

RESEARCH PAPER

Nutrient additions in pristine Patagonian *Sphagnum* bog vegetation: can phosphorus addition alleviate (the effects of) increased nitrogen loads

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ABSTRACT

Sphagnum-bog ecosystems have a limited capability to retain carbon and nutrients when subjected to increased nitrogen (N) deposition. Although it has been proposed that phosphorus (P) can dilute negative effects of nitrogen by increasing biomass production of *Sphagnum* mosses, it is still unclear whether P-addition can alleviate physiological N-stress in *Sphagnum* plants. A 3-year fertilisation experiment was conducted in lawns of a pristine *Sphagnum magellanicum* bog in Patagonia, where competing vascular plants were practically absent. Background wet deposition of nitrogen was low ($\sim 0.1\text{--}0.2\text{ g}\cdot\text{N}\cdot\text{m}^{-2}\cdot\text{year}^{-1}$). Nitrogen ($4\text{ g}\cdot\text{N}\cdot\text{m}^{-2}\cdot\text{year}^{-1}$) and phosphorus ($1\text{ g}\cdot\text{P}\cdot\text{m}^{-2}\cdot\text{year}^{-1}$) were applied, separately and in combination, six times during the growing season. P-addition substantially increased biomass production of *Sphagnum*. Nitrogen and phosphorus changed the morphology of *Sphagnum* mosses by enhancing height increment, but lowering moss stem density. In contrast to expectations, phosphorus failed to alleviate physiological stress imposed by excess nitrogen (e.g. amino acid accumulation, N-saturation and decline in photosynthetic rates). We conclude that despite improving growth conditions by P-addition, *Sphagnum*-bog ecosystems remain highly susceptible to nitrogen additions. Increased susceptibility to desiccation by nutrients may even worsen the negative effects of excess nitrogen especially in windy climates like in Patagonia.

INTRODUCTION

Peatlands store substantial amounts of carbon (Gorham 1991; Yu *et al.* 2010) and therefore their conservation is considered of global importance. Cycling of carbon in peatlands is mainly controlled by peatland hydrology (Belyea & Baird 2006; Couwenberg *et al.* 2010), which is largely driven by the vegetation and litter formed therein (Ivanov 1981; Couwenberg & Joosten 1999; Fritz *et al.* 2008). *Sphagnum* mosses are important peat-forming plants because of their high capacity to retain water and nutrients, which results in waterlogged conditions and recalcitrant litter (Van Breemen 1995; Rydin & Jeglum 2006). Under pristine conditions *Sphagnum* mosses can outcompete vascular plants through monopolising the nutrient pool which is primarily derived from atmospheric inputs (Clymo & Hayward 1982; Curtis *et al.* 2005; Phuyal *et al.* 2008; Wiedermann *et al.* 2009a). As peatlands dominated by *Sphagnum* mosses are adapted to extremely nutrient-poor conditions they are highly threatened by increased atmospheric nitrogen (N) deposition (Lamers *et al.* 2000; Rydin & Jeglum 2006; Limpens *et al.* 2011).

High nitrogen loads exceed the natural absorption (filter) capacity of *Sphagnum* mosses (Lamers *et al.* 2000; Bobbink & Hettelingh 2011). This results in leaching of nitrogen through the upper moss layer which increases the availability of nitrogen to vascular plants (Limpens *et al.* 2003a; Tomassen *et al.* 2004). As a consequence, *Sphagnum*-dominated ecosystems change into non-peat-forming phanerogam-dominated systems (Gunnarsson *et al.* 2008; Limpens *et al.* 2008; Juutinen *et al.* 2010). This already occurs at nitrogen (N) loads higher than $1\text{ g}\cdot\text{N}\cdot\text{m}^{-2}\cdot\text{year}^{-1}$ (Bobbink & Hettelingh 2011), but in several parts of the world atmospheric nitrogen deposition have exceeded $4\text{ g}\cdot\text{N}\cdot\text{m}^{-2}\cdot\text{year}^{-1}$ (Galloway *et al.* 2008). The decline of *Sphagnum*-dominated vegetation is further stimulated by damage of *Sphagnum* mosses due to ammonium toxicity (Baxter *et al.* 1992; Rudolph *et al.* 1993). Increased susceptibility to pests has also been found after nitrogen enrichment (Limpens *et al.* 2003b; Wiedermann *et al.* 2007).

The most detrimental effects of increased nitrogen deposition are probably due to imbalances in nutrient stoichiometry (Bragazza *et al.* 2004; Güsewell 2004; Limpens *et al.* 2011).

Under high nitrogen deposition, a lack of phosphorus seems to limit *Sphagnum*-dominated vegetation (Aerts *et al.* 1992; Lund *et al.* 2009). *Sphagnum* mosses subsequently saturate with nitrogen as a result of limited biomass production (Lamers *et al.* 2000; Limpens *et al.* 2011). Lamers *et al.* (2000) expected that negative effects of nitrogen can be diluted by stimulated biomass production after adding limiting nutrients. It is still debated whether a better balanced nutrient stoichiometry (cf. Bragazza *et al.* 2004) can alleviate negative effects of excess nitrogen. Field experiments have indicated that addition of phosphorus can increase biomass production of (*Sphagnum*) mosses which may alleviate the detrimental effects of nitrogen (Pilkington *et al.* 2007; Limpens *et al.* 2011). Other field studies observed only modest and temporary effects of P-addition (Aerts *et al.* 1992, 2001) or did not observe alleviating effects of phosphorus (Juutinen *et al.* 2010). The present paper therefore emphasises the role of phosphorus (P) in improving the nutrient balance and alleviating stress imposed by excess nitrogen on *Sphagnum* mosses.

Increased availability of (airborne) nitrogen substantially changes the physiology of (*Sphagnum*) mosses (Rudolph *et al.* 1993; Soares & Pearson 1997; Pearce *et al.* 2003). High uptake rates can cause a rapid saturation of the nitrogen metabolism in *Sphagnum* mosses (Rudolph *et al.* 1993; Wiedermann *et al.* 2009), which is often followed by growth reduction (Baxter *et al.* 1992; Gunnarsson & Rydin 2000; Limpens *et al.* 2011). Physiological parameters (e.g. nitrogen concentration, amino acid concentration and photosynthetic efficiency) have been suggested as early indicators for increasing metabolic stress preceding impeded growth and a decreased cover of (*Sphagnum*) mosses (Baxter *et al.* 1992; Arroniz-Crespo *et al.* 2008; Wiedermann *et al.* 2009; Limpens *et al.* 2011). The formation of amino acids is thought to prevent an accumulation of toxic ammonium caused by a saturation of nitrogen in mosses (Rudolph *et al.* 1993; Wiedermann *et al.* 2009). In contrast, growth of vascular plants is stimulated by enhanced nitrogen uptake (Tomassen *et al.* 2004).

Increased nitrogen deposition also stimulates the formation of chlorophyll in *Sphagnum* mosses (Tomassen *et al.* 2003; Granath *et al.* 2009). Upland mosses receiving high nitrogen loads increased chlorophyll concentrations, but surprisingly the biomass production declined (Arroniz-Crespo *et al.* 2008). Arroniz-Crespo *et al.* (2008) suggested that photo-inhibition caused damages to the chloroplast, which reduced the efficiency of photosystem II (PSII). Photo-inhibition can be related to increased chlorophyll levels: Photo-inhibition occurs when the photosynthetic system becomes 'over-excited' when the amount of photons absorbed exceeds photosynthesis, which can be limited by other stress factors (e.g. shortage of CO₂ under wet conditions or water shortage during desiccation). Under such conditions a high chlorophyll content makes plants more susceptible to damage caused by photo-inhibition if the concentration of xanthophyll and other pigments preventing photostress is insufficient. Raven (2011) summarised how photo-respiration and photo-inhibition may consequently prevent an increase of net C-sequestration (biomass production or growth) despite increased chlorophyll concentrations, in particularly in areas where a cold climate (like in Alaska and Patagonia) limits photo-respiration (Murray *et al.* 1993). In summary, nitrogen stress indicators (e.g. nitrogen, amino acids, photosynthesis) may

be used to predict detrimental effects of elevated nitrogen deposition (Tomassen *et al.* 2003; Arroniz-Crespo *et al.* 2008; Wiedermann *et al.* 2009).

Our study focuses on the potential of phosphorus (P) to alleviate excess nitrogen stress by increased biomass production. We conducted a fertilization experiment in the field to unravel the effects of P-addition on the physiology of *Sphagnum* plants that were stressed by high loads of nitrogen. It was tested whether biomass production lowered the concentration of nitrogen stress indicators. The experimental design of the present study follows Limpens *et al.* (2004) and Pilkington *et al.* (2007) to facilitate comparisons with these earlier experiments that found alleviating effects of phosphorus on biomass production. Experiments were conducted in a pristine bog in southernmost Patagonia with a very low atmospheric nitrogen deposition (Godoy *et al.* 2003; Kleinebecker *et al.* 2008; Schmidt *et al.* 2010), and consequently with very few vascular plants (cover <2%; Fritz *et al.* 2011), thus avoiding interactions with higher plants (Malmer *et al.* 2003).

MATERIAL AND METHODS

Study site, N-deposition and experimental design

A 3 year-fertilisation experiment was conducted in a *Sphagnum*-dominated bog in the Andorra valley mire, Patagonia, Argentina (54°45' S; 68°20' W, 200 m a.s.l.) within the deciduous forest zone (Grootjans *et al.* 2010). Growing season lasts from October till April. Average daily air temperatures are 4–5 °C with cold summers (maximum average summer temperature 10 °C) characteristic for this type of boreal climate (Iturraspe *et al.* 1989). Annual precipitation exceeds 600 mm, evenly distributed over the year, providing wet conditions and high air humidity levels (Iturraspe *et al.* 1989). Groundwater level fluctuations during the growing season range around 25 cm, with lowest levels found in late summer. Strong desiccating winds are characteristic to Patagonian peatlands (Kleinebecker *et al.* 2007).

In the present study bulk nitrogen deposition was collected in black polyethylene bottles equipped with funnels (0.01 m²). Adding 1 mg HgCl₂ inhibited microbial alteration of samples. Total inorganic N in rain water averaged 8 µmol·N·l⁻¹ (SD 4; n = 10) during two growing seasons (2007 and 2008) suggesting a nitrogen deposition as low as 0.1–0.2 g·N·m⁻²·year⁻¹. Such low concentrations are often found in Southern Patagonia with estimated bulk N-deposition below 0.1 g·N·m⁻²·year⁻¹ (Godoy *et al.* 2003). In general, Patagonian peatlands show very low nutrient concentrations and have preserved their pristine character with little human influence (Kleinebecker *et al.* 2008).

The bog vegetation was dominated by *Sphagnum magellanicum* Brid. with <2% cover of vascular plants (e.g. *Rostkovia magellanica*, *Empetrum rubrum*, *Nothofagus antarctica*, *Carex magellanica*, *Marsipospermum grandiflorum* and *Tetroncium magellanicum*). Aboveground biomass of vascular plants was low (15 g·DW·m⁻²; SD 7.7; n = 10) which equals approximately 1% of living *Sphagnum* biomass. Interestingly, *S. magellanicum* occupies all hydrological niches from pool to hummock, which may rise up to 1 m above the summer water table. Other *Sphagnum* like *Sphagnum falcatulum* (*S. cuspidatum* coll.) and *Sphagnum cuspidatum* co-exist only in

pools (cf. Kleinebecker *et al.* 2007). In poor fens *Sphagnum fimbriatum* can be found next to *S. magellanicum* (Grootjans *et al.* 2010 and literature therein).

Experimental design

The effects of applied nitrogen ($4 \text{ g}\cdot\text{N}\cdot\text{m}^{-2}\cdot\text{year}^{-1}$, NH_4NO_3 , N-treatment) were studied in comparison with a Control (bog water only Control-treatment) and addition of phosphorus ($1 \text{ g}\cdot\text{P}\cdot\text{m}^{-2}\cdot\text{year}^{-1}$, NaH_2PO_4 , P-treatment). Additionally we also applied both nitrogen and phosphorus ($1 \text{ g}\cdot\text{P}\cdot\text{m}^{-2}\cdot\text{year}^{-1}$, NaH_2PO_4 and $4 \text{ g}\cdot\text{N}\cdot\text{m}^{-2}\cdot\text{year}^{-1}$, NH_4NO_3 , NP-treatment) to unravel effects of phosphorus on N-addition (N \times P interaction effect). Fertilisers were dissolved in 10 l sieved (1 mm mesh size) bog water which was sprayed over the randomly assigned $1 \times 1 \text{ m}$ plots ($n = 5$ per treatment, resulting in 20 plots randomly assigned to treatments). Fertilisers were applied six times (monthly) during each growing season (usually November to April). One hour after fertilising, *Sphagnum* heads were gently washed with 5 l of sieved bog water to reduce possible toxic effects of concentrated salts (Wilcox 1984; Tomassen *et al.* 2004). The experiment lasted from December 2006 to April 2009.

Field measurements and chemical analysis

Height increase of *Sphagnum* mosses was measured by marking ($n = 50$ per treatment, $n = 10$ in two subplots $0.1 \times 0.1 \text{ m}$ per plot) the first branch below the capitulum with a small cable tie in March 2008. One year later the distance between the first branch below the capitulum and the cable tie was measured according to Krebs & Gaudig (2005). The bulk density of the two subplots was determined after harvesting. Annual biomass production of *S. magellanicum* in the third year of the experiment was estimated by multiplying height increment by bulk density of moss stems (first 40 mm below capitulum). Cover of vascular plants was estimated as percentage cover before the experiment started and after each year. In February 2010 we harvested vascular plants to determine dry weight of biomass (48 h at 70°C) in the control-treatment and the NP-treatment.

Net photosynthesis of the *S. magellanicum*-dominated plots was measured on 6 March 2009. We used a clear Plexiglas® climate-controlled chamber (Workshop University Groningen, The Netherlands) occupying a surface of 0.063 m^2 and a volume of 8.4 dm^3 . With an infrared gas analyser (Vaisala CarboCap GMP343; Vanta, Finland) we recorded changes in CO_2 concentration in 15 s intervals in the static chamber that was carefully placed 15 min prior to a measurement cycle. At every plot CO_2 exchange was measured for 5 min under light and under complete darkness in duplicates. Light levels were around $300 \mu\text{mol}\cdot\text{PAR}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, close to light-saturated photosynthesis in *Sphagnum* sp. (Petersen 1984; Harley *et al.* 1989). The light level was continuously measured with a Li-Cor quantum photometer (Model LI-250; Lincoln, NE, USA) and filter sheets were used to maintain light levels constant ($300 \mu\text{mol}\cdot\text{PAR}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ or complete darkness). The change of CO_2 concentrations over time suggested a linear relationship that was tight in all measurements ($r^2 > 0.8$). Chambers warmed by about 5°C during light measurements. Water level was 15–20 cm below the surface.

All chemical analyses were performed on material harvested in March 2009, at the end of the 3rd growing season after. In each plot mosses were harvested at three randomly assigned 10 by 10 cm subplots with a total volume of 1 l. Before drying the *Sphagnum* mosses were divided in three functional fractions: capitula (average length 8 mm, photosynthetically active tissue), stem below capitula (40 mm, living moss tissue) and decaying tissue (first 50 mm below stem section, litter). Capitula were picked manually ($300\text{--}500 \text{ dm}^{-2}$). The decaying tissue fraction mostly coincided with signs of disintegration and pale colour of the *Sphagnum* tissue. Vascular plants were harvested in 0.5 m^2 per plot. All plant samples were dried (48 h at 70°C) and ground with liquid nitrogen. For amino acid and chlorophyll analysis, fresh capitula material was used. We placed sods of intact bog vegetation ($20 \times 20 \times 20 \text{ cm}$) in chilled opaque containers and transported them back to the laboratory.

Porewater of the living *Sphagnum* layer (uppermost 50 mm) was collected by connecting syringes (60 ml) to soil moisture samplers ($n = 2$ per plot, 10 cm Rhizon SMS; Eijk-elkamp Agrisearch Equipment, Giesbeek, the Netherlands). The two subsamples were pooled and pH was measured. Part of the sample was stored after addition of HgCl_2 to a final concentration of $5 \text{ mg}\cdot\text{l}^{-1}$ to prevent denitrification. All water samples were stored in polyethylene bottles (20 ml) at -18°C until further analysis.

Nitrogen and carbon concentrations were measured with a CNS analyser (Model NA 1500; Carlo Erba Instruments, Milan, Italy). Homogenised portions of 200 mg dried material were digested with 4 ml HNO_3 (65%) and 1 ml H_2O_2 (30%), using an Ethos D microwave labstation (Milestone srl, Sorisole, Italy). Digests were diluted and concentrations of P and (micro-) nutrients (Al, Ca, Fe, Mg, Mn, Na, S, Si, Zn) were determined by an ICP spectrometer (IRIS Intrepid II; Thermo Electron Corporation, Franklin, USA). Potassium in digests and porewater was measured on a FLM3 Flame Photometer (Radiometer, Copenhagen, Denmark). Concentrations of nitrate, ammonium and phosphate in rain water and porewater were measured colourimetrically on a Traacs 800 + auto-analyser as described by Tomassen *et al.* (2003).

Recovery of nutrients was determined separately per fraction (capitula, stem and decaying tissue). We multiplied density of a fraction with the difference in nutrient concentrations between a treatment plot and the means of the control treatment rendering $\text{g}\cdot\text{N}\cdot\text{m}^{-2}$ and $\text{g}\cdot\text{P}\cdot\text{m}^{-2}$. The sum of all three fractions (capitula, stem and decaying tissue) was divided by the total amount of nutrients applied over 3 years ($12 \text{ g}\cdot\text{N}\cdot\text{m}^{-2}$ and $3 \text{ g}\cdot\text{P}\cdot\text{m}^{-2}$).

Free amino acids were extracted from fresh capitula in duplicate using ethanol according to Van Dijk & Roelofs (1988). Twenty amino acids were quantified by measuring fluorescence after precolumn derivation with 9-Fluorenylmethyl-Chloroformate (FMOC-Cl) and measured using HPLC (with a Star 9050 variable wavelength UV-VIS and Star 9070 fluorescence detector; Varian Liquid Chromatography, Palo Alto, USA) with norleucine as the internal standard (Tomassen *et al.* 2003).

Leaf pigment concentrations were determined in frozen and homogenized fresh capitula tissue shaken for 24 h (4°C) with 96% ethanol. Leaf pigment concentrations in the supernatant fraction were measured spectrophotometrically

according to Lichtenthaler (1987). For 'specific photosynthetic rates' we divided photosynthetic rates ($\mu\text{mol CO}_2\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) measured under field condition by chlorophyll a and chlorophyll b ($\text{mmol Chl}\cdot\text{m}^{-2}$) following Petersen (1984). Chlorophyll was determined 3 weeks after taking photosynthesis measurements.

The effects of N and P were tested using linear models in R 2.92 (R development core team 2009) which was followed by model justification procedures (Crawley 2007). If fitted models were not justified, generalised linear models (glm) with different error structures and distributions were fitted, or data were log-transformed before analysis. Next, we conducted regression analysis to test the potential of biomass production to dilute the parameters of N metabolism. We included only plots receiving additional N ($n = 10$) for this analysis as they revealed a large range in productivity ($243\text{--}657\text{ g}\cdot\text{DW}\cdot\text{m}^{-2}\cdot\text{year}^{-1}$) and signs of stress induced by excess nitrogen.

RESULTS

Ecosystem effects of nutrient addition

Sphagnum absorbed the majority of nutrients added. In contrast, vascular plants did not show substantial changes in cover or nutrient content after 3 years of nutrient addition. After application, nutrients depleted quickly in the porewater already after few days but showed a high variation. After 1 week only $\sim 1\%$ of the added ammonium (leading to a slight elevation of the concentration to $23\ \mu\text{mol}\cdot\text{l}^{-1}$) could be demonstrated. In contrast, nitrate and phosphate remained longer available at increased levels. Even fourteen days after application we found elevated nitrate concentrations ($31\ \mu\text{mol}\cdot\text{l}^{-1}$) in treatments receiving N and substantial concentrations of ortho-phosphate ($26\ \mu\text{mol}\cdot\text{l}^{-1}$) in treatments where phosphorus (P) was added (Fig. S1, S2). After 2 months, porewater concentrations were below $5\ \mu\text{mol}\cdot\text{l}^{-1}$ for ammonium and nitrate and below $1\ \mu\text{mol}\cdot\text{l}^{-1}$ for total phosphorus.

A large fraction of the $12\text{ g}\cdot\text{N}\cdot\text{m}^{-2}$ applied during the entire experiment was found in living *Sphagnum* tissue (53–59%) and the decaying fraction (15–18%) which stored together $8.5\text{--}8.8\text{ g}\cdot\text{N}\cdot\text{m}^{-2}$ (SD 0.78) more than the control. The total recovery of phosphorus was lower, averaging 47–53% of total P applied.

Cover and composition of vascular plants remained unaffected by nutrient addition. *Rostkovia magellanica* (Lam.)

Hook. f. a common rush in Patagonian peatlands, dominated the assemblage of vascular plants in all plots. Nutrient additions did not affect nitrogen concentrations in foliar tissue (mean $14.5\text{ mg}\cdot\text{N}\cdot\text{g}^{-1}\cdot\text{DW}$) of *R. magellanica*. The addition of phosphorus slightly increased P tissue concentrations (P-effect; $df = 1$, $F = 6.7243$, $P = 0.018$) from $1.17\text{ mg}\cdot\text{P}\cdot\text{g}^{-1}\cdot\text{DW}$ (SD 0.49) in the Control-treatment to $1.56\text{ mg}\cdot\text{P}\cdot\text{g}^{-1}\cdot\text{DW}$ (SD 0.17) in the P-treatment and $1.54\text{ mg}\cdot\text{P}\cdot\text{g}^{-1}\cdot\text{DW}$ (SD 0.02) in the NP-treatment. In contrast, P tissue concentration in *S. magellanicum* increased by a factor 5–7 upon P-addition (Table 1). N:P ratios of *R. magellanica* declined upon P-addition (P-effect; $df = 1$, $F = 4.7975$, $P = 0.042$). In the Control-treatment N:P ratios were higher ($13.1\text{ g}\cdot\text{g}^{-1}$) than in the P-treatment ($9.4\text{ g}\cdot\text{g}^{-1}$) and NP-treatment ($10.2\text{ g}\cdot\text{g}^{-1}$).

Nutrient stoichiometry and growth response

The addition of nutrients changed both the total concentrations of nutrients in *S. magellanicum* plants and their distribution between capitula and stem tissue. Intermediate N:P ratios ($10\text{--}14\text{ g}\cdot\text{g}^{-1}$) in capitula tissue were associated with high biomass production in the NP-treatment (Fig. 1). In contrast, the control treatment showed higher N:P ratios ($26.5\text{ g}\cdot\text{g}^{-1}$), which were increased when fertilising with nitrogen (Table 1; $df = 1$, $F = 64.284$, $P < 0.001$). The addition of P increased the tissue P and lowered the N:P ratio, which was lowest in the P-treatment (Table 1; $df = 1$, $F = 448.819$, $P < 0.001$). Also in the plots receiving the NP-treatment the N:P ratios were significantly lower than in the control, but still higher than the N:P ratio (4:1) of the nutrient solution added. N:P ratios in stem tissue were similarly affected by nutrient additions (Table 1) but showed generally lower ratios than found in capitula tissue. Only the N-treatment revealed higher N:P ratios in stem tissue than in capitula tissue.

The addition of $1\text{ g}\cdot\text{P}\cdot\text{m}^{-2}\cdot\text{year}^{-1}$ stimulated height increment (growth) of *S. magellanicum* by 4 mm in the third year (Table 1). Addition of N had little effects on growth rates. In contrast, highest growth rates (mean $32\text{ mm}\cdot\text{year}^{-1}$, SD 3 mm) occurred in the NP-treatment which suggests a significant interaction effect between N and P ($df = 1$, $F = 10.065$, $P = 0.006$). Together with the positive effect on plant height, P also stimulated net biomass production (Table 1; $df = 1$, $F = 13.659$, $P = 0.002$). In both the NP-treatment ($549\text{ g}\cdot\text{DW}\cdot\text{m}^{-2}\cdot\text{year}^{-1}$; SD 77) and the P-treatment ($405\text{ g}\cdot\text{DW}\cdot\text{m}^{-2}\cdot\text{year}^{-1}$; SD 79) more biomass was produced than in the con-

Table 1. Nutrient concentration and growth of *Sphagnum magellanicum* in plots over three field seasons of treatments. For treatment details, see Fig. 1. Mean of five plots per treatment. Results from GLMs are indicated: ns, not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

	treatment				P-value		
	C	N	P	NP	N effect	P effect	N × P
P content capitula ($\text{mg}\cdot\text{P}\cdot\text{g}^{-1}\cdot\text{DW}$)	0.27	0.57	1.84	1.32	ns	***	***
N:P ratio capitula ($\text{g}\cdot\text{g}^{-1}$)	26.5	27.8	4.0	11.4	***	***	***
N:P ratio stem ($\text{g}\cdot\text{g}^{-1}$)	15.9	33.9	2.9	6.5	***	***	ns
Growth rate ($\text{mm}\cdot\text{year}^{-1}$)	14	15	18	32	**	***	**
Biomass production ($\text{g}\cdot\text{DW}\cdot\text{m}^{-2}\cdot\text{year}^{-1}$)	316	311	405	549	ns	**	ns
Capitula biomass ($\text{g}\cdot\text{DW}\cdot\text{m}^{-2}$)	266	290	306	309	ns	**	ns
Photosynthesis ($\mu\text{mol CO}_2\text{ m}^{-2}\cdot\text{s}^{-1}$)	2.40	2.42	2.62	2.87	ns	*	ns
Stem density ($\text{g}\cdot\text{l}^{-1}$)	23.3	20.2	21.6	16.8	***	*	ns

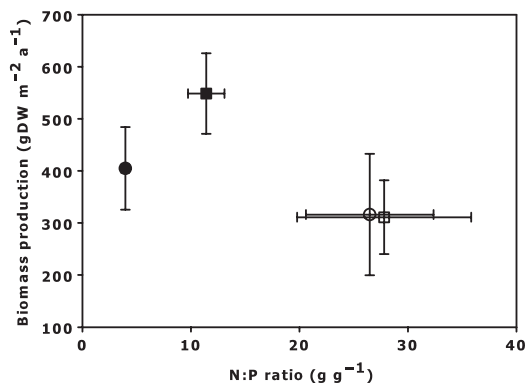


Fig. 1. Biomass production of *Sphagnum* moss in relation to N:P ratio, means and SD are represented for each treatment: P (1 g·P·m⁻²·year⁻¹), filled circle; NP (1 g·P and 4 g·N·m⁻²·year⁻¹): filled square; N (4 g·N·m⁻²·year⁻¹): open square; control (without nutrient addition): open square. Five plots per treatment were installed at the field experiment in a *Sphagnum* bog in Tierra del Fuego.

trol-treatment (316 g·DW·m⁻²·year⁻¹; SD 117) (Fig. 1, Table 1). The increase of capitula biomass (mean 40 g·DW·m⁻² over 3 years) following P-addition (df = 1, F = 8.3817, P = 0.0096) was small compared to the increase in total biomass production. Net photosynthesis at nearly light saturation was increased by P-addition, resulting in a 14% higher photosynthetic rate (Table 1; df = 1, F = 7.9836, P = 0.011). Photosynthesis on the basis of capitula biomass was similar in all treatments and averaged 89 nmol·CO₂·g⁻¹·DW·s⁻¹ (SD 12).

The addition of nutrients changed the morphology of *S. magellanicum*. The density of the *Sphagnum* layer affects both water transport and water conservation. A decrease in bulk density reduces the upward water transport by capillary flow (Clymo 1973). Both nutrients caused stem density to decrease (Table 1). Growth in length of *Sphagnum* mosses correlated well with the observed decrease in stem density ($r^2 = 0.50$, df = 1,18; F = 19.97, P < 0.001). In contrast, mosses in the N-treatment revealed only a small decrease in stem density without additional growth compared to the control (Table 1). The increased growth of *S. magellanicum* was associated with a large drop in bulk density when both N and P were added. In the NP-treatment mean density of the stem section was 16.8 g·l⁻¹ while in other treatments the density was always above 20 g·l⁻¹ (Table 1). We observed indicators for desiccation (white-colouration of capitula) in the NP-treatment after water tables had dropped to 35 cm below the surface in January 2009 (Fig. 2).

Indicator of excess N-stress in *Sphagnum* mosses

After 3 years of N-addition we observed substantial changes in physiological parameters that are related to stress because of excess N in mosses (Table 2). Addition of P had no significant effect on the concentration of excess N-stress indicators. N concentrations in capitula doubled by 7.2 mg·g⁻¹·DW upon N-addition (Table 2; df = 1, F = 413.96, P < 0.001). In stem tissue we observed a similar increase. Doubling N concentrations in capitula coincided with a sevenfold increase in concentration of free amino acids (df = 1, F = 1526.6,

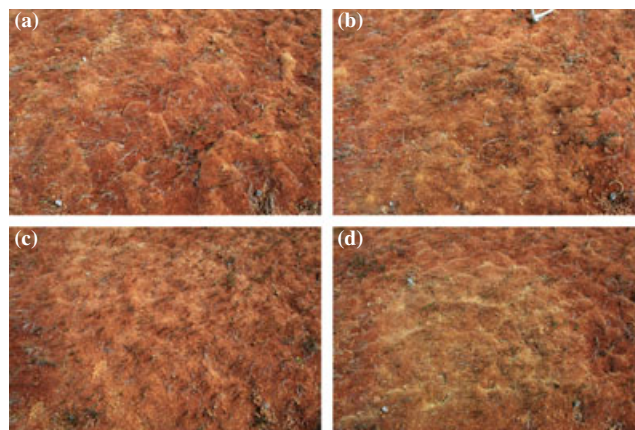


Fig. 2. Visible effects of treatments [Control (a), N-treatment (b), P-treatment (c), NP-treatment (d)] on *Sphagnum magellanicum*-dominated plots. Oblique photographs were taken after a dry spell in January 2009, when water levels were 35 cm below the surface, 15 cm lower than the average summer water level. For treatment details see Fig. 1.

P < 0.001). Especially amino acids with a low C:N ratio (arginine, asparagine) accumulated substantially. The concentrations of glutamine, serine, aspartic acid and glutamic acid increased twofold (Table 2), similar to the observed increase in total N.

N-addition also doubled chlorophyll and carotenoids (Table 2), while the Chl_a to Chl_b ratio increased from 1.08 to 1.25. Although nitrogen stimulated the production of photoactive pigments, the photosynthetic rate per capitulum (gram dry weight) did not increase. As a result, specific photosynthesis (e.g. photosynthesis per unit Chl) was negatively affected by N-addition (df = 1, F = 72.611, P < 0.001) in both the N-treatment and the NP-treatment. Treatments receiving nitrogen showed increased potassium concentrations in the porewater (mean >50 μmol·l⁻¹) compared to the Control-treatment and P-treatment (mean below <20 μmol·l⁻¹). However, tissue concentrations of potassium remained unaffected (df = 1, F = 2.1738, P = 0.160) by nitrogen addition (Table 2).

Alleviation of N-stress through biomass

Based on the assumption that increased biomass production can, by dilution, mitigate stress induced by excess nitrogen, we expected lower concentrations of N-stress indicators (amino acids, photosynthesis) in the NP-treatment compared to the N-treatment. However, the increased biomass production following P-addition failed to reduce concentrations of N-stress indicators. No significant correlation was found between N stored in amino acids (N_{AA}) and biomass production in plots of NP- and N-treatment (df = 1,8; F = 0.1917, P = 0.6731). Other N-stress indicators (total N, chlorophyll) were also not correlated with biomass production (data not shown). Neither did we find an interaction effect between nitrogen and phosphorus for N-stress indicators (Table 2). In contrast to an expected dilution, we even found asparagine concentrations to increase with biomass production (df = 1,8; F = 6.306, P = 0.036). While N-metabolism remained unaffected by additional P, increased biomass

production resulted in a 'dilution' of moss stem density which was associated with a moisture deficit in capitula after an episode of low water levels (Fig. 2).

Effects of nutrient addition on micro-nutrients

In capitula tissue the addition of P doubled concentrations of elements such as Mg, Na, Si, Zn and S (Table 3). The increase upon N addition was less pronounced. The NP-treatment revealed element concentrations lower than the P-treatment and N-treatment but exceeding those of the Control-treatment. For all elements the interaction between N and P reduced element concentrations (Table 3). We furthermore found a biomass dilution effect (negative correlation between biomass and element concentration) for Al, Ca, Fe, Mg, S, Si and Zn. For example, S concentrations declined with increasing biomass ($df = 1,8$; $F = 10.1$, $P = 0.013$).

DISCUSSION

This is the first study on the effects of N and its interaction with P in pristine bog vegetation in the Southern Hemisphere. Natural nutrient concentrations in *S. magellanicum* where comparable with other Patagonian bogs (Schmidt *et al.* 2010). Low P availability in porewater next to $<10 \mu\text{mol P}\cdot\text{g}^{-1}\text{DW}$ and N:P ratios close to 30 in moss tissues highlight the scarcity of P. This is in accordance with the stimulated growth and biomass production following the addition of P (Fig. 1). P-limitation has also been suggested for bogs in New Zealand (Clarkson *et al.* 2005).

Despite scarce nutrients, growth rates observed in the control plots were similar to the global average of *S. magellanicum* (reviewed in Gunnarsson 2005). Physiological parameters (e.g. nitrogen, amino acids, photosynthetic rates) were also similar to Northern Hemispheric *Sphagnum* mosses (Tomassen *et al.* 2003; Rydin & Jeglum 2006; Rice *et al.* 2008). From litter production in control plots we estimate a loss of $0.36 \text{ g}\cdot\text{N}\cdot\text{m}^{-2}\cdot\text{year}^{-1}$, which suggests an additional nitrogen source next to a bulk deposition of $0.1 \text{ g}\cdot\text{N}\cdot\text{m}^{-2}\cdot\text{year}^{-1}$ found in this experiment. Micro-organism, such as

(methanotrophic bacteria and cyanobacteria), can fix nitrogen at substantial rates ($0.2 \text{ g}\cdot\text{N}\cdot\text{m}^{-2}\cdot\text{year}^{-1}$) in North American *Sphagnum* bogs (Hemond 1983; Markham 2009) which may result in high N:P ratios even in pristine bogs. Bacteria having the potential for nitrogen fixation were also found at our experimental site (Kip *et al.* 2011).

Increased loads of N ($4 \text{ g}\cdot\text{N}\cdot\text{m}^{-2}\cdot\text{year}^{-1}$) resulted in a doubling of tissue N concentrations to $14.5 \text{ mg}\cdot\text{N}\cdot\text{g}^{-1}\text{DW}$ and increased N:P ratios. At this concentration saturation of nitrogen metabolism is known to occur resulting in impeded growth (Nordin & Gunnarsson 2000; Limpens & Berendse 2003; Tomassen *et al.* 2003). Several physiological parameters (e.g. amino acids, specific photosynthesis) indicated that the *Sphagnum* mosses were stressed by nitrogen addition. The accumulation of N resulted in an increased production of metabolically costly (cf. Rudolph *et al.* 1993) amino acids, predominantly arginine and asparagine, and chlorophyll (Table 2). The accumulation of free amino acids is associated with a suppressed productivity and cover in *Sphagnum* mosses (Nordin & Gunnarsson 2000; Wiedermann *et al.* 2009).

Assuming that increased biomass production potentially alleviates nitrogen stress, we expected lower concentrations of nitrogen and nitrogen-related stress indicators (Table 2) in mosses receiving additional P (NP treatment). Although N:P ratios were low ($10\text{--}14 \text{ g}\cdot\text{g}^{-1}$) and biomass production increased, we found, however, strong indications of excess nitrogen stress in NP-treated mosses, similar to N-treated mosses. In agreement with the expected dilution through biomass production a lowering of element concentrations was observed for micro-nutrients (e.g. Ca, Fe and S; Table 3). In contrast to other elements, the nitrogen metabolism apparently remained unaffected by the addition of P, although biomass production was stimulated. Other studies also found that *Sphagnum* mosses became saturated by nitrogen irrespective of P-addition (Limpens & Heijmans 2008; Bu *et al.* 2011). These earlier findings and the present study, therefore, suggest an accumulation of stress by excess nitrogen that may exceed a toxic threshold given a sufficiently long exposure time (Wiedermann *et al.* 2009).

Table 2. Indicators for stress induced by excess nitrogen in tissues of *Sphagnum magellanicum*. For treatments details, see Fig. 1. Results from GLMs on data for three field seasons are given: ns, not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

	treatment				P-value		
	C	N	P	NP	N effect	P effect	N × P
N content capitula ($\text{mg}\cdot\text{N}\cdot\text{g}^{-1}\text{DW}$)	7.0	14.5	7.2	14.4	***	ns	ns
N content stem ($\text{mg}\cdot\text{N}\cdot\text{g}^{-1}\text{DW}$)	3.6	10.0	3.8	10.7	***	ns	ns
Chl a ($\text{mmol}\cdot\text{g}^{-1}\text{DW}$)	0.71	1.65	0.72	1.52	***	ns	ns
Chl b ($\text{mmol}\cdot\text{g}^{-1}\text{DW}$)	0.67	1.25	0.66	1.25	***	ns	ns
Specific photosynthesis ($\mu\text{mol CO}_2 \text{ mmol Chla}+b^{-1}\cdot\text{s}^{-1}$)	6.6	2.9	6.8	3.4	***	ns	ns
Amino acid content ($\mu\text{mol}\cdot\text{N}\cdot\text{g}^{-1}\text{DW}$)	18.6	122.3	18.0	129.4	***	ns	ns
Arginine ($\mu\text{mol}\cdot\text{N}\cdot\text{g}^{-1}\text{DW}$)	4.93	81.57	5.39	71.12	***	ns	ns
Asparagine ($\mu\text{mol}\cdot\text{N}\cdot\text{g}^{-1}\text{DW}$)	3.31	19.29	3.04	35.47	***	ns	*
Glutamine ($\mu\text{mol}\cdot\text{N}\cdot\text{g}^{-1}\text{DW}$)	3.70	7.33	3.49	8.31	***	ns	ns
Serine ($\mu\text{mol}\cdot\text{N}\cdot\text{g}^{-1}\text{DW}$)	0.88	1.66	0.75	2.07	***	ns	ns
Aspartic acid ($\mu\text{mol}\cdot\text{N}\cdot\text{g}^{-1}\text{DW}$)	0.65	1.26	0.65	0.97	***	ns	ns
Glutamic acid ($\mu\text{mol}\cdot\text{N}\cdot\text{g}^{-1}\text{DW}$)	1.22	2.40	1.20	2.13	***	ns	ns
Potassium content capitula ($\text{mg}\cdot\text{K}\cdot\text{g}^{-1}\text{DW}$)	2.9	2.9	3.3	3.9	ns	**	ns

Table 3. Concentrations of micronutrients in capitula tissue of *Sphagnum magellanicum*. For treatment details, see Fig. 1. Results from GLMs on data for three field seasons are given: ns, not significant; *P < 0.05; **P < 0.01; ***P < 0.001.

	treatment				P-value		
	C	N	P	NP	N effect	P effect	N × P
Al ($\mu\text{mol}\cdot\text{g}^{-1}\cdot\text{DW}$)	2.01	3.46	3.83	2.42	ns	ns	***
Ca ($\mu\text{mol}\cdot\text{g}^{-1}\cdot\text{DW}$)	3.57	3.98	6.44	2.58	***	ns	***
Fe ($\mu\text{mol}\cdot\text{g}^{-1}\cdot\text{DW}$)	2.66	3.91	4.61	2.96	ns	ns	***
Mg ($\mu\text{mol}\cdot\text{g}^{-1}\cdot\text{DW}$)	8.25	10.78	18.32	10.02	*	***	***
Mn ($\mu\text{mol}\cdot\text{g}^{-1}\cdot\text{DW}$)	0.45	0.45	0.88	0.38	**	ns	**
Na ($\mu\text{mol}\cdot\text{g}^{-1}\cdot\text{DW}$)	26.08	34.95	53.88	36.82	ns	***	***
S ($\mu\text{mol}\cdot\text{g}^{-1}\cdot\text{DW}$)	5.57	9.94	11.42	7.99	ns	**	***
Si ($\mu\text{mol}\cdot\text{g}^{-1}\cdot\text{DW}$)	1.36	2.78	3.16	2.08	ns	**	***
Zn ($\mu\text{mol}\cdot\text{g}^{-1}\cdot\text{DW}$)	0.09	0.18	0.22	0.14	ns	***	***

At our Patagonian site N addition doubled chlorophyll levels without effects on photosynthetic rates. Increased chlorophyll levels have also been found in experiments applying various N-levels (Tomassen *et al.* 2003; Granath *et al.* 2009). Increased chlorophyll levels do not automatically result in increased net photosynthesis as they may result in photo-inhibition at low CO₂-availabilities, which are typical for wet pristine bogs (Rice 2000; Smolders *et al.* 2001). Low temperatures can increase negative effects due to photo-inhibition as shown for *Sphagnum* vegetation in Alaska (Murray *et al.* 1993). Also Arroniz-Crespo *et al.* (2008) linked detrimental effects of excess nitrogen to damage caused by photo-inhibition in mosses. Field evidence is needed to test the hypothesis whether nitrogen depresses growth by exacerbating negative effects of photo-inhibition in cold climates of Patagonia. Micro-nutrients may also become limiting after N addition as they seem to become diluted by increased biomass production in the NP-treatment (Table 3). The accumulation of ammonium in the porewater (Fig. S1) can facilitate the wash-out of cations like potassium, magnesium and iron, all important nutrients. Moisture availability and low temperatures during the growing season may furthermore lower growth of *Sphagnum* mosses.

We found that adding nutrients also changed the growth form of *Sphagnum* plants. Increased N availability lowered the bulk density of moss stems, which suggests an impeded branch formation or etiolation of the branches (Fig. 1, Table 1). This negative effect of extra nutrients on stem density was also found in nitrogen addition experiments in *Sphagnum* bogs (Gunnarsson & Rydin 2000; Manninen *et al.* 2011) and along a wide gradient of nitrogen deposition rates in European bogs (Bragazza *et al.* 2004) and Northern American bogs (Aldous 2002).

As *Sphagnum* lacks internal water-conducting tissues, water transport takes place via capillary action, which strongly depends on stem density (Clymo 1973). Lower stem density reduces the vitality of *Sphagnum* vegetation by lowering both the water-holding capacity (Hayward & Clymo 1982; Titus & Wagner 1984; Thompson & Waddington 2008) and water transport (Clymo 1973; Price & Whittington 2010) resulting in reduced photosynthesis of mosses (Schipperges & Rydin 1998; Robroek *et al.* 2009). In the longterm, mosses growing in low densities are more susceptible to damage during droughts (Robroek *et al.* 2007). In conclusion, water shortage resulting from changes in architecture of the moss carpets will impose constraints on *Sphagnum* mosses vitality in addition

to the physiological stress of excess nitrogen. We found evidence of severe desiccation in the NP-treatment at the end of a 3-week dry spell 2 years after the experiment ended. Mosses in the NP-treatment showed capitula water contents (3.1 g-FW·g⁻¹·DW) at which photosynthesis in *S. magellanicum* is impaired due to desiccation (Schipperges & Rydin 1998). In contrast, the denser mosses in the Control-treatment showed higher capitula water contents (6.8 g-FW·g⁻¹·DW; df = 8, t = 4.2327, P = 0.0029), suggesting near-optimal photosynthetic rates (cf. Schipperges & Rydin 1998). Stem density and water levels in 2011 were similar to values found in 2009.

Increased stem biomass production is insufficient to mitigate the negative effects of excess N on the physiology of *Sphagnum magellanicum*. The absence of an effect of dilution of N by the increase in biomass of the stem only, could be due to the lack of a substantial increase in capitulum biomass. Our data suggest that the metabolic burden of nitrogen cannot be alleviated by increased biomass production stimulated by P-addition alone. This is in agreement with long-term studies showing a sudden decline of *Sphagnum* cover when experiments lasted more than 5 years (Wiedermann *et al.* 2007; Juutinen *et al.* 2010). We therefore expect mosses to become poisoned by nitrogen when exposed to additional stressors, which limit photosynthesis and the N-metabolism (e.g. droughts). Strong growth reduction has been observed in several experiments after increased water losses due to droughts or increased temperatures (Aerts *et al.* 2001; Gerdol *et al.* 2008; Breeuwer *et al.* 2009). This raises the question whether the alleviating effects of phosphorus found in earlier studies (Limpens *et al.* 2004; Pilkington *et al.* 2007), were only temporary. We hypothesize that *Sphagnum magellanicum* is not able to increase its net photosynthetic rate sufficiently under natural CO₂-availability and therefore is not able to profit from an increased availability of nutrients. The pristine Patagonian bogs experience an additional stressor in the form of desiccating strong winds.

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SUPPORTING INFORMATION

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

Figure S1. Porewater nitrogen concentrations.

Figure S2. Porewater phosphorus concentrations.

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