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Chronic Atmospheric Reactive Nitrogen Deposition Suppresses Biological Nitrogen Fixation in Peatlands

Ernesto Saiz, Fotis Sgouridis, Falko P. Driijfhout, Matthias Peichl, Mats B. Nilsson, and Sami Ullah*



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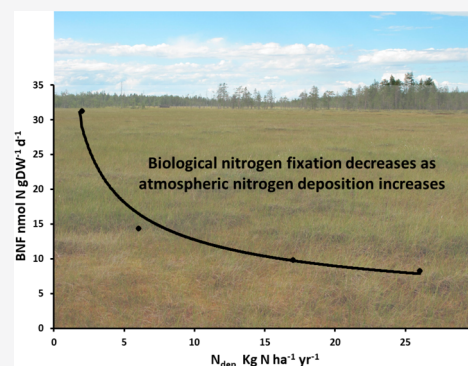
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ABSTRACT: Biological nitrogen fixation (BNF) represents the natural pathway by which mosses meet their demands for bioavailable/reactive nitrogen (Nr) in peatlands. However, following intensification of nitrogen fertilizer and fossil fuel use, atmospheric Nr deposition has increased exposing peatlands to Nr loading often above the ecological threshold. As BNF is energy intensive, therefore, it is unclear whether BNF shuts down when Nr availability is no longer a rarity. We studied the response of BNF under a gradient of Nr deposition extending over decades in three peatlands in the U.K., and at a background deposition peatland in Sweden. Experimental nitrogen fertilization plots in the Swedish site were also evaluated for BNF activity. In situ BNF activity of peatlands receiving Nr deposition of 6, 17, and 27 kg N ha⁻¹ yr⁻¹ was not shut down but rather suppressed by 54, 69, and 74%, respectively, compared to the rates under background Nr deposition of ~2 kg N ha⁻¹ yr⁻¹. These findings were corroborated by similar BNF suppression at the fertilization plots in Sweden. Therefore, contribution of BNF in peatlands exposed to chronic Nr deposition needs accounting when modeling peatland's nitrogen pools, given that nitrogen availability exerts a key control on the carbon capture of peatlands, globally.

KEYWORDS: ¹⁵N₂ assimilation method, biological nitrogen fixation, *Sphagnum* mosses, diazotrophs, Nr deposition, peatlands, nitrogen biogeochemistry



INTRODUCTION

Since the industrial revolution, the input of anthropogenic reactive nitrogen (Nr) to the land has more than doubled due to three principal activities: agricultural intensification, fertilizer production and fossil fuel combustion.^{1–3} This Nr consists of two major forms: reduced N (NH_x) mainly in the forms of NH₃ and NH₄⁺, and oxidized N (NO_y) mainly in the forms of NO₂ and particulate NO₃.⁴ Although in Western countries the Nr deposition rates are expected to continue declining during the next few decades,⁵ in the developing countries of Asia, Africa, and South America, Nr deposition is expected to rise further by 20% between 2010 and 2100.⁶ In the U.K., the Nr deposition rates in peatlands range from <10 kg N ha⁻¹ yr⁻¹ in the north of Scotland to more than 30 kg N ha⁻¹ yr⁻¹ in the Northwest of England.⁷ Payne (2014)⁸ remarked that it is highly likely that the UK areas with the lowest rates of Nr such as Forsinard in Scotland will still suffer an increase from about 6 kg N ha⁻¹ yr⁻¹ to 9 kg N ha⁻¹ yr⁻¹ by 2030 due to the lack of synchronization between emission and deposition.

Peatlands, often dominated by *Sphagnum* mosses,^{9,10} rely on biological nitrogen fixation (BNF) from moss associated and free-living diazotrophic organisms^{11,12} for the N nutrition as an additional source to complement atmospheric Nr deposition to meet their metabolic N demands.¹³ The process of fixing atmospheric N₂ is energy intensive, requiring 16 molecules of adenosine triphosphate (ATP) to fix 1 mol of N₂.¹⁴

Therefore, high rates of Nr deposition could potentially negate the need for a “costly” investment on BNF by peatlands. However, experimental Nr addition experiments reported contradictory impacts on BNF in peatlands. For example, in a boreal bog dominated by *Sphagnum* mosses, BNF was progressively inhibited following five years of experimental N fertilization at rates ranging from 5 to 25 kg N ha⁻¹ yr⁻¹.¹⁵ Alternatively, in plots with *Sphagnum* mosses subjected to long-term experimental Nr deposition (32 kg N ha⁻¹ yr⁻¹) van den Elzen et al. (2018)¹⁶ observed no impact on BNF activity. These contrasting results could be due to methodological anomalies as the former study, that of Wieder et al. (2019),¹⁵ quantified BNF activity through the indirect/surrogate acetylene reduction assay (ARA) technique, which is not a robust technique compared to the direct ¹⁵N assimilation method for assessing BNF activity of mosses in peatlands.¹⁷ The direct interference of acetylene with microbial activities including inhibition of nitrification, nitrous oxide reduction, and methane oxidation can result in under or overestimation of BNF activity.¹⁷ In addition to methodological

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Table 1. Mean Annual Temperature, Precipitation, Reactive Nitrogen (Nr) Deposition, and NH_x/NO_y Ratio at the Study Sites^a

site	mean annual temperature (°C)	mean annual precipitation (mm)	atmospheric Nr deposition ($\text{kg N ha}^{-1} \text{ yr}^{-1}$)	NH_x/NO_y ratio in atmospheric deposition
Degerö ^b (Sweden)	1.8	614	2	1.1
Forsinard (Scotland)	6.9	1104	6	1.4
Migneint (Wales)	7.3	2236	17	1.9
Fenn's & Whixall (England)	9.5	747	27	6.9

^aSource: Met Office (U.K.), Air Pollution Information System (APIS), European Monitoring and Evaluation Programme (EMEP).^{24,25,28} ^bMAT and MAP are the 30 years long-term average 1981–2010.

uncertainties, to our knowledge, no studies evaluated the response of BNF activity in peatlands under a gradient of decades of chronic Nr deposition across a wider geographic region to elucidate the response of BNF activity in intact peatlands.

Since the experimental Nr addition studies points to contradictory shifts in a key biogeochemical process, thus a need exist for extensive spatial evaluation of BNF across the contemporary Nr deposition gradients to enable a more realistic assessment of BNF under in situ conditions. This evaluation is imperative given that coupled N and C cycle models (e.g., N14CP model) simulating the C capture response of terrestrial natural ecosystems, including peatlands, to Nr deposition^{18,19} assume zero contribution of BNF into peatlands when background Nr deposition thresholds are exceeded. This assumption of zero BNF contribution may lead to over or underestimation of the total N budget and its implications for C capture by peatlands given that even in Europe, under high Nr deposition, peatlands are not completely overtaken by vascular plants and thus *Sphagnum* mosses with the associated and free-living diazotrophs may still be performing this important ecological function. Also Nr deposition constitute both oxidized and reduced mineral N species (NH_x and NO_y), and their relative proportions depend on source proximity (agriculture vs fossil fuel), thus the composition and dynamics of Nr deposition may have differing impacts on BNF activity. This is important given that BNF generates NH_4^+ and if Nr deposition includes both NH_4^+ and NO_3^- , then the impact of these two main species might be different on BNF activity.

The analysis of the natural abundance of the ^{15}N isotope in *Sphagnum* mosses provides information about the N sources used for growth.²⁰ If *Sphagnum* mosses take up N through BNF, then their $\delta^{15}\text{N}$ signature would be close to zero, similar to the atmospheric $^{15}\text{N}_2$ isotopic signal.²¹ Conversely, the type of atmospheric Nr deposition also affects the $\delta^{15}\text{N}$ signature, with elevated rates of NH_x deposition resulting in depleted values of $\delta^{15}\text{N}$ while elevated rates of NO_y forms resulting in enriched $\delta^{15}\text{N}$ values in plant tissues.^{20,22} Fractionation of N isotope originating from the mineralization of peat will lead to a $\delta^{15}\text{N}$ decrease in plant tissue.^{12,23} Thus, an opportunity exists to quantify BNF activity in the field using the $^{15}\text{N}_2$ assimilation method and corroborate the findings using the $\delta^{15}\text{N}$ natural abundance in mosses and bulk peat to elucidate the impacts of Nr deposition on BNF activity in peatlands.

Our objectives in this study were to use the $^{15}\text{N}_2$ assimilation method (1) to evaluate the effects of decades long chronic Nr deposition upon rates of BNF in peatlands across a large geographic region; (2) to investigate the effects of decades long experimental Nr and sulfur (S) fertilization and elevated temperature on BNF in experimental plots of a low-background peatland; and (3) to examine the source of Nr in *Sphagnum*

mosses and peat by investigating their natural abundance $\delta^{15}\text{N}$ signature across an Nr deposition gradient.

MATERIALS AND METHODS

Study Sites. Samples were collected from four different peatlands which represent an atmospheric Nr deposition gradient. Three sites were in the U.K.: Fenn's & Whixall ($52^\circ 92' \text{ N } 2^\circ 72' \text{ W}$) in England, Migneint ($52^\circ 97' \text{ N } 3^\circ 83' \text{ W}$) in Wales, and Forsinard ($58^\circ 38' \text{ N } 3^\circ 92' \text{ W}$) in Scotland; and one, Degerö Stormyr ($64^\circ 11' \text{ N } 19^\circ 33' \text{ E}$), located in northern Sweden (Figure S1 of the Supporting Information, SI). The latter was selected as a reference site due to its low background Nr deposition rates. The four sites had different patterns of precipitation, temperature, Nr deposition, and NH_x/NO_y ratio (Table 1). The Nr deposition rates for each of the U.K. sites were obtained through the Air Pollution Information System (APIS) that used the Fine Resolution Atmospheric Multipollutant Exchange (FRAME) model to produce a three year average estimation (2013–2015) of the wet and dry N deposition (NH_x and NO_y).²⁴ The three years (2014–2016) Nr deposition data for Degerö were obtained from the European Monitoring and Evaluation Programme (EMEP).²⁵ For a full description of the EMEP MSC-W version see Simpson et al. (2012).²⁶

Sampling Campaigns. Two main sampling campaigns were carried out during the growing season (in June in the U.K. sites and July in Sweden, 2016–2017) in the study sites during which in situ incubations were undertaken, except in Forsinard and for the experimental fertilization treatment plots in Degerö that were sampled and incubated in situ only in 2017. Four dominant *Sphagnum* moss species as well as bulk peat (0–15 cm) from hollows and hummocks were collected for in situ incubations (in Degerö treatment plots only two moss species from hollows). Two species usually located in hollows (in pools or wet areas), *Sphagnum cuspidatum* and *S. fallax*, and two species that usually form hummocks (elevated and less wet areas), *S. capillifolium* and *S. papillosum*. In Degerö it was not possible to find the exact same species, except for *S. papillosum*, therefore similar ones were sampled:²⁷ in hollows *S. majus* and *S. balticum*; and in hummocks *S. fuscum*.

Degerö Stormyr Treatment Plots. At the Degerö peatland site, an experiment started in 1995 to evaluate the effects of increased air temperature (T) combined with increased nitrogen (N) and sulfur (S) deposition on peatland biogeochemistry and ecology. Plots ($2 \times 2 \text{ m}^2$) with two levels of temperature (with, $+1.5^\circ \text{C}$, and without polycarbonate shelter) and three levels of S, and N (no addition, 10/15 and 20/30 $\text{kg ha}^{-1} \text{ yr}^{-1}$ of S and N, respectively) were established following a full factorial design, giving a total of 20 plots. Thus, the number of replicates for evaluating the main, two way, and three way interaction effects, respectively, were 8, 4, and 2, i.e., two plots exposed to three

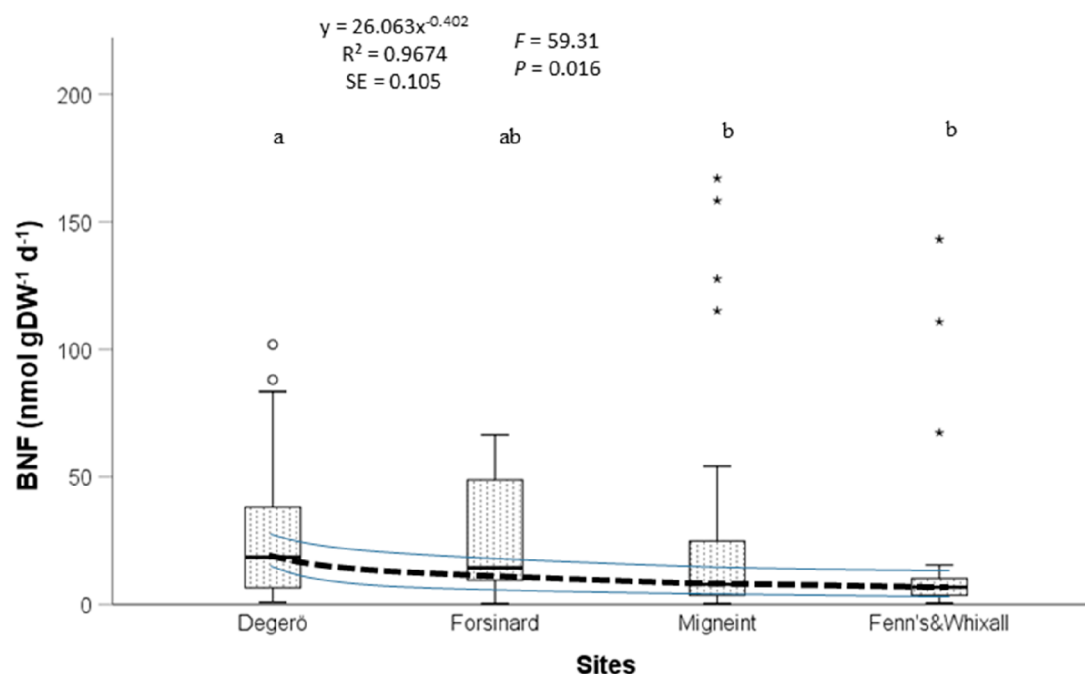


Figure 1. Boxplot of BNF rates ($\text{nmol gDW}^{-1} \text{d}^{-1}$) of all species at each sampling site: Migneint, Fenn's & Whixall, Forsinard, and Degerö ($n = 39$, except Forsinard $n = 15$). The box shows the median (central line), the 25th (lower part), and 75th (upper part) percentiles with whiskers indicating the minimum and maximum values. The white dots show outliers ($1.5\text{--}3$ IQR) and the stars extreme values (>3 IQR). Sites with different letters have significantly different BNF rates. Kruskal–Wallis Test: $H(3) = 11.499$, $P = 0.009$, with mean rank of 57.0 for Fenn's & Whixall, of 63.5 for Migneint, of 84.0 for Forsinard, and of 84.8 for Degerö. The dotted line shows the power regression line between BNF (median values) at each site and atmospheric N_r deposition in those sites (2, 6, 17, and 27 $\text{kg N ha}^{-1} \text{yr}^{-1}$, respectively), and the blue lines show the upper and lower limits of 95% confidence intervals.

treatment combinations (SNT), 10 plots exposed to two treatment combinations (4 ns, 2-NS, 2-NT, 2-ST), six plots exposed to one treatment (2-N, 2-S, 2-T), and two control plots under ambient conditions with no fertilization or temperature treatment. At each plot, 5 replicate samples were incubated. The treatment additions were applied as one-third after the snowmelt, and the rest of the fertilization was undertaken every month from June to September in one-sixth doses dissolved in surface mire water. They were N as ammonium nitrate (NH_4NO_3), and S as sodium sulfate (Na_2SO_4). No additions of N and S meant that water from the mire was used, and the deposition was the natural background recorded for the area, 3 $\text{kg ha}^{-1} \text{yr}^{-1}$ for S and 2 $\text{kg ha}^{-1} \text{yr}^{-1}$ for N. The temperature was a qualitative variable. Table S1 shows the description of the treatments for each plot. A detailed explanation of the experimental design and manipulations can be found in Granberg et al. (2001).²⁹

Biological Nitrogen Fixation ($^{15}\text{N}_2$ Assimilation Method). To measure BNF rates in situ the $^{15}\text{N}_2$ assimilation method was used as per Saiz et al. (2019).¹⁷ The incubated samples consisted of about 20 shoots (5 cm upper part) for each of the *Sphagnum* species, and about 10 g of peat (homogenized through a 2 mm sieve) that were placed, separately, into 50 mL glass serum vials. At each sampling site there were four incubation replicates and one control for each of the *Sphagnum* species and peat. Immediately after the insertion of the samples in the vials they were capped using rubber septa, and 5 mL of air (10% of the headspace) was replaced with $^{15}\text{N}_2$ gas (98 atom % ^{15}N Cambridge Isotope Laboratories Inc., U.S.A.). The gas was previously checked for contamination,³⁰ and the data for BNF calculation corrected accordingly (see SI). Then the vials were placed upside-down (to avoid cap shade) in the same spot where

the samples were collected. In the case of the peat samples, they were located under the moss carpet. After 24 h of incubation, the vials were opened and ventilated to flush out the remaining gas. The samples were transferred to the laboratory (see detailed protocols in Saiz et al. 2019), dried (calculating bulk density and gravimetric moisture), pulverized and packed into tin capsules and sent to the UK Centre for Ecology and Hydrology (Lancaster U.K.), where the samples were analyzed for ^{15}N content in peat and moss tissues by an Isotope Ratio Mass Spectrometer (IRMS). The analytical precision of the IRMS was 0.36 ‰. The analysis of all the samples (control and enriched) was done in duplicate,³¹ and if the difference between samples was greater than $\sim 0.5\text{‰}$ the analysis was repeated. To calculate the BNF rates, the following formula was used:³²

$$Y = \left(\frac{\text{atom\% } ^{15}\text{N}_{\text{excess}}}{100} \right) \times \left(\frac{\text{total } N_{\text{sample}} \times 10^9}{t \times 28} \right) \times \left(\frac{100}{\text{‰}^{15}\text{N}_{\text{air}}} \right)$$

where Y ($\text{nmol N gdw}^{-1} \text{h}^{-1}$) is the molar amount of N_2 fixed during the experiment, $\text{atom\% } ^{15}\text{N}_{\text{excess}}$ is the difference between $\text{atom\% } ^{15}\text{N}_{\text{sample}}$ and $\text{atom\% } ^{15}\text{N}_{\text{control}}$, $\text{total } N$ is the total amount of nitrogen in the sample ($\text{g N } 100 \text{ gdw}^{-1}$), t is the incubation time, 28 is the molecular weight of N_2 (g/mol), and $\text{‰}^{15}\text{N}_{\text{air}}$ is the percentage of ^{15}N out of the total amount of N gas in each incubation vial.

Information about the gas contamination correction, elemental analyses in *Sphagnum* tissue and peat, and ancillary measurements in the field are available in the SI section.

Statistical Analysis. We performed the statistical analysis using IBM SPSS Statistics for Windows software, version 24 (IBM Corp., NY, U.S.A.). We tested the data for normality (Shapiro–Wilk) and for homogeneity of variance (Levene's test) and they resulted to be non-normal and/or nonhomogeneous,

even transforming the data. Consequently, the statistical analysis was done using nonparametric tests, in which all data was included.³³ To test correlations between two variables among paired samples we used the Spearman's rank-order correlation. The bootstrapped *t* test was used to look for differences in paired samples. The differences by site and the differences by species or by treatments in the same site were measured using the Kruskal–Wallis test, followed by pairwise comparisons. Significant differences were considered at $P < 0.05$.

RESULTS

BNF across an Nr Deposition Gradient. Median BNF rates across the two growing seasons (2016–2017) were significantly different among sites ($P < 0.01$), while there was a significant inverse correlation between BNF and Nr deposition ($P < 0.01$; Spearman's $\rho = -1.000$) (Figure 1). The decrease in the median BNF rates under increasing Nr deposition followed a power relationship (Figure 1) and was consistent for each year, i.e., 2016 and 2017. Using contemporary Nr deposition data of the ratios of reduced and oxidized mineral N (NH_x and NO_y ; Table S2) we observed a significant ($P < 0.01$; Spearman's $\rho = -1.000$) negative correlation between NH_x/NO_y ratios and BNF rates among sites, i.e., the higher the relative proportion of NH_x , the lower the BNF rates.

The BNF suppression ratios (Table 2) obtained (rate of BNF reduced, per unit of Nr deposition, in $\text{mg N m}^{-2} \text{d}^{-1}$) for each of

Table 2. Suppression Ratio^a, Following an Increasing Nr Deposition^b Gradient ($\text{mg N m}^{-2} \text{d}^{-1}$), for each of the British Sites Looking at the Rate of BNF^c Reduced Considering 2017 Medians ($\text{mg N m}^{-2} \text{d}^{-1}$; the Reference for the First Site is Degerö) per Unit of Nr Deposition

Site	BNF ($\text{mg N m}^{-2} \text{d}^{-1}$)	Nr deposition ($\text{mg N m}^{-2} \text{d}^{-1}$)	Suppression ratio
Degerö	0.15	0.55	-
Forsinard	0.07	1.6	0.08
Migneint	0.05	4.7	0.006
Fenn's and Whixall	0.03	7.4	0.005

^aIt is calculated as the ratio of BNF with the preceding site with lower Nr deposition ($(\text{BNF}_{\text{ref}} - \text{BNF}_i) / (\text{Nr dep}_i - \text{Nr dep}_{\text{ref}})$) with (i) representing each of the British sites, starting with Degerö–Forsinard, then Forsinard–Migneint, and finally Migneint–Fenn's & Whixall. ^bNr deposition considered as “average” for the whole year. ^cBNF per surface area calculated after knowing the surface of incubated *Sphagnum* and peat.

the British sites while using the Swedish Degerö peatland as reference (under background Nr deposition), we observed that the suppression effect was 13.3 times higher in the Forsinard than in the Migneint, and 1.2 times higher in Migneint than in the Fenn's & Whixall peatland. We observed a very high suppression effect of Nr deposition on BNF in the area of Britain, where the Nr deposition was the lowest and the suppression effect decreased as Nr deposition increased.

BNF rates were significantly different among species ($P = 0.006$) with *S. fallax* inhabiting hollows showing the highest rate ($16.6 \pm \text{median absolute deviation-MAD } 13.6 \text{ nmol N gDW}^{-1} \text{d}^{-1}$) followed by *S. cuspidatum* (Figure 2). Mosses (including that from Degerö species: median BNF rates of $11.2 \pm \text{MAD of}$

$8.2 \text{ nmol N gDW}^{-1} \text{d}^{-1}$) showed higher BNF rates than peat (median of $6.8 \pm \text{MAD of } 2.8 \text{ nmol N gDW}^{-1} \text{d}^{-1}$). The results also showed that *Sphagnum* species in hollows (*S. cuspidatum* and *S. fallax*) fixed 69% more (median of $15 \pm \text{MAD } 13.4 \text{ nmol N gDW}^{-1} \text{d}^{-1}$) than the ones in hummocks (*S. capillifolium* and *S. papillosum*; median of $8.9 \pm \text{MAD } 6.1 \text{ nmol N gDW}^{-1} \text{d}^{-1}$).

Environmental Factors Affecting BNF. We found a significant negative correlation ($P = 0.029$; Spearman's $\rho = -0.655$) between BNF and NH_4^+ in peat while a weak but significant ($P = 0.042$; Spearman's $\rho = 0.351$) positive correlation between BNF and pore water NO_3^- concentration Table S3. Among the range of macro and micronutrients that we analyzed in moss tissues and peat (Tables S4 and S5), we only found a significant positive correlation between BNF and calcium (Ca; $P = 0.046$; Spearman's $\rho = 0.296$) and a negative correlation with manganese (Mn; $P = 0.004$; Spearman's $\rho = -0.551$; Tables S4 and S5). Interestingly, to be considered as a trend, we found a significant ($P < 0.01$; Spearman's $\rho = 1.000$) positive correlation between Nr deposition and the concentration of Ni, Cu, Mo, and P at each site, and also a negative one ($P < 0.01$; Spearman's $\rho = -1.000$) with the C:P ratio.

Variability in $\delta^{15}\text{N}$ Signature. The $\delta^{15}\text{N}$ values decreased with the increase of Nr deposition in the UK from a median value of -1.49 ‰ in Forsinard with a rate of Nr deposition of $6 \text{ kg N ha}^{-1} \text{y}^{-1}$, to -5.73 ‰ in Fenn's & Whixall with an Nr deposition of $27 \text{ kg N ha}^{-1} \text{y}^{-1}$ (Table S2), and we found a significant negative correlation ($P < 0.01$; Spearman's $\rho = -1.000$) between Nr deposition and $\delta^{15}\text{N}$. The median $\delta^{15}\text{N}$ value found in Degerö was -2.26 ‰ , slightly lower than that of Forsinard. Regarding the NH_x/NO_y ratio (Table S2), we found a significant negative correlation ($P < 0.05$; Spearman's $\rho = -0.372$) with the $\delta^{15}\text{N}$ signature, as the ratio decreased (F&Whixall > Migneint > Forsinard < Degerö), the $\delta^{15}\text{N}$ values, in general, increased.

The *Sphagnum* species forming hummocks, *S. capillifolium* (including *S. fuscum*), and *S. papillosum* had a median $\delta^{15}\text{N}$ value of -4.72 ‰ and -4.18 ‰ , respectively, which were the lowest. The median $\delta^{15}\text{N}$ signature for the species in hollows *S. cuspidatum* (including *S. majus*) and *S. fallax* (including *S. balticum*) was -2.63 ‰ and -2.92 ‰ correspondingly. The peat from hollows and from hummocks had values closer to 0 : -0.08 ‰ and -0.59 ‰ , respectively (Figure 3).

Degerö Treatment Plots. The results of the Degerö treatment plot incubation (Figure 4) show that after more than two decades of N, S, and T treatments (Table S1), BNF did not shut down although it was reduced. The treatments with a significant reduction compared to the control plots (median of $31.3 \text{ nmol N gDW}^{-1} \text{d}^{-1}$) were SN T, NS, ns and N, with median rates of 2, 3.3, 3.9, and $11.2 \text{ nmol N gDW}^{-1} \text{d}^{-1}$ respectively. Other treatments resulted also in a considerable decrease such as T with a rate of 8.8, ST of 8.9, and NT of $10.1 \text{ nmol N gDW}^{-1} \text{d}^{-1}$. In addition, regarding S, although BNF rates were overall lower than the control ones, in one of the two plots with the S treatment (there were at least two plots for each treatment), the rates were higher than the median of the control plots. The median BNF rates of the three treatments of N, S, and T (considering 8 plots with the high levels of each treatment, and 4 plots for the two way combined treatments—n and s low level treatment) were significantly lower than the control ($P < 0.05$), but no significant difference was found among them nor considering all possible combinations ($P > 0.05$).

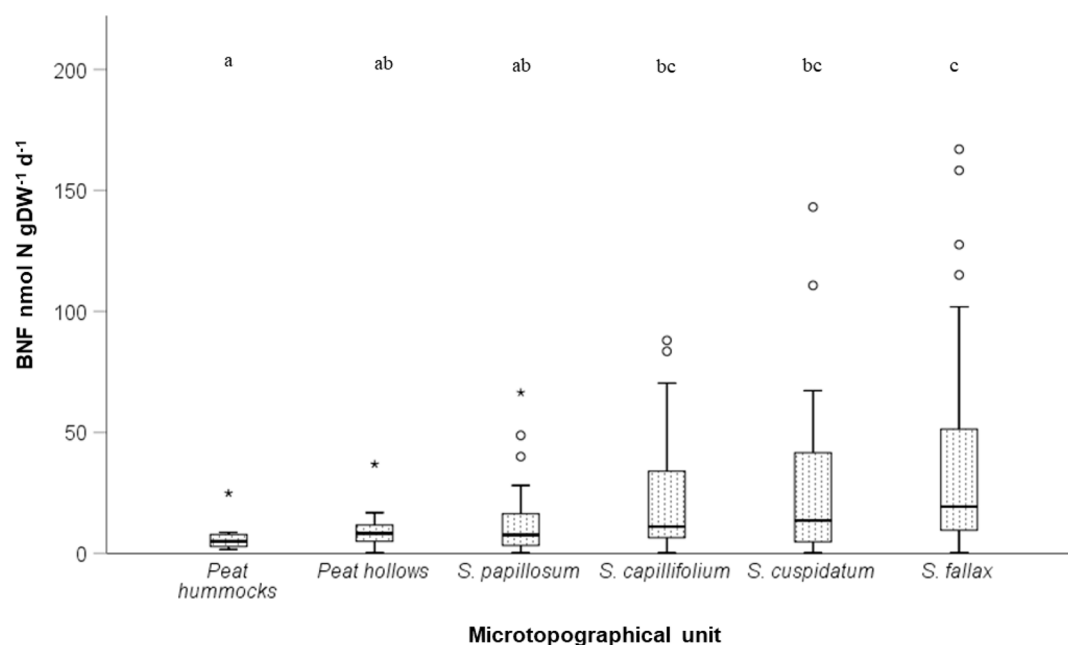


Figure 2. Boxplot of the overall (2016–2017) BNF rates (nmol gDW⁻¹ d⁻¹) per *Sphagnum* moss species and peat ($n = 27$). Also included is the data from Degerö *Sphagnum* species as follows: *S. fuscum* in *S. capillifolium*, *S. majus* in *S. cuspidatum*, and *S. balticum* in *S. fallax*. The box shows the median (central line), the 25th (lower part), and 75th (upper part) percentiles with whiskers indicating the minimum and maximum values. The open circles show outliers (1.5–3 IQR), and the stars extreme values (>3 IQR). Kruskal–Wallis Test: $H(5) = 16.295$, $P = 0.006$. Species with different letters have significantly different BNF rates.

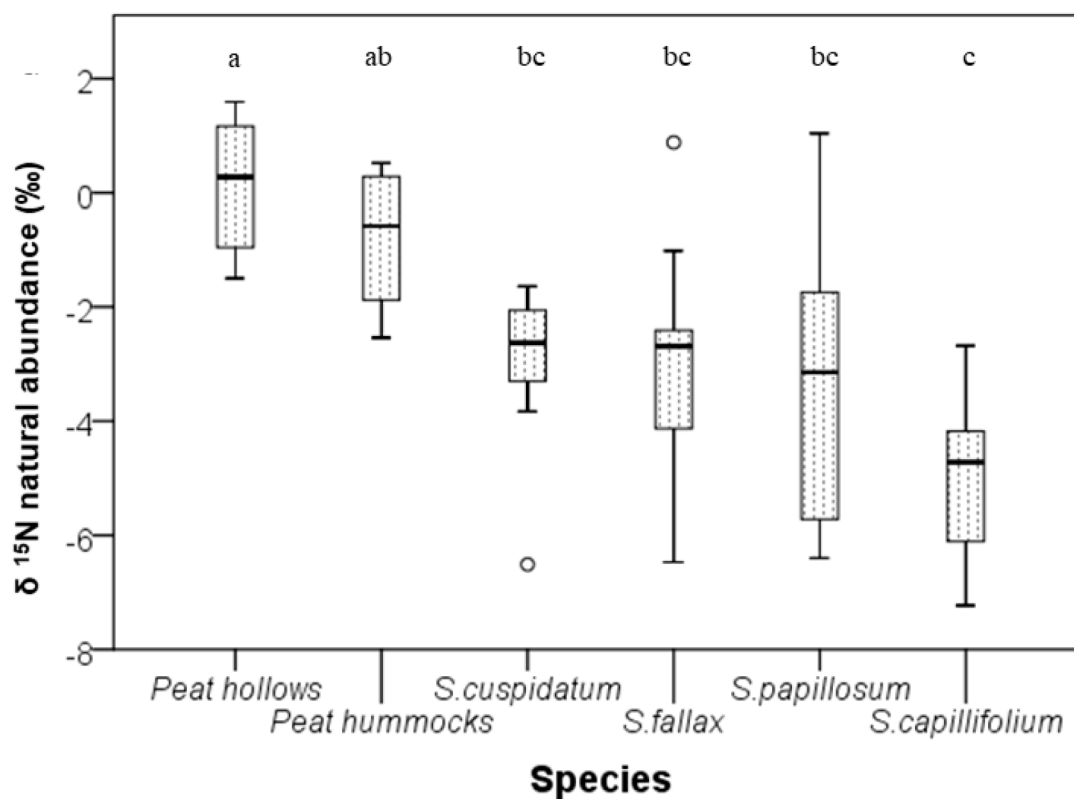


Figure 3. Boxplot of the $\delta^{15}\text{N}$ natural abundance in ‰ ($n = 7$) of the six different species of *Sphagnum* and peat studied in the four sites (including Degerö species). The box shows the median (central line), the 25th (lower part), and 75th (upper part) percentiles with whiskers indicating the minimum and maximum values. The dots show outliers (1.5–3 IQR).

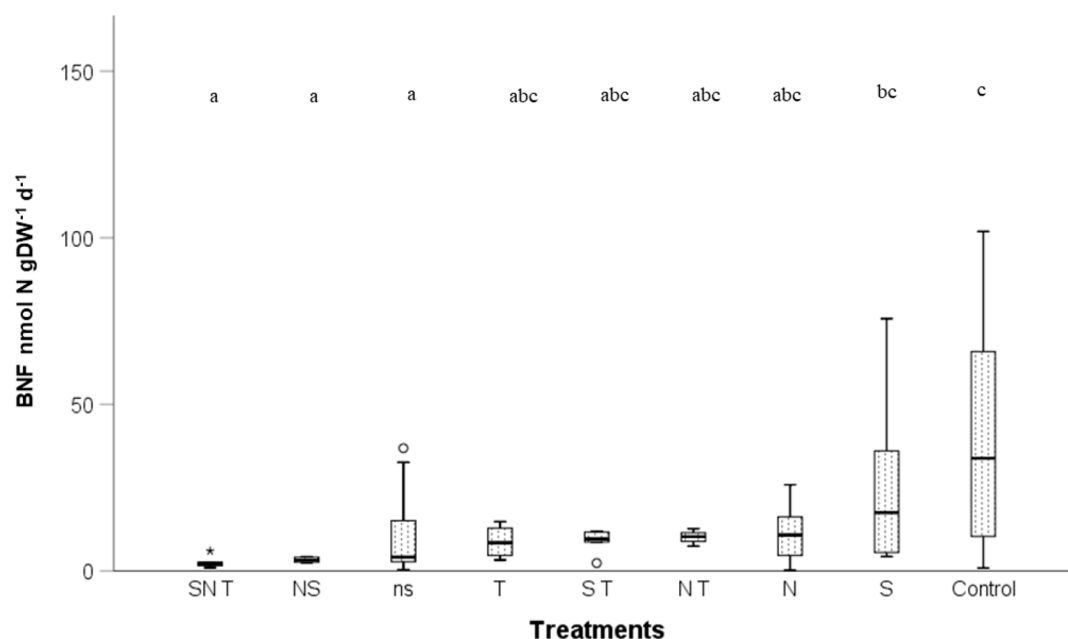


Figure 4. BNF rates ($\text{nmol N gDW}^{-1} \text{d}^{-1}$) of the *Sphagnum* spp. in response to the different experimental factors ($n = 8$; except ns $n = 16$). The box shows the median (central line), the 25th (lower part), and 75th (upper part) percentiles with whiskers indicating the minimum and maximum values. The dots show outliers (1.5–3 IQR) and the stars extreme values (>3 IQR). Treatments with different letters have significantly different BNF rates. (S, sulfur; N, nitrogen; T, temperature; and the combined treatments).

DISCUSSION

BNF rates in peatlands (mosses and bulk peat) decreased along an increasing gradient of Nr deposition, showing a significant negative correlation (Figure 1); however a complete shutdown was not observed. The suppression effect of Nr deposition on BNF was higher (per unit of Nr deposition) in areas with lower Nr deposition rates (e.g., Forsinard) than in areas with high Nr deposition rates (e.g., Fenn's & Whixall) (Table 2). This suggests that BNF activity is more sensitive to Nr deposition in areas with a low Nr deposition rate, i.e., more pristine areas, and as the Nr deposition rate increases (more Nr pollution) the suppression ratio decreases, suggesting the development of diazotrophic microbes tolerance to high rates of Nr deposition. Overall, on the basis of the $^{15}\text{N}_2$ assimilation method, BNF activity in peatlands was suppressed under chronic and excessive Nr deposition rates (above the typical ecological threshold of $10 \text{ kg N ha}^{-1} \text{yr}^{-1}$)³⁴ but not completely shut down. The ecological Nr deposition threshold defines the limit beyond which vascular plants dominates over mosses in peatlands.³⁴ Under such circumstances, pockets of mosses in the wet areas of the peatlands tend to sustain their BNF activity as has been observed in our most polluted peatland of Fenn's & Whixall in England where the majority of the peatland is taken over by cotton grass (*Eriophorum* spp) and heather (*Calluna vulgaris*).

The gradual increase over decades in Nr deposition rates above the natural background may have affected the diazotrophic microbial population by making them less sensitive to high rates of Nr deposition. Compton et al. (2004)³⁵ found, in a study of microbial communities in pine and hardwood stands under different chronic Nr additions, that the gene for N_2 -fixation was present in the two forest soils. However, compared to hardwood forests, the gene in the pine soils was rare under Nr deposition suggesting a reduction of the diazotrophs and hence of the fixation gene expression. We found a similar percentage of suppression in the median BNF rates for 2016 and 2017 in the Fenn's & Whixall peatland (63%) compared to the experimental

fertilization plots in the Degerö peatland after more than 20 years of Nr addition at $30 \text{ kg N ha}^{-1} \text{yr}^{-1}$ (64%) which is close to the Nr deposition rates of the former. These results suggest that irrespective of differences in abiotic factors across the wider geographic regions, Nr deposition induced suppression of BNF activity both across the field sites and within the same site under experimental fertilization (Degerö peatland), which is commensurate with the findings of van den Elzen et al. (2018)¹⁶ under 11 years of experimental N fertilization of a peatland in Scotland.

The median BNF rates found in Degerö ($18.51 \text{ nmol N gDW}^{-1} \text{d}^{-1}$) were within the range of those reported in a low background oligotrophic fen in Finland ($14.4\text{--}163 \text{ nmol N gDW}^{-1} \text{d}^{-1}$).³⁶ In Fenn's & Whixall with an Nr deposition of $\sim 27 \text{ kg N ha}^{-1} \text{yr}^{-1}$ and Migneint bog with $\sim 17 \text{ kg N ha}^{-1} \text{yr}^{-1}$, the median of BNF rates were 6.8 and $7.9 \text{ nmol N gDW}^{-1} \text{d}^{-1}$, respectively, which were far lower than the rates found by van den Elzen et al. (2017)³⁷ ranging between 517 and $1651 \text{ nmol N gDW}^{-1} \text{d}^{-1}$ in *Sphagnum* mosses collected from a fen in The Netherlands with a Nr deposition rate of $25 \text{ kg N ha}^{-1} \text{yr}^{-1}$. However, these high BNF rates were obtained in a mesocosm experiment in the laboratory under optimal controlled temperature set at 18°C at vegetation level with a daily light regime of 16 h which may have induced higher BNF activity compared to our median values based on incubations under field conditions. The mean BNF rate of $12.2 \text{ nmol N gDW}^{-1} \text{d}^{-1}$ found in laboratory incubations of peat from a forested peatland of Austria with an Nr deposition of $21 \text{ kg N ha}^{-1} \text{yr}^{-1}$, fell within the range of the BNF rates of peat we found in Fenn's & Whixall and Migneint ($0.3\text{--}37 \text{ nmol N gDW}^{-1} \text{d}^{-1}$). Looking at the median BNF rates by species (Figure 2) we found BNF values close to those reported by van den Elzen et al. (2020)³⁸ from samples collected in different peatland habitats of southern Sweden regarding *S. capillifolium* subsp. *rubellum*/*S. fuscum* (open bog $0.4\text{--}17.5 \text{ nmol N gDW}^{-1} \text{d}^{-1}$) and *S. fallax* (Lagg fen $17.5\text{--}66.3 \text{ nmol N gDW}^{-1} \text{d}^{-1}$). Across the moss species, *S. fallax* had the highest BNF rates, which is commensurate with the findings of

van den Elzen et al. (2020).³⁸ Both *Sphagnum* mosses and peat collected from hollows, had higher BNF rates than species in hummocks (70% and 67%, respectively). These results are in agreement with those of other studies that have measured BNF rates in flarks/hollows and hummocks in peatlands in Finland,³⁶ or in hollows and hummocks of a bog located in an experimental boreal peat-forest mosaic in Minnesota.³⁹ The reason for larger BNF rates in hollows seems to be driven by the fact that wet conditions results in anoxic conditions which is conducive to the N fixation activity of the nitrogenase enzyme. Moreover, hollows with higher moisture content may be furnishing relatively more mineral nutrients to the N fixers thus promoting BNF activity.^{39,40}

We found that more than two decades of high doses of N and S together (30 and 20 kg ha⁻¹ yr⁻¹) suppressed BNF by 89% in the Degerö treatment plots which is a higher suppression than when N and S applied separately (Figure 4). However, BNF was not shut down. Possible explanation for this more detrimental effect of the combined N and S additions on BNF could be due the high levels of NH_x and NO_y, which reduces BNF activity directly and indirectly through the inhibition of CH₄ oxidation by NH₄⁺ given that it is a strong inhibitor of methane monooxygenase enzyme.^{41–43} A reduction in methanotrophy in the presence of NH₄⁺ means a reduction in BNF activity as methanotrophy induced BNF activity contributes about 40% of the total N₂ fixation in peatlands.³⁶ Moreover, methanotrophy in the oxic layers of peatlands depends on the rate of production of CH₄ in the anoxic layers and a reduction of CH₄ production in the presence of SO₄ as alternative electron acceptors for anaerobic respiration can reduce methanogenesis, which eventually can result in downregulating methanotrophy^{44,45} and hence BNF rates.³⁶ This finding corroborates the finding of Novak et al. (2016) who reported that the δ¹⁵N signature of moss tissues indicated the contribution of BNF under historically high N and S deposition.²³

We found a significant negative correlation between BNF and extractable NH₄⁺ in peat while a positive correlation between BNF and NO₃⁻ in pore water. As plants including mosses preferentially take up NH₄⁺ rather than NO₃⁻ (~8 times faster),^{46,47} this observation shows that higher availability of NH₄⁺ to mosses downregulate BNF. The high preference of mosses for NH₄⁺ is further substantiated by the fact that NO₃⁻ assimilation by mosses is limited under low pH conditions.⁴⁸ The observation that NH₄⁺ reduces BNF is further corroborated by the findings of a significant negative correlation of BNF with the contemporary NH_x/NO_y ratio of the atmospherically deposited Nr across our study sites. Interestingly, the percentage of the reduced form of Nr (NH_x) in the deposited Nr decreases in the order of Fenn's & Whixall > Migneint > Forsinard > Degerö (Table S2). For this reason BNF activity was lowest in the Fenn's & Whixall and highest in the Degerö peatland. The composition of Nr deposition is highly variable among regions based on land use and fossil fuel use patterns. Agricultural activities are the main sources of NH_x emission into air, while NO_y emissions emanates from fossil fuels combustion.⁴ Therefore, future changes and/or emission reduction strategies of Nr from agriculture and fossil fuel into air could affect the role of BNF in peatlands and hence their ecology. A positive correlation of NO₃⁻ with BNF seems to be a function of inverse collinearity of NH₄⁺ with NO₃⁻ rather than a promoter of BNF in peatlands. One plausible pathway of NO₃⁻ induced enhancement of BNF may due to the fact that sequential respiratory reduction of NO₃⁻ through denitrification,⁴⁹ particularly of N₂O into N₂ has

been shown to support BNF. For example, respiratory reduction of N₂O to N₂ and its subsequent fixation by diazotrophs in pure bacterial cultures has been reported.^{50,51} We, therefore, recommend further studies to elucidate the role of dissimilatory reduction of NO₃⁻ by denitrifiers in influencing BNF in peatlands.

The δ¹⁵N natural abundance values found in each site showed a significant negative correlation with the atmospheric Nr deposition where the values increased (on average from -5.73 ‰ in Fenn's & Whixall to -2.26 ‰ in Degerö) as the Nr deposition decreased (from 27 in Fenn's & Whixall to 2 kg N ha⁻¹ yr⁻¹ in Degerö), which is in line with the findings of Zivkovic et al. (2017)²⁰ in Canada, where a closer to 0 ‰ δ¹⁵N value shows an increasing contribution of BNF to the N nutrition of mosses given that the atmospheric δ¹⁵N of N₂ is 0. Additionally, we found a significant negative correlation between the NH_x/NO_y ratio of the deposited Nr and the δ¹⁵N signature at all the sites which is in agreement with the findings of Bragazza et al. (2005).²² Our results suggest that the higher Nr deposition rates implies a higher availability of NH_x that is initially filtered by the mosses and this source of N being a depleted one results in more negative δ¹⁵N values in mosses. This clearly reveals that Nr deposition dominates over BNF as a N source of the mosses in Fenn's & Whixall and Migneint peatlands compared to the Forsinard and Degerö peatland mosses and these trends are similar to those reported by Moore and Bubier (2020).²¹ In the Degerö peatland where atmospheric Nr deposition is the lowest of the all the sites, the relatively lower δ¹⁵N values in mosses than in Forsinard, could be due to the combined contribution of BNF and mineralized N uptake from peat decomposition where preferential uptake of light N can result in a relatively depleted δ¹⁵N in mosses.^{12,20}

Our results demonstrate that BNF did not shut down in peatlands exposed to a gradient of decades of excessive atmospheric Nr deposition and that the suppression of BNF is driven mainly by the amount of ammonia compared to nitrate. The observation of suppression of BNF under decades of Nr deposition across this wider geographic peatland sites was corroborated by similar suppression of BNF under experimental fertilization for over two decades in northern Sweden. Thus, it is imperative to consider the role of BNF in the nitrogen budgets of peatlands under Nr deposition scenarios knowing that N availability exerts a key control on C capture by the global peatlands.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.est.0c04882>.

Materials and methods: checks on ¹⁵N₂ gas for contamination; elemental analyses in *Sphagnum* tissue and peat; ancillary measurements in the field; location of the sampling sites; description of the treatments of the experimental plots; Tables: nitrogen deposition by its two major forms and related data; environmental variables for pore water and peat; elements in *Sphagnum* mosses; and elements in peat (PDF)

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Notes

The authors declare no competing financial interest.

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