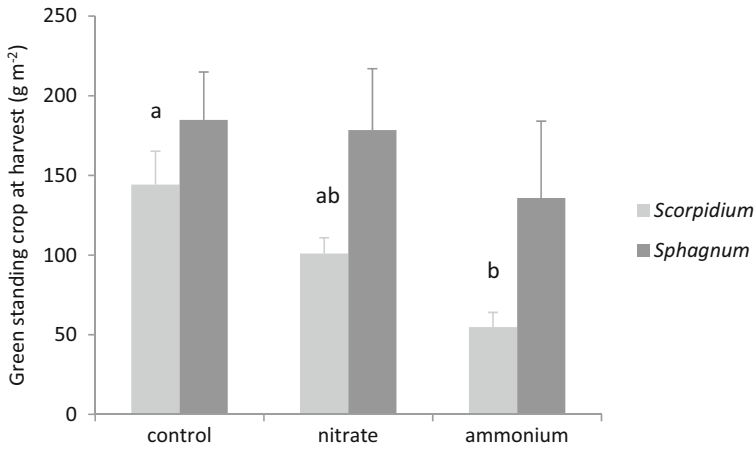

Fig. 4 Green biomass of *Scorpidium revolvens* and *Sphagnum contortum* in natural bryophyte gradients after 4 years of nitrate or ammonium addition (5 g N m^{-2} $year^{-1}$). *Bars* represent treatment means+1 SE, with N=4 for control, N=5 for nitrate and N=5 (*Scorpidium*) and N=4 (*Sphagnum*) for ammonium, respectively. Separate ANOVA tests were run for *Scorpidium* and *Sphagnum*. *Different letters* indicate significant differences (P<0.01) between N treatments

hand, can be toxic at high concentrations. Yet, in our treatment, sodium was bound to nitrate. Our results show no signs of any significant growth-inhibiting effect of application of a $NaNO_3$ solution. Possibly, the Atlantic climate at the experimental site, with very frequent precipitation events, has favoured mitigation of potential direct negative effects of high treatment ion concentrations.

Halfway through the experiment, after 2 years, there were no significant N treatment effects on bryophyte cover. However, phosphatase activity in response to N addition appeared to be an early indicator that *Scorpidium*, but not *Sphagnum*, would on the longer term be negatively affected by ammonium enrichment. Both PMEase and PDEase activity in our experiments were higher in *Scorpidium* than in *Sphagnum*. This may be linked to differences in internal P relocation efficiency between the species, as efficient P relocation reduces the need for high surface phosphatase activity. It is known that relocation of P and other elements from the lower (i.e. older) part of the shoots to the capitula can be quite efficient in *Sphagnum* (Rydin and Clymo 1989). Such relocation is probably high in summer, i.e. at the time of our phosphatase measurements (cf. Turner et al. 2003). This could explain the lower phosphatase activities in *Sphagnum*. Although internal relocation of nutrients has also been shown to occur in pleurocarpous bryophytes (e.g. Eckstein and Karlsson 1999; Bates 2000). this process may be less efficient in *Scorpidium*, increasing the need for high surface phosphatase activity in this brown moss.

Our PMEase and PDEase activity values were only partially in range with year-round activity ranges as measured in eight terrestrial and semi-terrestrial bryophyte species in upland northern England (Turner et al. 2003). PMEase activity values found for *Scorpidium* and *Sphagnum* in our study were mostly at the lower end of the ranges measured by Turner et al. (2003), or up to 50 % lower than their values. The same applies to PDEase activity of *Sphagnum* in our study, but PDEase activity of *Scorpidium* seemed to be, overall, more in range with values measured by Turner et al. (2003). These authors tested different species, which may explain why our PMEase and PDEase activity values were consistently lower, even in the high N treatments, than those found by Turner et al. (2003). An alternative explanation for the higher PMEase and PDEase activity values that Turner et al. (2003) measured may be acclimatization to chronically higher N deposition rates (estimated at 2–4 g N m^{-2} $year^{-1}$) in their upland sites than in our study site (0.7–1.0 g N m^{-2} $year^{-1}$; cf. Aherne and Farrell 2002).

The cover of the two main bryophyte species changed significantly during the 4-year experimental period. *Sphagnum* cover had increased significantly in all treatments at the expense of *Scorpidium* cover. We attribute this to autonomous succession within our research site. Increasing *Sphagnum* dominance is the long-term fate of many minerotrophic peatlands, in N-polluted as well as untouched regions. However, the succession rates may vary greatly between eutrophied and unpolluted rich fens, generally with higher rates in the former (Gorham et al. 1987; Kooijman and Bakker 1995; Vitt 2000; Paulissen et al. 2014).

After 4 years of N addition, we did not record an effect of N treatment on percentage cover of the co-dominant bryophyte species. However, the significant reduction of mass growth that we observed in

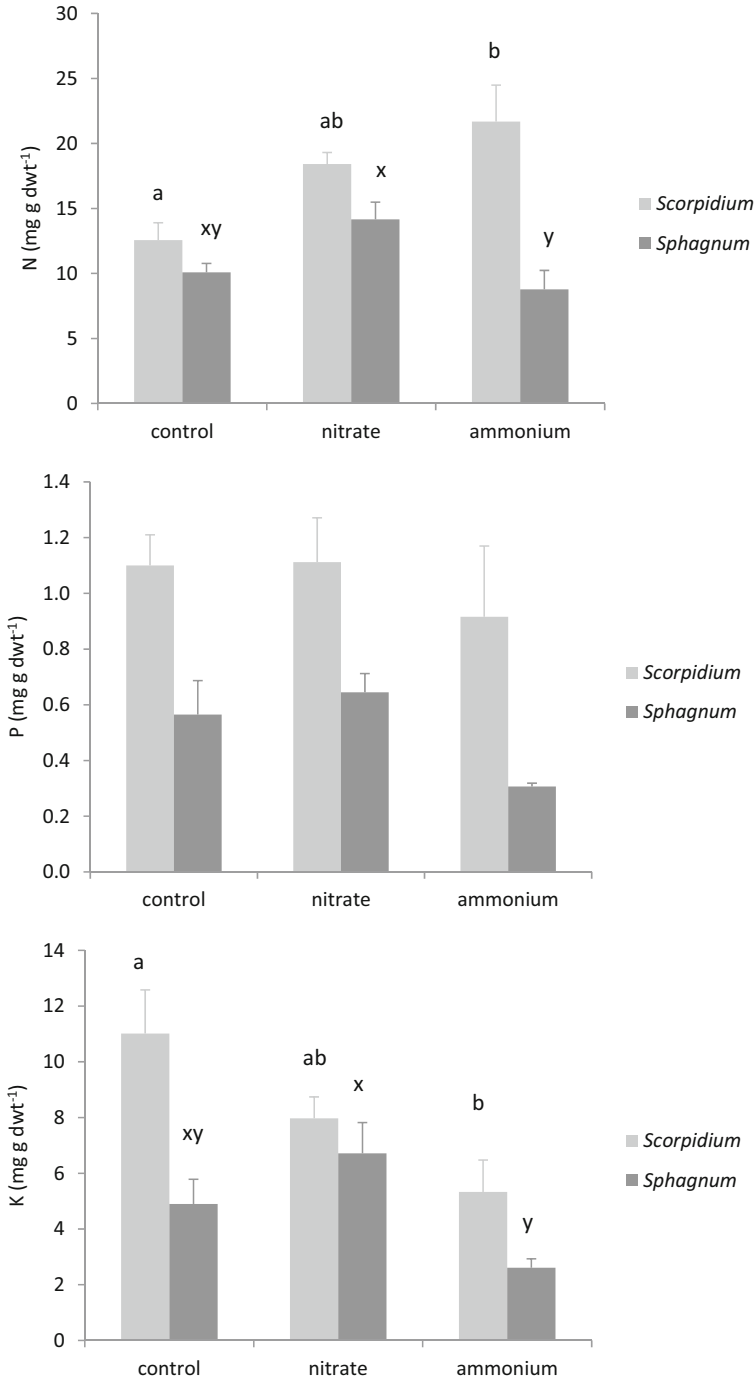

Fig. 5 Tissue N, P and K concentrations in living tissue of *Scorpidium revolvens* and *Sphagnum contortum*, after 4 years of N addition as either NO_3^--N or NH_4^+-N (5 g N m^{-2} $year^{-1}$). *Bars* represent treatment means+1 SE, with N=3 (control), N=5 (nitrate) and N=5 (ammonium) for *Scorpidium* and N=4 (control), N=4 (nitrate) and N=3 (ammonium) for *Sphagnum*. Separate ANOVA tests were run for *Scorpidium* and *Sphagnum*. *Different letters* indicate significant differences (P<0.05) between N treatments

Scorpidium following ammonium addition could well lead to more space for bryophyte species that are less sensitive to ammonium, such as *Sphagnum*. For the Dutch context, there is evidence that high ammonium deposition levels have promoted the fast transition from rich fens to floristically impoverished poor fens (Paulissen et al. 2004, 2005; Kooijman and Paulissen 2006). Unlike the situation in the valley fen where our experiment was carried out, the (often floating) fens in the Dutch polders are not naturally flooded by base-rich water in winter. Cusell et al. (2013) showed that artificially provoked inundation of rich fens with base-rich

Table 2 Concentrations (μmol g^{-1} dry wt) of the quantitatively most important free amino acids in living tissue of *Scorpidium revolvens* and *Sphagnum contortum*, after 4 years of N addition

	Control		NO_3^--N		NH_4^+-N	
	Scorpidium	*Sphagnum*	*Scorpidium*	*Sphagnum*	*Scorpidium*	*Sphagnum*
Alanine ($C_3H_7NO_2$)	10.3±5.1†	2.0±0.2a	4.3±0.6‡	2.9±0.1b	20.4±16.6	3.1±0.3b
Arginine ($C_6H_{14}N_4O_2$)	28.4±25.6^{A}†	1.8±0.5a	79.4±35.4^{AB}‡	4.8±0.8ab	234.3±27.7b	120.4±44.7b
Asparagine ($C_4H_8N_2O_3$)	77.4±42.0†	6.3±0.9a	76.2±39.0‡	159.3±25.4ab	47.3±25.5	250.1±62.8b
Aspartic acid ($C_4H_7NO_4$)	4.6±1.2†	3.6±0.5	4.7±0.8‡	4.3±0.4	3.9±0.3	3.9±0.7
Glutamic acid ($C_5H_9NO_4$)	8.3±5.6†	5.0±1.1	2.3±0.5‡	5.5±0.5	2.3±0.6	6.0±1.2
Glutamine ($C_5H_{10}N_2O_3$)	3.7±1.1†	3.0±0.8a	5.9±0.6‡	5.4±0.9ab	32.7±27.8	64.8±37.9b
Serine ($C_3H_7NO_3$)	2.5±0.8†	1.9±0.3a	4.5±0.5‡	2.4±0.2ab	3.5±0.9	3.8±0.3b
Threonine ($C_4H_9NO_3$)	1.2±0.4†	1.0±0.1	1.1±0.2‡	1.5±0.3	1.1±0.2	1.4±0.1

N was added as either NO_3^--N or NH_4^+-N, at a rate of 5 g N m^{-2} $year^{-1}$. Mean±1 SE; N=5, except † N=4, and ‡ N=3. Different letters indicate significant differences (P<0.05) between N treatments. Separate Kruskal–Wallis tests were run for *Scorpidium* and *Sphagnum*

and nutrient-poor water in summer may be beneficial to the endangered brown moss communities in the Netherlands by increasing the alkalinity. In winter, penetration of inundation water into the water-saturated shallow soil is lower, while the risk of accumulation (following flooding) of ammonium to levels toxic to brown mosses is higher than in summer. However, a pre-requisite for success is that P eutrophication due to external loading through inundation water or due to internal P mobilization in soils with a high total P content and low Fe/P ratio can be ruled out (Cusell et al. 2013).

Our study highlights the risk that increased deposition of reduced N poses to characteristic rich-fen mosses. However, our data also show that a negative effect of increased deposition of oxidised N on rich fens cannot be ruled out. Addition of oxidised rather than reduced N tended to promote vascular plant cover in our experiment. This can indirectly hamper moss growth through shading by vascular plant growth stimulation. On the longer term, it could possibly promote high-productive vascular plant species in particular. However, in an experiment that additionally focused on vascular plants and had a larger plot size than our study, Verhoeven et al. (2011) did not find an effect of nitrate addition on biomass or species number of rich-fen vascular plants and mosses. The overall nutrient status of a rich fen, including P availability, seems important in determining the effect of increased NO_x deposition (Kooijman and Paulissen 2006; Verhoeven et al. 2011). In situations where N is relatively scarce due to high P availability and/or annual mowing, increased deposition of NO_x (and NH_y) may promote vascular plant biomass to the detriment of the moss layer.

5 Conclusion

Our study clearly confirms that it is ecologically relevant to consider the specific form in which N enrichment occurs, i.e. the ratio of reduced versus oxidised nitrogen. We have shown in this field experiment that increased ammonium inputs are negative for the development of the characteristic brown moss species of rich-fen habitats, while the co-dominant *Sphagnum* species is not affected. Furthermore, N addition may also indirectly hamper moss growth through shading by vascular plant growth stimulation. In our experiment, this effect was somewhat more pronounced in response to nitrate than to ammonium addition. We conclude that, on the longer term, increased NH_y deposition may accelerate *Sphagnum* dominance, turning rich fens into poor fens. It is thus especially of high importance for the conservation of rich fens to control the emissions of reduced N from agricultural sources.

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