Too Much of a Good Thing? Inorganic Nitrogen (N) Inhibits Moss-Associated N_2 Fixation But Organic N Can Promote It

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Abstract

Moss-associated nitrogen (N_2) fixation is one of the main inputs of new N in pristine ecosystems that receive low amounts of atmospheric N deposition. Previous studies have shown that N_2 fixation is inhibited by inorganic N (IN) inputs, but if N_2 fixation in mosses is similarly affected by organic N (ON) remains unknown. Here, we assessed N_2 fixation in two dominant mosses in boreal forests (*Pleurozium schreberi* and *Sphagnum capillifolium*) in response to different levels of N, simulating realistic (up to 4 kg N ha^{-1} yr^{-1}) and extreme N deposition rates in pristine ecosystems (up to 20 kg N ha^{-1} yr^{-1}) of IN (NH_4NO_3) and ON (alanine and urea). We also assessed if N_2 fixation can recover from the N additions. In the realistic scenario, N_2 fixation was inhibited by increasing NH_4NO_3 additions in *P. schreberi* but not in *S. capillifolium*, and alanine and urea stimulated N_2 fixation in both moss species. In contrast, in the extreme N additions, increasing N inputs inhibited N_2 fixation in both moss species and all N forms. Nitrogen fixation was more sensitive to N inputs in *P. schreberi* than in *S. capillifolium* and was higher in the recovery phase after the realistic compared to the extreme N additions. These results demonstrate that N_2 fixation in mosses is less sensitive to organic than inorganic N inputs and highlight the importance of considering different N forms and species-specific responses when estimating the impact of N inputs on ecosystem functions such as moss-associated N_2 fixation.

Introduction

Nitrogen (N) is usually the limiting nutrient for plant productivity in pristine ecosystems such as boreal forests and arctic tundra. In these ecosystems, N_2 fixation by moss-cyanobacteria associations can contribute > 2 kg N ha^{-1} yr^{-1} to new N input, being one of the major sources of N (DeLuca et al. 2002; Rousk et al. 2017). Nitrogen fixation by moss-associated bacteria (diazotrophs) has been shown to be sensitive to inorganic N inputs through N deposition (Ackermann et al. 2012; Rousk et al. 2013; Salemaa et al. 2019; Zheng et al. 2019). However, we still know little about how the projected increase in N input affect moss-associated N_2 fixation in pristine ecosystems. More importantly, most studies on the effects of increased N availability or deposition have focused on inorganic N (e.g. ammonium), while the effects of organic N on N_2 fixation in mosses is unknown.

Because N_2 fixation is an energy costly process, N_2 fixation associated with moss-cyanobacteria declines with increasing environmental N levels, such as along busy roads with high N deposition (Ackermann et al. 2012), along natural N deposition gradients (Rousk et al. 2013; Salemaa et al. 2019) and experimentally fertilized ecosystems (Sorensen et al. 2012). However, N_2 fixation in moss-cyanobacteria associations is not inhibited at all N input rates and the response among moss species differs. For instance, N_2 fixation is even promoted in the moss *Hylocomium splendens* in the laboratory when adding 5 kg N ha^{-1} yr^{-1} (Rousk & Michelsen 2016), unresponsive in *Sphagnum magellanicum* and *S. papillosum* after adding 8-32 kg N ha^{-1} yr^{-1} in peat bogs (van den Elzen et al. 2018), and inhibited in *Pleurozium schreberi* (Brid.) Mitt. by 3 kg N ha^{-1} yr^{-1} N deposition in boreal forests of northern Sweden (Gundale et

al. 2011). These different findings among studies could be the result of different moss species investigated. Diazotrophs associated with e.g. *Sphagnum* mosses reside inside specialized cells (hyalocytes), while diazotrophs associated with other mosses such as *P. schreberi*, live epiphytically on the moss leaves (Bragina et al. 2012; DeLuca et al. 2002). This leaves *Sphagnum*-associated diazotrophs more protected against environmental fluctuations, including N deposition, compared to diazotrophs associated with *P. schreberi*. Thus, although N input can impede N_2 fixation in mosses, the thresholds of N input above which N_2 fixation is inhibited is not well defined and likely differs among species.

Besides the input from N_2 fixation, mosses can receive N via atmospheric deposition (Zechmeister et al. 2007), which contain both inorganic and organic N forms (Cornell et al. 2003; Jia et al. 2016). Organic N deposition can contribute ~20 % to total N deposition (Cornell 2011; Violaki et al. 2010). Even though mosses prefer chemically simpler inorganic N, i.e. NH_4^+ to organic N (Forsum et al. 2006; Liu et al. 2013), mosses are able to take up and use organic N forms such as amino acids and urea (Krab et al. 2008; Nasholm et al. 2009; Persson & Nasholm 2001; Rousk et al. 2013; Witte 2011). While inorganic N can suppress N_2 fixation associated with mosses (see above), how organic N affects N_2 fixation by cyanobacteria on mosses has not been explored to date.

Though N_2 fixation can be inhibited by N inputs, moss-cyanobacteria associations can show resilience towards increased N deposition. For instance, negative effects of high N inputs (>12 kg N ha^{-1} yr^{-1}) on moss-associated N_2 fixation disappeared quickly after N input ceased (within 2 weeks) (Rousk et al. 2014a) and no effects on N_2 fixation in mosses by long-term N additions (0-50 kg N ha^{-1} yr^{-1}) were found (Gundale et al. 2013). Recovery of moss-associated N_2 fixation has even been found after adding very high N loads (5-320 kg N ha^{-1} yr^{-1}), providing only 2 weeks of recovery (Rousk & Michelsen 2016). However, these studies focused on inorganic N, but if N_2 fixation can recover from organic N input is unknown.

Hence, the aim of this study was to investigate how moss-associated N_2 fixation responds to and recovers from different forms and rates of N, and how this differs between physiologically contrasting moss species. To do this, we measured N_2 fixation rates of two dominant boreal moss species, *P. schreberi* and *Sphagnum capillifolium*, after the addition of three forms of N, one inorganic (ammonium nitrate, henceforth NH_4NO_3) and two organic forms (alanine and urea), with low N additions (“realistic”) for several weeks, followed by a period of N deprivation (recovery period). We then added extreme N rates and concluded with another period of N deprivation to assess if different N rates affect recovery from N stress. We also assessed moss pH, N content as well as cyanobacterial biomass to assess if the N additions lead to changes in those factors that could affect N_2 fixation. We hypothesized that (H1) moss-associated N_2 fixation rates decrease with increasing N addition; (H2) thresholds for inhibition depend on the forms of added N, following this sequence from largest inhibition to smallest inhibition of N_2 fixation: NH_4NO_3 > alanine > urea; (H3) N_2 fixation rates will be higher during recovery after the realistic N loads compared to rates after the extreme N loads; (H4) N_2 fixation associated with *P. schreberi* is more

sensitive to N additions than *S. capillifolium* due to the different locations of diazotrophs in the two moss hosts.

Material And Methods

Sampling

Moss material was collected in August 2020 from a boreal forest site (65°55'10.62"N, 19°42'22.38"E) close to Arvidsjaur, Northern Sweden. The mean annual temperature and precipitation are 1°C and 570mm, respectively. The dominant vegetation was composed of *Vaccinium vitis-idaea*, *V. myrtillus*, *Empetrum hermaphroditum*, *Hylocomium splendens*, *Pleurozium schreberi* as well as *Picea abies*, *Pinus sylvestris*. Individual moss shoots of *P. schreberi* and *S. capillifolium* were collected from ca. 10 patches within a sampling plot (n=6). Replicate plots were at least 2 m apart from each other. The moss samples were kept in transparent ziplock bags and transported to Copenhagen. Here, the samples were kept at 4°C in the dark until the start of the experiment (ca. 4 months). Mosses can handle this type of storage and still maintain normal function when re-exposed to light (Rousk et al. 2014b)

Experimental setup

To ensure optimal conditions after storage, moss samples were water saturated by soaking in double distilled water (ddH_2O) for ca. ten minutes. Eight shoots of *P. schreberi* and 10 shoots of *S. capillifolium*, representing a ground area of approx. 10 cm^2 were weighted and transferred into 20ml glass tubes. Glass tubes with mosses were kept in a growth chamber at 10°C for 18h in light and at 6°C for 6h in darkness for one week before the N addition and throughout the whole experiment.

Effects of three N forms (NH_4NO_3, alanine, urea) on N_2 fixation were tested at realistic rates (0, 0.4, 2, 4 kg N ha^{-1} yr^{-1}) and extreme rates (0, 2, 10, 20 kg N ha^{-1} yr^{-1}). The realistic N addition rates were based on background total N deposition rates of ca. 2 kg N ha^{-1} yr^{-1} in these systems (Pihl-Karlsson et al. 2012), and a doubling of that (4 kg N ha^{-1} yr^{-1}). The 0.4 kg N ha^{-1} yr^{-1} corresponds to the proportion of organic N in total background N deposition (Cornell 2011; Violaki et al. 2010). For the extreme rates, we added 5 times the “realistic” rates as this would exceed the suggested threshold of 15 kg N ha^{-1} yr^{-1} for ecosystem processes (Bobbink et al. 2010) and allow us to explore a wide range of rates.

Treatments with N began in January 2021. At each N addition event, 0.5 ml N solutions or ddH_2O were added to the mosses and the control, respectively, with six replicates for each N addition, N form and moss species (totaling to 120 samples). The N solutions were added weekly over a period of two weeks, i.e., ½ rate of the final rate. This was done to not stress moss and associated diazotrophs too much. Hence, the realistic rates were added to the mosses in week1 and week2, followed by two weeks (week3 and week4) of recovery time with ddH_2O addition only to keep the mosses moist, if necessary. After the first recovery period, the extreme rates were added over the course of two weeks (week5 and week6). The experiment was concluded with another recovery period of 1 week (week7).

Acetylene reduction assay

Nitrogen fixation rates were measured using the acetylene reduction assay (ARA) as previously described (Zackrisson et al. 2004). The ARA is based on the preferential reduction of acetylene to ethylene by the nitrogenase enzyme. Hence, it is a direct measure of the nitrogenase enzyme activity, is non-destructive and thus allows measurements over time on the same sample. Acetylene reduction was measured immediately after each N addition and during the recovery weeks, 7 times in total. For the incubations, each 20 ml tube was sealed and 10% of the headspace was replaced with acetylene gas. Moss samples were incubated for 24h in the growth chamber with the settings described above. Ethylene generated in the headspace was measured by gas chromatography on an Agilent 8890 GC coupled to a headspace sampler and fitted with a FID (Agilent Technologies, Santa Clara, California, USA). For testing the natural production of ethylene by mosses, moss samples without acetylene gas were also incubated under the same conditions as described above. No natural production of ethylene was detected.

pH

To assess if the added N solutions lead to changes in moss pH, half of the moss shoots from each sample were moved into 50ml centrifuge tubes after the final ARA, and 10ml ddH_2O was added. The tubes were shaken for 1h and the pH of the solution was measured using a pH meter (Sevencompact S220, Mettler Toledo, Shanghai, China). All moss shoots, including those used for pH measurement were dried at 45℃ for >24h to measure total dry weight.

Phycocyanin and N content

The remainder of the moss shoots not used for pH measurements were ground and used for phycocyanin and total N measurements. Phycocyanin pigment extraction and quantification is an easy and efficient way to estimate moss-associated cyanobacterial biomass as phycocyanin is a cyanobacterial specific pigment (Renaudin et al. 2021). Approximately 0.08-0.1g of dry moss sample were placed in 50ml centrifuge tubes with 10ml of sterilized sodium phosphate buffer (0.025M, pH=7). Then, the moss samples were mixed by vortexing and shaking manually for 10 s. Samples were then subjected to two freeze-thaw cycles (2h at -20°C followed by 1h at room temperature) and mixed by vortexing for 10s between cycles to break the cyanobacteria cell membrane. After sonication for 5min, samples were centrifuged at 4 100 × g at 8°C for 15min. Finally, 5-8ml supernatants were transferred to 15 ml tubes covered with aluminum foil and stored in dark at -80°C until analysis. Phycocyanin was quantified using a Synergy HT Microplate Reader (BioTek Instruments, Inc, Winooski, VT, USA) with excitation at 590 nm and emission at 645nm. For the analysis of total N of moss tissue, 4-5mg dry ground samples were weighed into tin capsules and analyzed on an elemental analyzer (Euro EA 3000 Elemental Analyzer, Eurovector SPA., Milano, Italy)

Data Analysis And Statistics

Differences in moss N content, cumulative N_2 fixation rates, pH and phycocyanin content between N addition rates, forms and moss species were tested with three-way ANOVAs followed by Tukey's Post Hoc Test. All data was log transformed to conform with the assumptions of homoscedasticity. The relationships between N addition rates, moss N content, pH and phycocyanin content and cumulative N_2 fixation rates were tested with regression analyses. Except for cumulative N_2 fixation rates, values for the regression analyses were final values, measured at the end of the experiment, i.e., after the extreme N addition scenarios. We also determined the pH optimum for N_2 fixation using a nonlinear regression (bell-shaped curve), and how the N additions affect this optimum. Acetylene reduction response ratios were calculated as N_2 fixation rates at different rates and forms of N addition divided by the rates in the control samples (Zheng et al. 2019). Mixed effect models were conducted to test the effects of N addition rates on acetylene reduction response ratios under each N form during the realistic or extreme rates scenarios. All analyses were performed in R (R version 3.6.3, R Core Team, 2020, Vienna Austria).

Results

Moss tissue N content

Moss tissue N content increased with increasing N additions for both moss species (Table S1; Fig. 1). However, this increase was more pronounced for *P. schreberi* below N additions of 10 kg N ha^{-1} yr^{-1}, where *P. schreberi* had lower N content than *S. capillifolium*. But when the N additions were higher than 10 kg N ha^{-1} yr^{-1}, *P. schreberi* showed higher N content than *S. capillifolium*. The form of N addition also influenced moss tissue N content and adding NH_4NO_3 lead to lower moss tissue N content compared to the organic N forms.

Effects of N addition rates and forms on nitrogenase activity

Under the realistic N addition scenario, nitrogenase response ratios at or above 1 in most treatments, showed that N addition rates mostly increased or had no effect on nitrogenase activity (acetylene reduction) in both moss species among N addition forms (Fig. 2). This was except for *P. schreberi* at 4 kg N ha^{-1} yr^{-1} of NH_4NO_3 and for *S. capillifolium* at 0.4 kg N h^{-1} yr^{-1} as urea (Table 1, Fig. 2), where inhibiting effects were found. Positive effects of added N were mostly found at intermediate and high rates of organic N but not when inorganic N was added.

In the extreme scenario, N addition (after week 4) generally decreased nitrogenase activity (Table 1), as seen by the response ratios below 1 in most treatments (Fig. 2). For the NH_4NO_3 and urea additions, nitrogenase activity decreased with increasing N addition rates in both moss species. However, when alanine was added, acetylene reduction response ratios in *P. schreberi* were below 0 only in the two highest N addition rates (10 and 20 kg N h^{-1} yr^{-1}), while no inhibition of nitrogenase activity was found when adding alanine to *S. capillifolium*.

Both moss species recovered to some extent during the N deprivation phase after the realistic N addition rates (Figs. 2, S1). As such in *P. schreberi*, acetylene reduction rates almost doubled during the recovery phase in the NH_4NO_3 additions (1.29 ± 0.29 vs. 2.23 ± 0.31 nmol g dw^{-1} h^{-1}, averages across N addition rates), and in the urea additions (2.63 ± 0.42 vs. 4.71 ± 0.52 nmol g dw^{-1} h^{-1}), while activity remined similar after the alanine treatments (2.07 ± 0.41 vs. 2.97 ±0.42 nmol g dw^{-1} h^{-1}). For *S. capillifolium* nitrogenase activity was 2 to 3 times higher after N addition irrespective of N form. However, no recovery was found after the extreme N addition scenario (Fig S1), and acetylene reduction rates were significantly higher ($p < 0.001$) in both moss species in the recovery period after the realistic additions compared to the extreme additions, irrespective of N form (2.82 ± 0.31 vs. 1.54 ± 0.24 nmol g dw^{-1} h^{-1}, for *P. schreberi* and *S. capillifolium*, respectively).

Cumulative nitrogenase activity – response pattern over 7 weeks

The cumulative acetylene reduction (N_2 fixation rates from the N addition and N deprivation periods) after 7 weeks differed between the two moss species (Table S1) and was higher in *S. capillifollium* than in *P. schreberi* in the NH_4NO_3 ($p < 0.05$) and alanine additions ($p < 0.1$), but not in the urea additions (Fig. 3). Also, the effects of addition rate depended on the N form (Table S1). As such, NH_4NO_3 decreased acetylene reduction, while alanine increased cumulative acetylene reduction ($R^2 = 0.87$, $p = 0.06$; $R^2=0.99$, $p = 0.07$; for *P. schreberi* and *S. capillifolium*, respectively, Fig. 3).

Moss pH

Moss pH changed with N addition rate but the specific effect depended on species and N form (Table S1, Fig. 4). Moss pH increased with increasing alanine and urea additions in both moss species, and here *P. schreberi* had higher pH than *S. capillifolium* (Fig. 4). However, when NH_4NO_3 was added, moss pH decreased and this was more pronounced in *P. schreberi* than in *S. capillifolium*. The optimum pH for N_2 fixation decreased with N additions in *P. schreberi* from 5.33 (control samples) to 5.03 (N addition samples) but increased from 4.83 to 5.18 in *S. capillifolium*. But overall, N_2 fixation in *P. schreberi* (5.06) had a higher optimum pH than *S. capillifolium* (4.91, Fig S2).

Phycocyanin

Phycocyanin concentration, which is a measure of cyanobacterial biomass, was significantly affected by N addition rates, forms and moss species (Table S1). Phycocyanin concentrations increased with increasing alanine and urea additions in *S. capillifolium* ($R^2 = 0.73$, $p = 0.094$; $R^2=0.89$, $p = 0.037$; Fig. 5), which were significantly higher than in *P. schreberi* ($p < 0.05$). The NH_4NO_3 addition did not lead to any changes in phycocyanin concentrations.

Discussion

Nitrogen fixation in mosses is the primary input of N in pristine ecosystems. Nitrogen deposition is expected to increase due to increased human influence with the presumed outcome that N_2 fixation will decrease. However, N deposition consists of ~20% organic N, and we have very limited knowledge about how this influences N_2 fixation in mosses. This study suggests that organic N has very different and often positive effects on N_2 fixation. Below we discuss the details and consequences of these findings.

Effects of N additions on moss-associated N_2 fixation

Nitrogen additions increased N content in the mosses, which indicated that the mosses absorbed the exogenous N. Hence, we can address our hypotheses. We hypothesized (H1) that N_2 fixation in two common moss species would decrease with increasing N additions. However, this was only partly confirmed, as N_2 fixation response to N additions was highly dependent on N form and moss species. The realistic NH_4NO_3 addition (0.4-4 kg N ha^{-1} yr^{-1}) followed the expected pattern with lower N_2 fixation rates with increasing rates as also seen in boreal forest (Gundale et al. 2011; Rousk & Michelsen 2016). Interestingly, addition of alanine and urea had positive or no effects on N_2 fixation in this experiment (see below). Usually, the nitrogenase enzyme is depressed by its end product, i.e., ammonia. Besides, NH_4NO_3 causes acidification (Tian & Niu 2015), and low pH can inhibit N_2 fixation in mosses (Alvarenga & Rousk 2021), as a result of suppressed nitrogenase enzyme activity (see below).

According to our second hypothesis (H2), more complex N forms, alanine and urea, showed a higher threshold for inhibiting N_2 fixation and often benefitted N_2 fixation at low rates compared to NH_4NO_3, as NH_4NO_3 inhibited activity already at 2-4 kg N ha^{-1} yr^{-1} for *P. schreberi* and around 10 kg N ha^{-1} yr^{-1} for *S. capillifolium*, while 10 and 20 kg N ha^{-1} yr^{-1} with alanine addition for *P. schreberi* and *S. capillifolium*, respectively, and urea inhibited N_2 fixation only at the highest rate (20 kg N ha^{-1} yr^{-1}) for both species (Figs. 2, S1). Surprisingly, realistic additions of alanine and urea did not inhibit N_2 fixation in either species, but rather, promoted N_2 fixation. A possible explanation is that the organic N forms were allocated towards growth by both cyanobacteria and moss and therefore, did not inhibit N_2 fixation (Krausfeldt et al. 2019; Liu et al. 2013; Rawson 1985). Indeed, phycocyanin concentration, as a measure of cyanobacterial abundance, did increase in both species after adding organic N (only a trend in *P. schreberi*) but not after adding inorganic N. Moreover, organic nitrogen could also act as a carbon source for cyanobacteria (Krausfeldt et al. 2019), which could save the cost for photosynthesis and allow more energy to be invested to fix N_2. Another positive, but indirect effect of organic N, is the increase in moss pH that can promote N_2 fixation activity (e.g. (Alvarenga & Rousk)). Further, amino acids can be absorbed and utilized directly by moss and cyanobacteria, and the assimilation cost of amino acids is considered to be lower than that of NH_4^+ and much lower than that of NO_3^- (Liu et al. 2013; Song et al. 2016). Urea likely offers the greatest energetic advantage because urea hydrolysis by urease results in the production of two N containing molecules (Herrero et al. 2001). Moreover, the breakdown of urea results in the release of CO_2 as a by-product, which can be incorporated into photosynthesis, reducing the reliance on

active uptake (Glibert et al. 2014). Hence, different uptake – and metabolism mechanisms lead to the diverse response patterns of N_2 fixation towards different types of N. This is also reflected in the higher moss tissue N content in both investigated moss species after organic than inorganic N additions, suggesting different uptake strategies or requirements for inorganic vs. organic N (Krab et al. 2008; Liu et al. 2013).

Under the extreme N addition scenario, all three N forms inhibited N_2 fixation, although inorganic N had the strongest effect. The inhibition could be explained by pH stress and toxicity caused by high N concentrations. In this study, high rates of N additions changed the pH optimum for N_2 fixation – above or below the pH optimum in the control samples, depending on moss species. The pH optimum for N_2 fixation in both species was lower than the pH of 5.9 ~ 6.2 found by Smith (1984) and may be due to different moss species investigated. High rates of added NH_4NO_3 decreased pH while alanine and urea increased moss pH. Rawson (1985) found that several amino acids affected nitrogenase and appeared to be toxic at high concentrations in culture (10mM). The hydrolysis of urea produces two ammonia molecules, which can be protonated to form two NH_4^+ molecules and cause an increase in pH (Carlini & Ligabue-Braun 2016; Herrero et al. 2001; Veaudor et al. 2019), thus under the extreme urea addition, high NH_4^+ accumulation and high pH (average pH in both species was 5.67 after urea addition) may account for inhibition of N_2 fixation. Even though we found inhibition of N_2 fixation by alanine and urea at high rates, in natural ecosystems, organic N contributes 20-30% to total N deposition (Cornell 2011; Violaki et al. 2010), which means in natural ecosystems organic N inputs are much lower than our extreme addition rates and could promote N_2 fixation. Besides, in natural environments available N is always complex and mixed. A preference to take up different N forms such as ammonium, nitrate, amino acids, urea should therefore be taken into account when considering the N addition effects on N_2 fixation (Andersen et al. 2020; Liu et al. 2013).

Recovery of N_2 fixation after N additions

Since organic N did not inhibit N_2 fixation in the realistic N addition scenario, recovery from the N stress can strictly speaking not occur. Nonetheless, given that we expected an inhibition of N_2 fixation by all N forms, we deprived the mosses of N for 2 weeks, and we found inhibition after the extreme N addition scenario, we still define the N_2 fixation rates during the N deprivation period as recovery rates. In line with our third hypothesis (H3), we found evidence for higher recovery of N_2 fixation after lower N additions than in the extreme N addition scenario. Also, a higher recovery rate was found with urea addition than with NH_4NO_3 or alanine addition in both moss species during the N deprivation after the realistic scenario but not after the extreme scenario, which may be because low rates of urea additions increased moss pH (5.22~5.52 for *P. schreberi* and 4.68~4.92 for *S. capillifolium*) more than NH_4NO_3 and alanine addition, creating an environment conducive to N_2 fixation. Urea also provides both C and N to cyanobacteria (Krausfeldt et al. 2019). Recovery after N additions under the realistic scenario suggests that the cyanobacteria may have down-regulated N_2 fixation during the N additions, since N_2 fixation is an energy

consuming process (Sohm et al. 2011; Turetsky 2003). As soon as N availability is decreasing (N deprivation phases), cyanobacteria start fixing N_2 again. After extreme N additions, however, N_2 fixation did not recover. Previous studies showed that recovery from high N loads needs a longer time or N needs to be actively removed via e.g. rinsing (Rousk et al. 2014a; Rousk & Michelsen 2016). Therefore, recovery from N loads is possible, if N input remains below a certain threshold.

Moss species-specific responses to N addition

Although acetylene reduction rates were higher in *S. capillifolium* than in *P. schreberi* in this study, the rates still likely underestimate the actual N_2 fixation as in *Sphagnum*, the most dominant diazotrophs are methanotrophs (Bragina et al. 2013; Leppänen et al. 2015), whose activity is suggested to be inhibited by acetylene. Yet, the high acetylene reduction rates in *Sphagnum* in our study indicate either a high abundance of cyanobacteria present, which are not inhibited by acetylene, or not all methanotrophs are inhibited by acetylene during the incubation period (Rousk et al. 2018).

Throughout the experiment, *P. schreberi* and *S. capillifolium* had comparable average nitrogenase activity in the control and urea treatments. However, nitrogenase activity in *P. schreberi* dropped 3 times after NH_4NO_3 addition and halved after alanine additions compared to *S. capillifolium*. The cumulative N_2 fixation rates in *P. schreberi* with NH_4NO_3 addition was suppressed but not in *S. capillifolium*, and we only found a positive relationship between phycocyanin and N addition rates in *S. capillifolium* in the alanine and urea treatment. All these results only partly supported H4, stating that *P. schreberi* would be more sensitive to N additions than *S. capillifolium*. Nevertheless, response differences between the moss species could be identified. The higher sensitivity of N_2 fixation in *P. schreberi* towards increased inorganic N may be due to the different colonization locations of cyanobacteria in the two moss hosts. Cyanobacteria colonize the leave surface of *P. schreberi* (DeLuca et al. 2002), causing them to be exposed to the environment directly. *Sphagnum* mosses harbor microorganisms both on the surface and inside their hyaline cells. Hyaline cells provide a relative stable living space where diazotrophs are protected from N stress (Bragina et al. 2012). Also, *S. capillifolium*, and *Sphagnum* species in general, are common in boreal peatlands with usually low pH (Kostka et al. 2016; Turetsky et al. 2012), which could explain why N_2 fixation in this species was less responsive to extreme NH_4NO_3 additions leading lower pH (Fig. S2) compared to *P. schreberi*. Higher regression slopes between N content and N addition rates found in *P. schreberi* also suggests that *P. schreberi* took up more of the added N and could be therefore more sensitive to N inputs than *S. capillifolium*, leading to *P. schreberi* had lower threshold toward N inputs (Fig. 1).

Conclusions

This study illustrates that different N forms have different impacts on moss- associated N_2 fixation with high sensitivity of N_2 fixation in *P. scherberi* towards low loads of inorganic N while organic N forms can promote N_2 fixation in both species. However, importantly we found that high N inputs -no matter in which form - inhibit N_2 fixation. Moss-associated N_2 fixation can recover from N stress, if N loads do not

exceed a certain threshold above which N_2 fixation is inhibited. Variation in N_2 fixation response patterns between the moss species, with inhibition of N_2 fixation in *P. schreberi* at 2-4, 10, 20 kg N ha^{-1} yr^{-1} by NH_4NO_3, alanine and urea addition, respectively, while *S. capillifolium* had higher thresholds for inhibition, at 10, 20, 20 kg N ha^{-1} yr^{-1}, is likely the result of differences in the location of the diazotrophs between the moss species (epiphytic vs. endophytic for *P. schreberi*, *S. capillifolium*, respectively).

This is the first study to assess the effects of organic N on moss-associated N_2 fixation. The results show that organic N can promote N_2 fixation at low rates and that the threshold for inhibiting N_2 fixation is higher than that for inorganic N. Hence, diverse N sources and species differences should be taken into account when estimating impacts of N inputs on moss-associated N_2 fixation in natural ecosystems.

Declarations

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Ethics declarations

The authors declare that they have no conflict of interest.

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Tables

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

Figures

Fig. 1

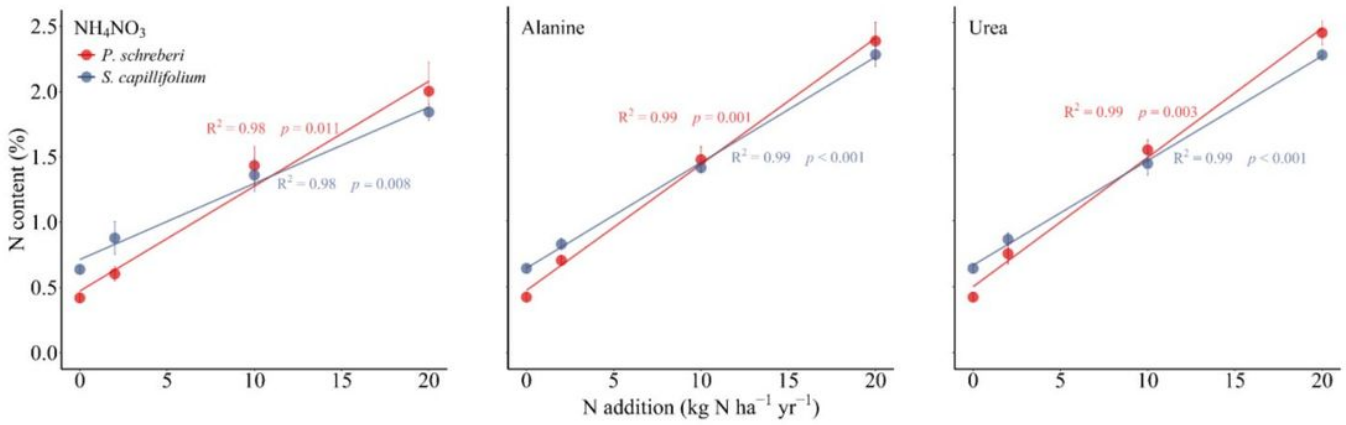

Figure 1

Moss tissue N content (%) in two moss species Pleurozium schreberi (red) and Sphagnum capilifollium (blue) at the end of the experiment after different N addition rates (extreme N addition rates: 0, 0.4, 2 and 4 kg N ha-1 yr-1) and N forms (NH4NO3, urea and alanine). Given are mean values ±SE (n = 6).

Fig. 2

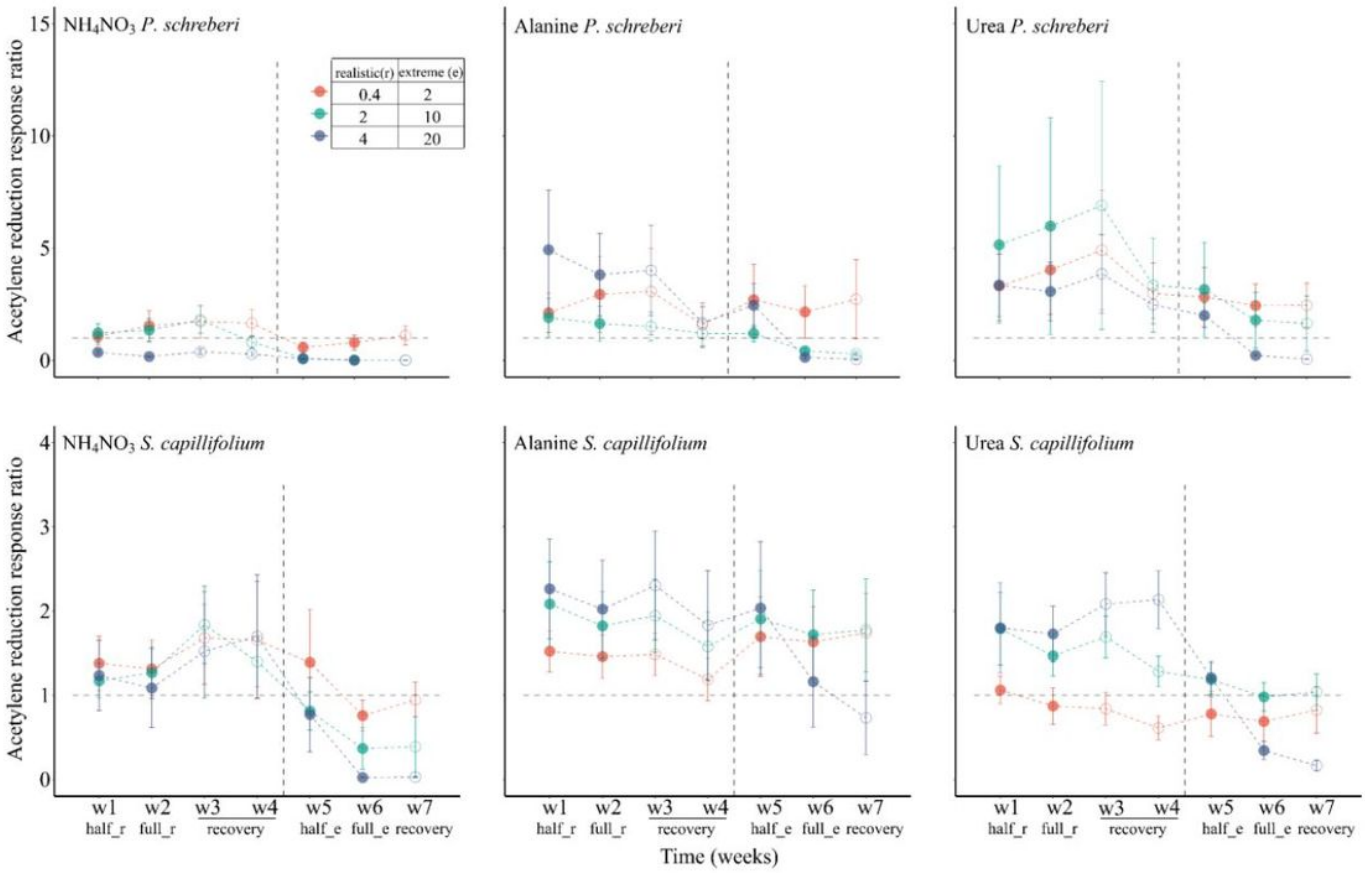

Figure 2

Acetylene reduction response ratio at different N addition rates and forms in two moss species (P. schreberi in red, S. capillifolium in blue) measured each week during the experiment relative to controls. Given are means ± SE (n = 6). On the x axes, half_r and full_r correspond to samples that received half or the full amount of the realistic N addition, and half_e and full_e represent samples that received half or the full amount of the extreme N addition. During recovery weeks, samples received only ddH2O. On the left side of the vertical dotted lines are the realistic N additions (0, 0.4, 2 and 4 kg N ha-1 yr-1) and on the right side are the extreme N additions (0, 2, 10, 20 kg N ha-1 yr-1). Full symbols indicate weeks where N was added, while open symbols indicate recovery weeks. Response ratios with error bars not overlapping RR = 1 (horizontal dotted line), indicate that the treatment significantly increased (above the line) or decreased (below the line) ethylene production relative to the control treatment.

Fig. 3

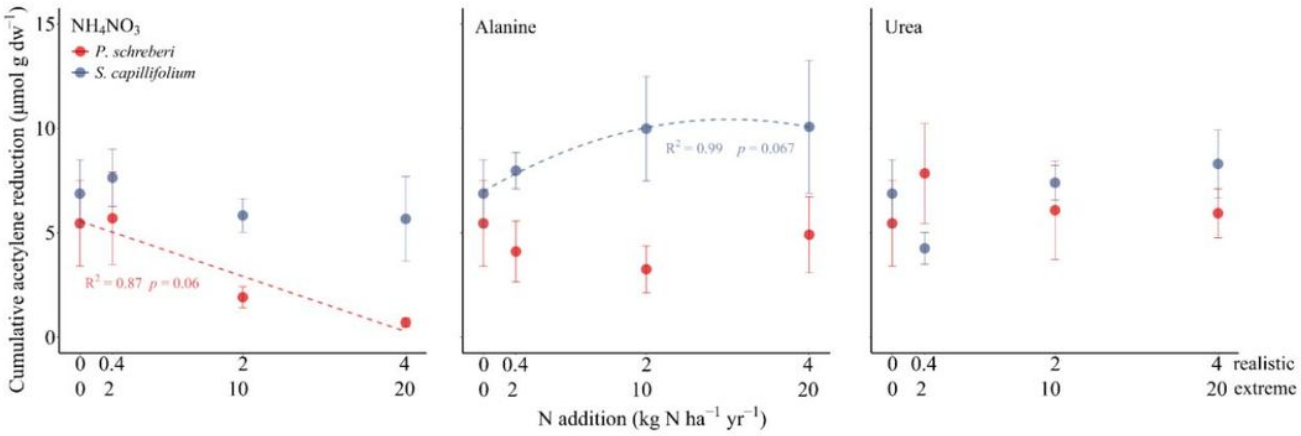

Figure 3

Cumulative acetylene reduction (μmol g dw-1) in moss samples in response to different N addition rates and forms in two moss species (P. schreberi, S. capillifolium) over a 7-week period. On the x axes, the first row of number represents the realistic N additions (0, 0.4, 2 and 4 kg N ha1 yr-1) and the second row are the extreme N additions (0, 2, 10, 20 kg N ha-1 yr-1). Given are means ± SE (n = 6). Solid lines indicate significant relationships at $p < 0.05$, while dotted lines indicated relationships at $p < 0.1$.

Fig. 4

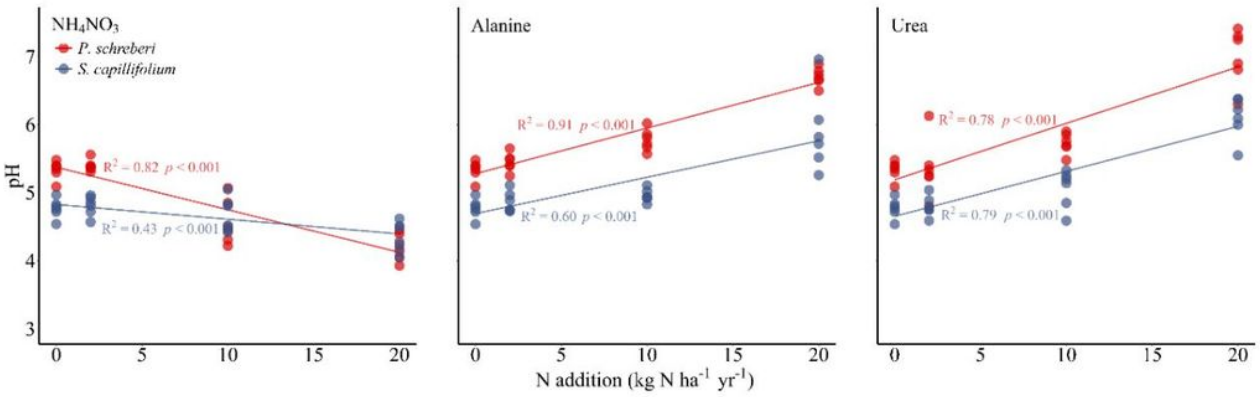

Figure 4

Moss (P. schreberi, S. capillifolium) pH (mean ±SE, n=6) exposed to different N rates and forms at the end of the experiment.

Fig. 5

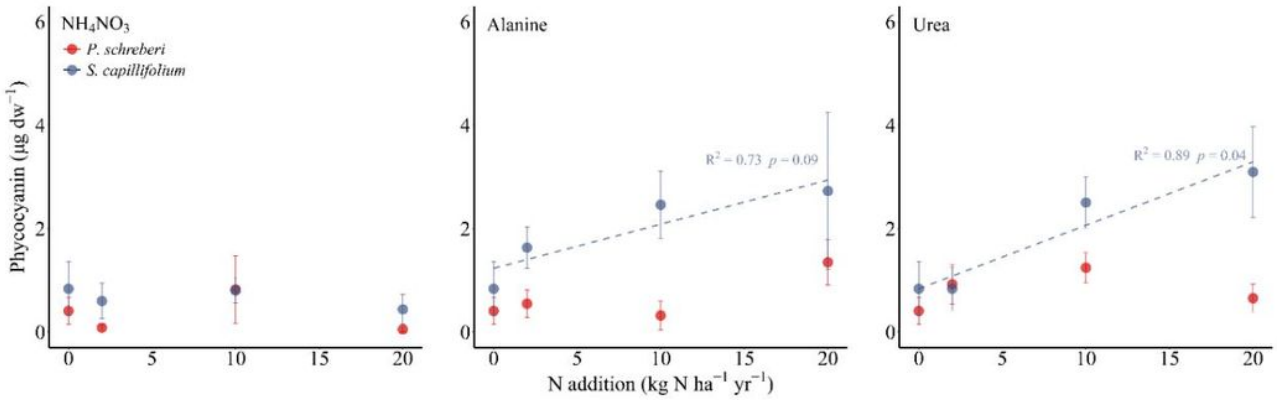

Figure 5

Phycocyanin content (μg dw-1) in moss samples in response to different N addition rates and forms in two moss species (P. schreberi, S. capillifolium). Given are means ± SE (n = 6). Solid lines indicated the significant relationships at $p < 0.05$, while dotted lines indicated the relationships at $p < 0.1$.

Supplementary Files

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