https://doi.org/10.5194/egusphere-2023-827
Preprint. Discussion started: 21 June 2023

Contrasting potential for biological N_2-fixation at three polluted Central European *Sphagnum* peat bogs: Combining the $^{15}N_2$-tracer and natural-abundance isotope approaches

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ABSTRACT

Availability of reactive nitrogen (N_r) is a key control of carbon (C) sequestration in wetlands. To complement the metabolic demands of *Sphagnum* in pristine rain-fed bogs, diazotrophs supply additional N_r *via* biological nitrogen fixation (BNF). Since breaking the triple bond of atmospheric N_2 is energy-intensive, it is reasonable to assume that increasing inputs of pollutant N_r will lead to BNF downregulation. Yet, recent studies have documented measurable BNF rates in *Sphagnum*-dominated bogs also in polluted regions, indicating adaptation of N_2-fixers to changing N deposition. Our aim was to quantify BNF at high-elevation peatlands located in industrialized Central Europe. A $^{15}N_2$-tracer experiment was combined with a natural-abundance N-isotope study at three *Sphagnum*-dominated peat bogs in the northern Czech in an attempt to assess the roles of individual BNF drivers. High short-term BNF rates (8.2 ± 4.6 g N m^2 d^{-1}) were observed at Male Mechove Jezirko receiving ~17 kg N_r ha^{-1} yr^{-1}. The remaining two peat bogs, whose recent atmospheric N_r inputs differed from Male Mechove Jezirko only by 1-2 kg ha^{-1} yr^{-1} (Uhlirska and Brumiste), showed zero BNF. The following parameters were investigated to elucidate the BNF difference: NH_4^+-N/NO_3^--N ratio, temperature, wetness, *Sphagnum* species, organic-N availability, possible P limitation, possible Mo limitation, SO_4^{2-} deposition, and pH. At Male Mechove Jezirko and Uhlirska, the same moss species (*S. girgensohnii)* was used for the $^{15}N_2$ experiment, and therefore host identity could not explain the difference in BNF at these sites. Temperature and moisture were also identical in all incubations and could not explain the between-site differences in BNF. The N:P stoichiometry in peat and bog water indicated that Brumiste may have lacked BNF due to P limitation, whereas non-detectable BNF at Uhlirska may have been related to 70 times higher SO_4^{2-} concentration in bog water. Across the sites, the mean natural-abundance $\delta^{15}N$ values increased in the order: atmospheric deposition (-

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5.3 ± 0.3 ‰) < *Sphagnum* (-4.3 ± 0.1 ‰) < bog water (-3.9 ± 0.4 ‰) < atmospheric N_2 (0.0 ‰). Only at Brumiste, N in *Sphagnum* was significantly isotopically heavier than in atmospheric deposition, possibly indicating a longer-term BNF effect. Collectively, our data highlight spatial heterogeneity in BNF rates under high N_r inputs and the importance of environmental parameters other than atmospheric N_r pollution in regulating BNF.

Keywords: Peat, *Sphagnum*, nitrogen deposition, pollution, biological nitrogen fixation, BNF controls, phosphorus limitation

1. Introduction

Nitrogen (N) is the limiting nutrient in most terrestrial environments. The amount and form of N available to organisms (reactive N, N_r) is controlled by biogeochemical processes (Vitousek and Howarth, 1991; LeBauer and Treseder, 2008; Zhang et al., 2020; Davies-Barnard and Friedlingstein, 2020). A growing body of research has focused on the role of biological N_2-fixation (BNF) as a source of N_r in pristine ecosystems, such as subarctic tundra and boreal forests, with special attention given to ombrotrophic peat bogs and minerotrophic fens (Hemond, 1983, Rousk et al., 2013, 2015; Larmola et al., 2014; Vile et al., 2014; Diakova et al., 2016; Stuart et al., 2021; Yin et al., 2022). Globally, peatlands store between 20 and 30 % of total soil carbon and approximately 15 % of total soil nitrogen (Wieder and Vitt, 2006; Gallego-Sala et al., 2018; Fritz et al., 2014). Microbial N_2-fixation helps to sustain C accumulation in peatlands and to remove carbon dioxide (CO_2) from the atmosphere (Vile et al., 2014, and references therein). Changes in BNF may affect the dynamics of climate change. A combination of high anthropogenic N_r inputs with sustained N_2- fixation may accelerate invasion of vascular plants into peat bogs leading to the reduction of the C–N stocks.

The nitrogen budget at the peat bog scale results from a balance between N inputs [atmospheric deposition of N_r, mostly nitrate (NO_3^-) and ammonium (NH_4^+), with a contribution of organic N and BNF) and N outputs [runoff dominated by dissolved, colloidal, and particulate N, and emissions of gaseous N forms, mainly nitrous oxide (N_2O), nitric oxide (NO), and N_2 as products of denitrification; Sgouridis et al., 2021]. The atmospheric lifetime of N_2O, a potent greenhouse gas, is relatively long (>100 yr; Frolking et al., 2011). In contrast, the atmospheric lifetime of NO, another greenhouse gas, is short (days), and, along with N_2 as the final product of denitrification with no warming potential, is not considered in climate warming scenarios. Atmospheric deposition of N_r in high-latitude pristine bogs is 0.5-1.0 kg $ha^{-1}yr^{-1}$ (Vitt et al., 2003). Bogs receiving less than 10 kg N_r $ha^{-1}yr^{-1}$ are defined as low-polluted (Lamers et al., 2000). Bogs receiving more than 18 kg N_r $ha^{-1}yr^{-1}$ are considered to be highly polluted. Reactive N deposited on the surface of ombrotrophic peat bogs is vertically mobile (Novak et al., 2014).

Nitrogen capture in rain-fed bogs is dominated by *Sphagnum* mosses (Limpens et al., 2006). Nitrogen-fixing microbes (diazotrophs) mostly reside inside specialized *Sphagnum* cells (hyalocytes), although the mosses'

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metabolic demands for N are supported also by free-living diazotrophs. In contrast, diazotrophs in feather mosses, common in boreal forests, live epiphytically on the leaves (DeLuca et al., 2002; Rousk et al., 2015). Endophytic diazotrophs are more protected against environmental fluctuations, including changes in N_r deposition. BNF in bogs is associated mostly with cyanobacteria and methanotrophs (Larmola et al., 2014; Vile et al., 2014; Leppanen et al., 2015; Holland-Moritz et al., 2021; Kolton et al., 2022). It follows that BNF may affect potential methane (CH_4) emissions in two opposing directions: while higher C accumulation due to efficient BNF may lead to higher CH_4 emissions during peat decomposition, N_2-fixing methanotrophs may reduce emissions of CH_4 by oxidizing this greenhouse gas.

Recent work in peatlands has quantified the relative roles of various biotic and abiotic controls over BNF. Leppanen et al. (2015) reported than BNF rates were independent of the diazotroph community structure. The effect of temperature was reviewed by Carrell et al. (2019), Zivkovic et al., (2022), and Yin et al. (2022). The optimal temperature for BNF is 20-30 °C (Zielke et al., 2005). Dry conditions are generally unfavorable for BNF, but the moisture–BNF correlation tends to be insignificant (Yin et al., 2022). The effect of phosphorus (P) as a limiting nutrient was evaluated by Limpens et al. (2004), Larmola et al. (2014), Ho and Bodelier (2015), van den Elzen et al. (2017, 2020), and Zivkovic et al. (2022). In an interplay with other environmental and chemical parameters, higher P availability may augment BNF. The role of the NH_4^+/NO_3^- ratio in atmospheric deposition as a BNF control was evaluated by Saiz et al. (2021). A higher NH_4^+ proportion relative to the total N_r deposition may result in lower BNF rates. Stuart et al. (2021) stressed a strong interaction between moss identity, temperature, moisture and pH as possible BNF drivers. Kox et al. (2018) reported higher BNF rates under oxygen (O_2) depletion. Wieder et al. (2019, 2020) and Kox et al. (2020) showed that BNF rates generally increase in the presence of light.

The rates of BNF are measured using an acetylene reduction assay (ARA), $^{15}N_2$ isotope-labelling incubations, or compound-specific amino acid ^{15}N probing (*e.g.,* Knorr et al., 2015; Chiewattanakul et al., 2022). Recent studies have stressed the need for caution in ARA studies (Vile et al., 2014; Saiz et al., 2019; Soper et al., 2021). Inhibition of the activity of methanotrophs by acetylene may lead to an underestimation of BNF rates. These methods of direct measurements inevitably choose specific experimental conditions and thus provide *potential instantaneous* BNF rates. A complementary, indirect evaluation of BNF can be based on natural-abundance $^{15}N/^{14}N$ isotope systematics (Novak et al., 2016; Zivkovic et al., 2017; Saiz et al., 2021; Stuart et al., 2021). *Sphagnum* taking up N through BNF would carry a $\delta^{15}N$ signature close to 0 ‰, a value characterizing atmospheric N_2 ($\delta^{15}N$ values are defined as a per mil deviation of the $^{15}N/^{14}N$ ratio in the sample from a standard; the widely used standard is atmospheric N_2). With increasing BNF rates, the $\delta^{15}N$ values of living *Sphagnum* converge from the often negative $\delta^{15}N$ value of atmospheric deposition to the 0 ‰ value of the source N_2. This simple approach is complicated by tight inner N cycling near the bog surface, involving open-system isotope fractionations. In particular, *Sphagnum* may additionally take up N_r resulting from mineralization of organic N. Because denitrification preferentially removes isotopically light N in a gaseous form, the residual N_r in bog water may become isotopically heavy and supply high-$\delta^{15}N$ nitrogen for assimilation. Mineralized N_r in bog water as another nutrient source may thus be isotopically similar to atmospheric N_2 (Novak et al., 2019; Stuart et al., 2021).

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BNF is an energy-intensive process requiring 16 adenosine-triphosphate (ATP) molecules to fix 1 mol of N_2. It follows that, with an increasing input of pollutant N_r *via* atmospheric deposition, BNF should be rapidly downregulated. However, experiments applying additional N_r to *Sphagnum* both in the laboratory and in the field have indicated contradictory impacts on BNF. Some studies have shown a decrease in BNF rates in the proximity of anthropogenic N_r sources (Wieder et al., 2019; Saiz et al., 2021), while others have indicated continuing BNF even at N-polluted sites (van den Elzen et al., 2018). BNF data from natural settings with known time-series of historical N_r deposition rates are rare (van den Elzen et al. 2018; Saiz et al., 2021). The aim of the current study was to quantify BNF at high-elevation *Sphagnum*-dominated peatlands in an industrial part of Central Europe, also known for intense agriculture. We combined $^{15}N_2$-tracer experiments with a natural abundance N-isotope study at three peat bogs situated in the northern Czech Republic to provide qualitative insights into the roles of individual BNF drivers. Our specific objectives were: (i) to investigate whether BNF rates at the study sites correlate with well-constrained NO_3^- and NH_4^+ deposition rates and P availability, and (ii) to compare the results of experiments investigating ^{15}N-assimilation by *Sphagnum* with the results of a natural-abundance $\delta^{15}N$ inventory of individual wetland pools and fluxes. We expected that convergence of *Sphagnum* N toward $\delta^{15}N_{N2}$ = 0 ‰ would corroborate the relative magnitude of instantaneous BNF rates in between-site comparisons.

2. Materials and methods

2.1. Study sites

The three studied *Sphagnum*-dominated peat bogs (Fig. 1, Tab. 1) are located in the northern Czech Republic, a highly industrialized part of Central Europe with numerous coal-burning power plants. In the 1970s-1990s, Norway spruce monocultures were affected by acid rain in the vicinity of Brumiste (BRU; Krusne Mts.) and Uhlirska (UHL; Jizerske Mts.). At UHL, most spruce stands died back and were harvested. The third site, Male Mechove Jezirko (MMJ; Jeseniky Mts.) is surrounded by relatively healthy mature spruce forests. The distance between adjacent study sites is 160-190 km (Fig. 1). The studied high-elevation catchments are drained by small streams. The studied peatlands are partly rain-fed, with a possible contribution of lateral water influx from the surrounding segments of the catchments. The bedrock is composed of granite at BRU and UHL, and phyllite at MMJ. The surface of each bog is characterized by a combination of hummock–hollows microtopography and lawns (Dohnal, 1965). Moss species at BRU include *S. cuspidatum*, common in hollows and pools, *S. magellanicum*, mostly occupying intermediate positions between the tops of the hummocks and the hollows, *S. rubellum*, typical of dense carpets in rain-fed bogs, and *S. papillosum*, forming low hummocks and mats in bogs and mires. At UHL and MMJ, the predominant moss species is shade-demanding *S. girgensohnii*, requiring slight base enrichment (Tab. S1 in the Supplement). The growing season is more than seven months long, from late March to early November. The measured density of living *Sphagnum* is 0.04 g cm^{-3}. More details on BRU are in Bohdalkova et al. (2013), and Buzek et al. (2019, 2020). Biogeochemical processes at UHL were studied by Novak et al. (2005), Sanda and Cislerova (2009), Bohdalkova et al. (2014), Marx et al. (2017), Oulehle et al. (2017, 2021a), and Vitvar et al. (2022). Further information on MMJ is in Novak et al. (2003, 2009).

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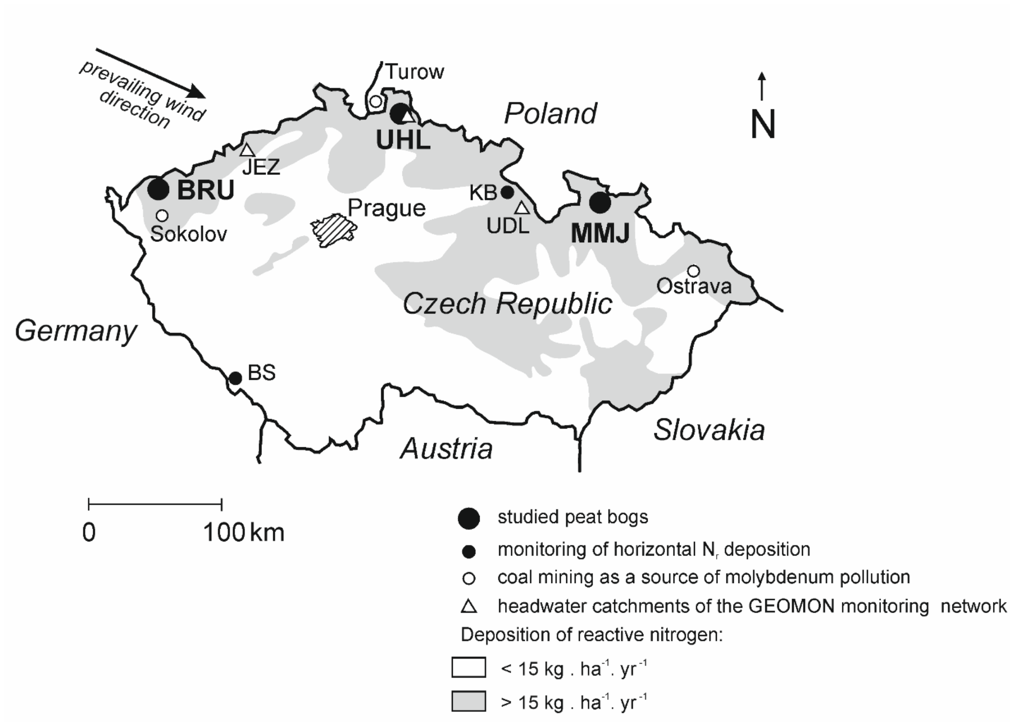

Fig. 1. Location of the studied *Sphagnum*-dominated peat bogs.

Table 1. Study site characteristics.

Site	Location	Elevation (m)	Long-term precipitation total (mm yr^{-1})	Mean annual temperature (ºC)	Bog area (ha)	Maximum peat depth (cm)	Atmospheric vertical N_r deposition (kg ha^{-1} yr^{-1})[1]	Total atmospheric N_r deposition (kg ha^{-1} yr^{-1})[2]	NH_4^+-N/NO_3^--N ratio
Brumiste, BRU	50º24´ N 12º36´ E	930	1080	4.5	17	200	12.7	16.5	1.2
Uhlirska, UHL	50º49´ N 15º08´ E	830	1230	4.0	50	< 200	15.5	20.2	1.2
Male Mechove jezirko, MMJ	50º13´ N 17º18´ E	750	1090	5.3	195	660	14.3	18.6	1.3

[1]long-term average according to Oulehle et al. 2016
[2]including 30 % of horizontally deposited N_r (Novak et al., 2015)

2.2. Sampling

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Samples of rain and snow for $\delta^{15}N$ determinations were collected between January 2016 and October 2019 using a simplified protocol of Fottova and Skorepova (1998). Open-area precipitation was sampled by two rain collectors placed five meters apart, 160 cm above ground. Spruce canopy throughfall was sampled using five (UHL) or three (BRU, MMJ) collectors installed 10 m apart. Deposition samplers were polyethylene (PE) funnels (surface area of 113 cm^2) fitted to 1-L bottles. In winter, cylindrical PE vessels (surface area of 167 cm^2) were used to collect snow. At the end of cumulative one-month sampling, open area precipitation and throughfall samples, respectively, were pooled prior to chemical and N-isotope analysis. One-liter samples of runoff were collected in ~30-day intervals at BRU over a 25-month period, samples of runoff were collected at UHL and MMJ in summer 2019 (*see* Tab. S2 for specific dates). Five replicates of surface bog water were collected throughout each study site in June 2019. The depth of the water pools was less than 20 cm. The total number of water samples for $\delta^{15}N$ analysis was 136.

A vertical peat core, 10-cm in diameter, 30-cm deep, was collected in a *Sphagnum*-dominated lawn at each of the study sites in October 2018, kept vertically at 6 ˚C for 12 hours and then frozen. At the same time, 12 samples of living *Sphagnum* were collected randomly throughout each bog for species identification and N isotope analysis. Additionally, 12 replicate samples of living *Sphagnum* were collected in various parts of each of the peat bogs for a $^{15}N_2$-labelling experiment. Each replicate sample consisted of 30 individual 5-cm long *Sphagnum* plants. *S. girgensohnii* was used in the UHL and MMJ experiments, a mix of *S. magellanicum, S. papillosum,* and *S. cuspidatum* was used in the BRU experiment (*cf.,* Tab. S1); *Sphagnum* samples were transported to the laboratory at a temperature of 6 ˚C.

2.3. $^{15}N_2$ Sphagnum incubation experiment

Measurements of potential N_2-fixation rates were performed using a modified protocol of Larmola et al. (2014). Four plant replicates per site were analyzed at time t = 0 without incubation (control no. 1). Eight replicates per site were closed in 200-mL transparent PE containers with 5 mL of bog water collected at BRU, UHL and MMJ, respectively. Twenty-four mL of headspace air were removed from four replicates in closed containers and replaced with 32 mL of $^{15}N_2$ tracer gas containing 98 atomic % of ^{15}N (Aldrich, Germany). The four remaining *Sphagnum* replicates with no $^{15}N_2$ addition served as a procedural control no. 2 to identify possible incubation artifacts. The ^{15}N-labelled and control-no.-2 replicates were incubated for 168 hours. Each day, the temperature in the growth chamber was kept at 18 ˚C for 16 hours at daylight, and at 10 ˚C for 8 hours under dark conditions. Following N-isotope analysis, BNF rates were calculated according to Vile et al. (2014) and Knorr et al. (2015):

$$N_{2fix} = \frac{\Delta\, at.\,\%\ ^{15}N_{Sph}}{\Delta\, at.\,\%\ ^{15}N_{gas}}\ x\ \frac{\text{total N }\%_{Sph}}{t * 100} \quad (\text{g N g DW}^{-1}\ \text{day}^{-1}), \qquad (1)$$

where N_{2fix} is the N_2-fixation rate in g N g DW^{-1}(*Sph)* day^{-1}, t is incubation time (days), total N% $_{Sph}$, Δ at. % $^{15}N_{Sphagnum}$ is the difference between atom % labeled and control sample, Δ at. % $^{15}N_{gas}$ is the difference between the concentration ^{15}N in the headspace and the natural abundance (at. %). The used *Sphagnum* density was 0.04 g cm^{-3}.

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We note that our $^{15}N_2$ experimental design used a longer incubation period (168 hr), compared to most previous studies (24 to 80 hr; *cf.,* Knorr et al., 2015). To minimize the effect of changing headspace concentrations of O_2 and CO_2 on the living moss and the microbiome, we used larger sealed containers, compared to most previous studies (200 *vs.* ≤ 125 mL). It also bears mention that Dabundo et al. (2014) found a deviation from the declared $^{15}N_2$ purity within commercially available tracer tanks. We did not study the tracer purity and hence the observed BNF rates might be viewed as maximum estimates. Because our incubation study was based on one-time measurements under laboratory conditions, in the current paper we chose not to upscale the BNF rates to the entire peat bog and an annual time span.

2.4. Chemical and isotope analysis

Frozen peat cores were sectioned to 2-cm thick segments. Samples of peat and *Sphagnum* were dried and homogenized. Nitrogen concentrations in peat and *Sphagnum* samples were determined on a Fisons 1180 elemental analyzer with a 1.5 % reproducibility (2σ). Ammonium and nitrate concentrations in water samples were determined spectrophotometrically with a reproducibility of 0.1 mg L^{-1}. About 0.5 L of each water sample were used to separate NH_4^+ and NO_3^- (Bremner, 1965). Nitrogen isotope composition was measured on a Delta V mass spectrometer and expressed in $\delta^{15}N$ notation. IAEA isotope standards N1 ($\delta^{15}N$ = 0.4 ‰) and N_2 ($\delta^{15}N$ = 20.3 ‰) were analyzed before every session, and two in-house standards (ammonium sulfate, $\delta^{15}N$ = -1.7 ‰, and glycine, $\delta^{15}N$ = 4.0 ‰) were analyzed after every six samples. The reproducibility of the $\delta^{15}N$ determinations was 0.30 and 0.35 ‰, for the liquid and solid samples, respectively. Methods of concentration analysis of other chemical species are given in *Appendix I.*

2.5. Historical rates of N_r deposition

Long-term data from 32 monitoring stations in the Czech Republic operated by the Czech Hydrometeorological Institute, Prague, were used to assess temporal and spatial variability of NH_4^+ and NO_3^- concentrations in vertical deposition using a model by Oulehle et al. (2016). Median *z*-score values of NH_4^+ and NO_3^- concentrations derived from observations at the monitoring stations and nation-wide emission rates, published by Kopacek and Vesely (2005), and Kopacek and Posh (2011), showed significant relationships at the $p < 0.001$ level. Using linear models, *z*-score values were expressed for the period 1900-2012 and then back-transformed to give concentration estimates for the study sites. Annual rates of vertically deposited NH_4^+ and NO_3^- were products of modelled concentrations and precipitation quantities at BRU, ULH and MMJ.

2.6. Statistical evaluation

Statistical analysis was performed using the R software (R Core Team, 2019) version 3.6.2, and its contributed packages *sandwich* (Zeileis, 2004) and *multcomp* (Hothorn et al., 2008). Comparisons of groups of N isotope and concentration data (see sections 2.3 and 2.4)

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were based on one-way analysis of variance with a sandwich estimator of covariance matrix to account for heteroscedasticity among the groups (MacKinnon and White, 1985). *Post-hoc* multiple comparisons of the same groups were then performed according to Hothorn et al. (2008). Because of the largely uneven number of runoff samples *per* site (50, 6, and 2 at BRU, UHL and MMJ, respectively), we did not include runoff $\delta^{15}N$ data in the statistical evaluation.

3. Results

3.1. Historical rates of atmospheric N_r inputs

Vertical deposition of NH_4^+ reached a maximum in 1980, remained almost unchanged until 1990, and decreased thereafter (Fig. 2a). Nitrate-N deposition exhibited a wider maximum between *ca.* 1970 and 1990 (Fig. 2b). In the oldest modelled time period (1900-1930), ammonium in deposition dominated over nitrate. During the deposition peak, the contributions of NH_4^+-N and NO_3^--N to total vertical N_r deposition were similar (8 to 13 kg ha^{-1} yr^{-1} at individual sites). Across the modelled years, the NH_4^+-N/NO_3^--N ratio in vertical deposition was similar at all three sites (1.2 to 1.3; Tab. 1). Since *ca.* 1950, pollution at the study sites *via* total vertical deposition of inorganic N_r increased in the order: BRU < MMJ < UHL (Fig. 2c). Fig. 2c shows that the between-site differences in the most recent years have been small (1-2 kg N ha^{-1} yr^{-1}).

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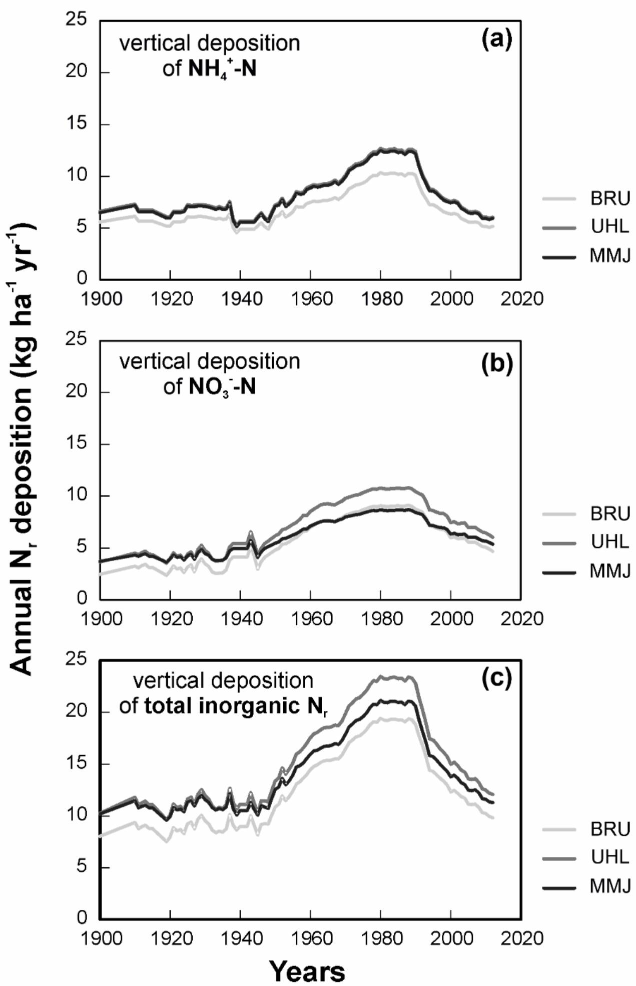

Fig. 2. Modelled long-term changes in atmospheric N_r deposition according to Oulehle et al. (2016).

3.2. $^{15}N_2$ incubation experiment

There were no statistically significant differences between $\delta^{15}N$ values of *Sphagnum* at time *t* = 0 and at time *t* = 168 hours following incubation in natural atmosphere (controls no. 1 and 2; Tab. 2; $p > 0.05$). Mean $\delta^{15}N$ values of the moss of the two controls were similar among the sites (-3.6 to -4.1 ‰). At the end of the $^{15}N_2$ *Sphagnum* incubation, there was no change in the N isotope signature of the moss at BRU and UHL ($p > 0.05$). In contrast, there was a large positive shift in $\delta^{15}N$ values of *Sphagnum* collected at MMJ (59.2 to 467 ‰; Tab. 2; Fig. 3). The N_2 fixation rate calculated from the N isotope systematics in the $^{15}N_2$ labelling experiment was 0 at BRU and UHL, and 4.11 µg N g^{-1} d^{-1}, or 8.20 mg N m^{-2} d^{-1} at MMJ.

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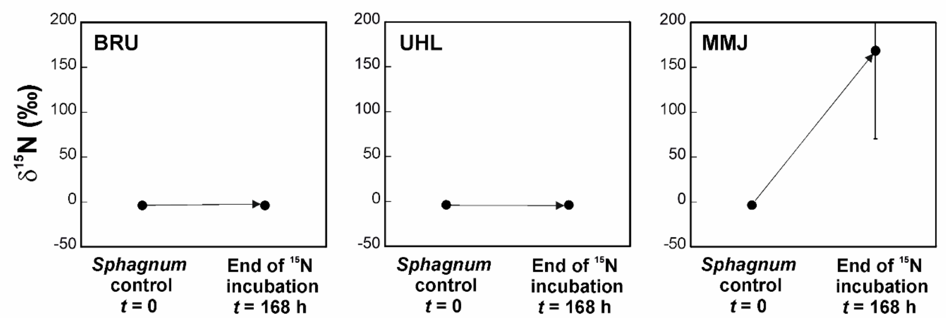

Fig. 3. Results of a $^{15}N_2$ incubation study using living *Sphagnum*. Means and standard errors are given.

Table 2. Positive $\delta^{15}N$ shift in total moss nitrogen following the $^{15}N_2$ assay incubation at MMJ.

Site	BRU			UHL			MMJ		
	$\delta^{15}N$ (‰)								
	Sphagnum control t_0	*Sphagnum* control t = 168 h	*Sphagnum* at the end of $^{15}N_2$ incubation t = 168 h	*Sphagnum* control t_0	*Sphagnum* control t = 168 h	*Sphagnum* at the end of $^{15}N_2$ incubation t = 168 h	*Sphagnum* control t_0	*Sphagnum* control t = 168 h	*Sphagnum* at the end of $^{15}N_2$ incubation t = 168 h
Replicate 1	-3.9	-4.0	-4.1	-3.7	-3.8	-3.9	-2.7	-2.7	467
Replicate 2	-3.9	-4.1	-3.9	-3.9	-3.7	-3.7	-4.0	-3.8	59.2
Replicate 3	-3.9	-4.2	-4.3	-4.4	-4.0	-4.2	-3.8	-4.0	68.8
Replicate 4	-3.5	-3.8	-3.6	-4.7	-4.6	-4.6	-3.8	-4.2	83.0
Mean ± SE	-3.8 ± 0.1	-4.0 ± 0.1	-4.0 ± 0.2	-4.1 ± 0.2	-4.0 ± 0.2	-4.1 ± 0.2	-3.6 ± 0.3	-3.7 ± 0.4	169 ± 99.2

3.3. Natural-abundance N-isotope systematics

3.3.1. Atmospheric deposition

Ninety-six per cent of the deposited inorganic N_r species had negative $\delta^{15}N$ values; *i.e.*, contained isotopically light N (Tab. S2; Fig. S1). The mean $\delta^{15}N$ value across all 181 samples of atmospheric deposition was -5.3 ± 0.3 ‰ (SE). Mean $\delta^{15}N$ values of both forms of atmospherically deposited N (NH_4^+ and NO_3^-) in an open area were slightly higher than those in throughfall at BRU and MMJ, and slightly lower than those in throughfall at UHL (Tab. 3). Nitrate-N in open-area deposition was on average slightly isotopically lighter than NH_4^+-N at all three sites. At the 0.05 probability level, however, the within-site differences among deposition sample types and among N species at BRU and MMJ were insignificant. The only statistically significant difference was found between $\delta^{15}N$ values of open-area NO_3^- and both N species in throughfall at UHL (*see* superscript letters in Tab. 3).

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Table 3. Multiple comparisons among $\delta^{15}N$ values of four sample types of atmospheric deposition. Different letters in superscript denote statistical difference ($p < 0.05$).

	mean $\delta^{15}N$ (‰) ± SD		
Site	BRU	UHL	MMJ
open-area NH_4^+	-5.18 ± 3.63^a	-5.84 ± 3.31^{ab}	-3.48 ± 6.01^a
open-area NO_3^-	-5.71 ± 2.82^a	-6.19 ± 2.34^b	-4.10 ± 3.18^a
throughfall NH_4^+	-6.86 ± 3.10^a	-3.15 ± 1.66^a	-6.57 ± 6.40^a
throughfall NO_3^-	-6.16 ± 2.29^a	-4.17 ± 0.58^a	-6.02 ± 4.14^a

3.3.2. Comparison of $\delta^{15}N$ values of *Sphagnum and atmospheric deposition*

The $\delta^{15}N$ values of living *Sphagnum* were between -6.2 and -1.9 ‰ (Tab. S1). The $\delta^{15}N$ values of living *Sphagnum* at BRU were statistically different from the $\delta^{15}N$ values of atmospheric deposition (means of -4.0 and -5.9 ‰, respectively; $p < 0.05$; Fig. 4). At UHL (means of -4.3 and -5.6 ‰, respectively;) and MMJ (means of -4.4 and -4.3 ‰, respectively), the differences between the $\delta^{15}N$ values of living *Sphagnum* and the $\delta^{15}N$ values of atmospheric deposition were insignificant ($p > 0.05$; Fig. 4). At BRU (but also at UHL), *Sphagnum* N was on average isotopically heavier than deposited N, *i.e.*, closer to the 0 ‰ value of atmospheric N_2. Nitrogen concentration in living *Sphagnum* was significantly higher at MMJ (mean of 1.0 wt. %) than at UHL (0.9 wt. %; $p < 0.05$; Fig. 5). The mean N concentration in BRU *Sphagnum* was 1.0 wt. %, indistinguishable from the other two study sites.

https://doi.org/10.5194/egusphere-2023-827
Preprint. Discussion started: 21 June 2023

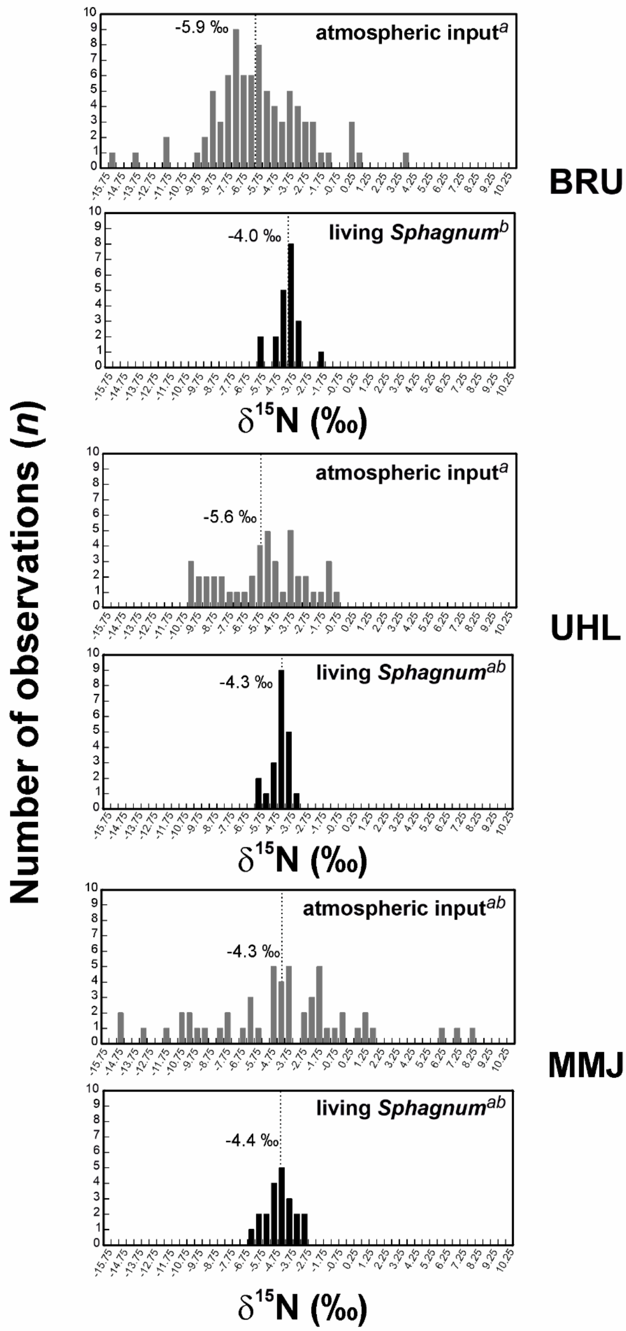

Fig. 4. Histograms of $\delta^{15}N$ values of atmospheric input of N_r and living *Sphagnum*. Different letters in superscript mark statistically different sample types ($p < 0.05$).

https://doi.org/10.5194/egusphere-2023-827
Preprint. Discussion started: 21 June 2023

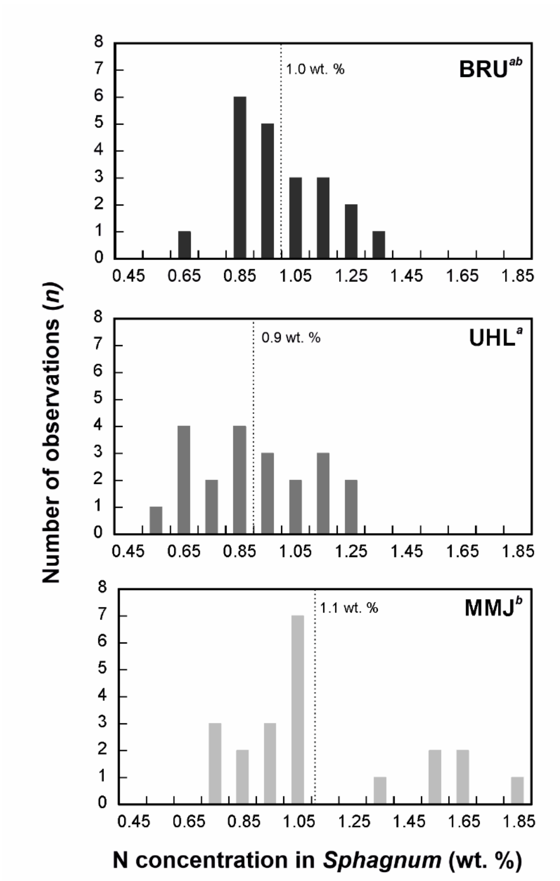

Fig. 5. Nitrogen concentrations in living *Sphagnum*. Different letters in superscript mark statistically different sample types ($p < 0.05$).

3.3.3. Multiple $\delta^{15}N$ comparisons among sample types

The mean $\delta^{15}N$ value of surface bog water was 0.9 ‰ at BRU, 1.8 ‰ at UHL, and -1.9 ‰ at MMJ. Nitrogen in surface bog water was isotopically significantly heavier than N in both *Sphagnum* and atmospheric input at all three sites (Fig. 6; $p < 0.05$). At BRU and UHL, the mean $\delta^{15}N$ value of surface bog water was higher than the 0 ‰ value of atmospheric N_2. At MMJ, the mean $\delta^{15}N$ value of surface bog water was lower than the N isotope signature of atmospheric N_2. In other words, all three sample types (deposition, *Sphagnum*, and bog water) at MMJ contained isotopically lighter N, compared to atmospheric N_2 (Fig. 6).

When averaged across all depths (0-30 cm), the mean $\delta^{15}N$ value in the peat core was -2.4 ‰ at BRU, -0.4 ‰ at UHL, and -1.9 ‰ at MMJ. At all three sites, the maturating peat in the vertical profile contained isotopically significantly heavier N compared to living *Sphagnum* ($p < 0.05$; Fig. 6; Tab. S2).

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The mean $\delta^{15}N$ value of runoff was -2.7 ‰ at BRU (combined NH_4^+ and NO_3^- data; number of observations n = 50), -5.3 ‰ at UHL (n = 6), and -5.1 ‰ at MMJ (n = 2; Tab. S1). The N isotope signature of runoff was higher compared to the atmospheric input at BRU, and similar with the atmospheric input at UHL and MMJ (small solid squares in Fig. 6). At all three sites, runoff contained isotopically lighter N compared to bog water (Fig. 6).

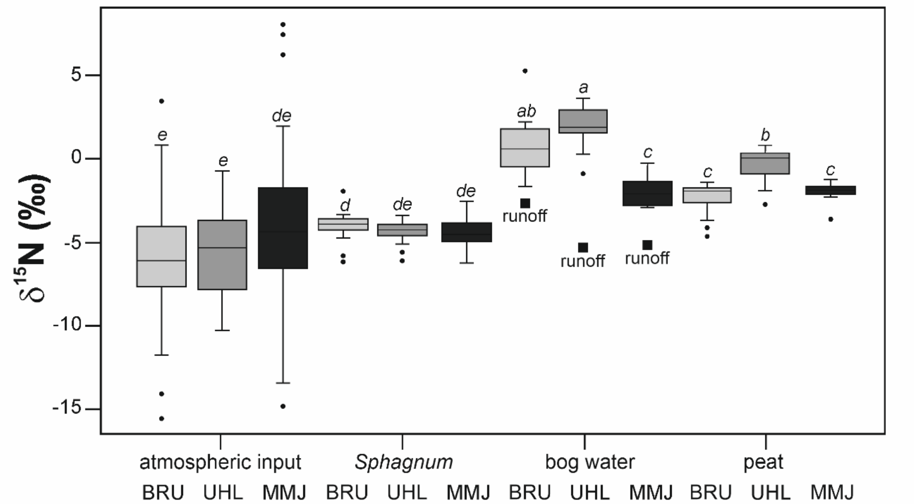


Fig. 6. Between-site comparisons of $\delta^{15}N$ values of studied N pools and fluxes. Horizontal lines in boxes correspond to median values. Different letters mark statistically different sample types ($p < 0.05$).

3.4. Chemistry of natural waters

Acidity. Surface bog water had lower pH than atmospheric deposition and runoff at all three sites. Mean bog water pH was 4.0 at UHL, 4.3 at BRU, and 4.9 at MMJ (Tab. S3). The pH of atmospheric deposition was lower than 5.0 only at UHL.

Nitrogen. The maximum NH_4^+-N concentration in open area precipitation was 1.7 mg L^{-1} (UHL) and the maximum NO_3^--N concentration in open area precipitation was 7.1 mg L^{-1} (BRU; Tab. S2). The maximum concentration of NH_4^+-N in throughfall was 3.9 mg L^{-1}(MMJ) and the maximum concentration of NO_3^--N in throughfall was 9.7 mg L^{-1} (BRU; Tab. S2). The maximum concentration of NH_4^+-N in bog water was 2.3 mg L^{-1}(UHL) and the maximum concentration of NO_3^--N in bog water was 2.7 mg L^{-1} (MMJ; Tab. S2). The maximum concentration of NH_4^+-N in runoff was 1.3 mg L^{-1} (BRU) and the maximum concentration of NO_3^--N in runoff was 7.1 mg L^{-1} (BRU; Tab. S2).

Phosphorus. The mean concentration of total P in atmospheric deposition increased in the order: BRU (below 6.0 µg L^{-1}) < UHL (9.3 µg L^{-1}) < MMJ (15.5 µg L^{-1}; Tab. S3). Phosphorus concentration in surface bog water was roughly 30 times higher than in atmospheric deposition at BRU, more than 50 times higher at UHL, and more than 10 times higher at MMJ (Tab. S3). The UHL bog water contained as much as 490 µg P L^{-1}. The mean P concentration in runoff increased in the order: MMJ (12.4 µg L^{-1}) < BRU (29.4 µg L^{-1}) < UHL (40.2 µg L^{-1}; Tab. S3).

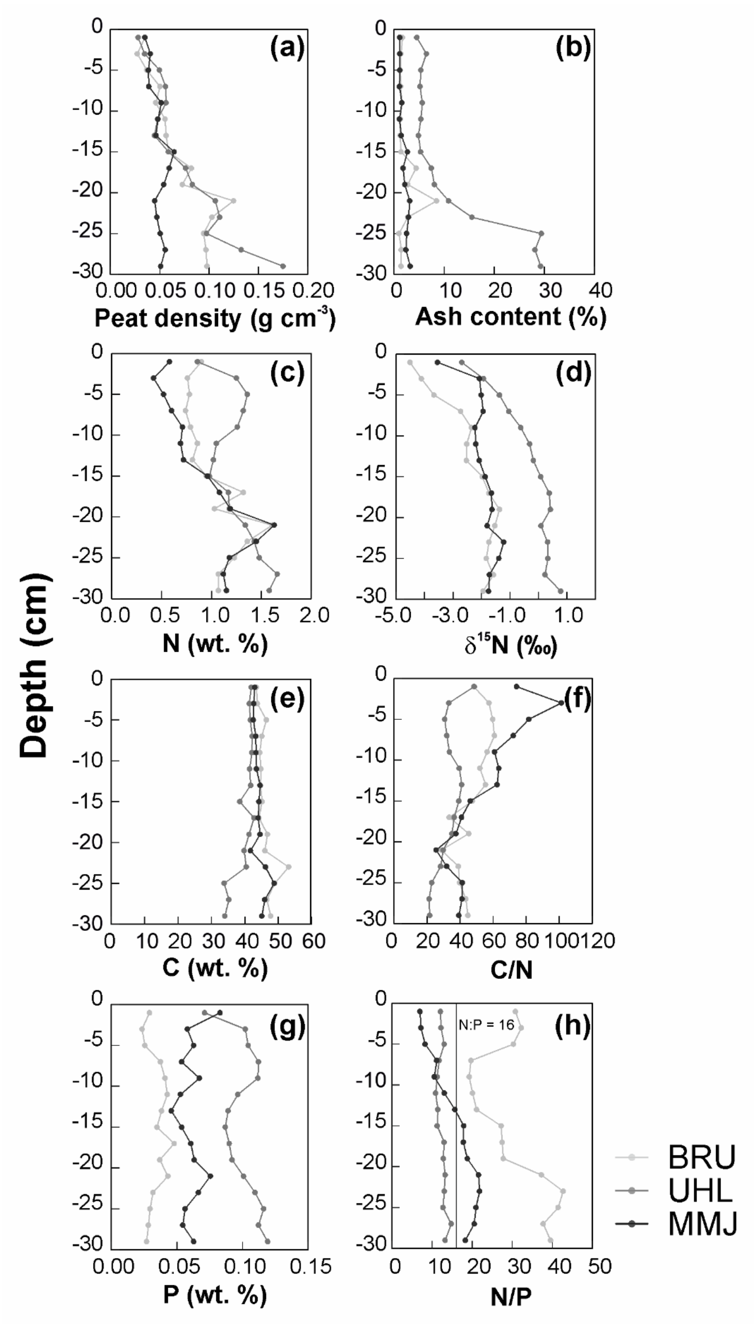
Other chemical species. Natural waters at UHL were richer in sulfate (SO_4^{2-}) than those at the remaining two sites (Tab. S3). UHL bog water and runoff contained as much as 47.4 and 33.7 mg SO_4^{2-} L^{-1}, respectively. Bog water was richer in potassium (K^+) at UHL (9.05 mg L^{-1}) compared to BRU and MMJ (1.85 and 1.97 mg L^{-1}, respectively). The concentration of DOC in atmospheric deposition was 2-4 times higher at MMJ than at the remaining two sites (Tab. S3). In contrast, surface bog water at MMJ had 1.4 to twice lower DOC concentrations, compared to the remaining two sites. Detailed water chemistry is in Tab. S3.

3.5. Vertical peat profiles

From peat surface to the depth of 15 cm, peat density exhibited a slight increase similar at the three sites (Fig. 7a). Deeper, peat density remained relatively low (~0.05 g cm^{-3}) at MMJ, and continued increasing irregularly at BRU and UHL. Ash content remained below 5 wt. % to a depth of 30 cm at MMJ, and, with one exception, also at BRU (Fig. 7b). The highest ash content was observed at UHL. Below the depth of 20 cm, it increased downcore to values greater than 10 wt. %. The total N concentrations in peat substrate increased downcore or exhibited a zigzag pattern (Fig. 7c). The UHL peat core was the richest in N in most 2-cm peat sections. Down to a depth of 15 cm, N concentration was the lowest in MMJ peat. At all three sites, the vertical $\delta^{15}N$ profile was characterized by a downcore increase near the surface flattening out in the deepest peat sections (Fig. 7d). Generally, the $\delta^{15}N$ values in peat cores increased in the order BRU < MMJ < UHL.

The nearly constant carbon (C) concentrations in peat were similar at all three sites to the depth of 20 cm, and became more variable deeper (Fig. 7e). The sharpest downcore decrease in the C:N ratio was found at MMJ, with the exception of the 0-to-4 cm depth where the C:N ratio increased (7f). Throughout the vertical peat profiles, P concentration was the lowest at BRU, and the highest at UHL (Fig. 7g). The N:P ratio was close to 12 throughout the UHL peat profile, increased downcore at MMJ from 10 to 20, and exhibited an irregular pattern at BRU, ranging between 20 and 40 (Fig. 7h). Further information on vertical changes in peat composition is in Tab. S4.

https://doi.org/10.5194/egusphere-2023-827
Preprint. Discussion started: 21 June 2023



392 **Fig. 7.** Vertical changes in physicochemical characteristics of *Sphagnum* peat.

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4. Discussion

4.1. The role of horizontal N_r deposition in peatlands

Using field experiments, we have recently shown a sizeable contribution of horizontally deposited N_r to total atmospheric deposition in Central European *Sphagnum* peat bogs (Novak et al., 2015b). During 80-90 days of the spring and fall foggy seasons, horizontal deposition added another 45 % to vertical deposition at Kunstatska Kaple Bog (KB), a mountain-top site in northern Czech Republic, and 14 % at Blatenska Slat (BS) in the less polluted southern Czech Republic (*see* Fig. 1 for location). Additionally, Hunova et al. (2023) reported a relatively high horizontal contribution of nitrate-N to winter-time atmospheric deposition in Czech mountains by analyzing ice accretions (mean of 29 ± 3 %; data for December–March; number of sites *n* = 10). As a first approximation, we suggest that the upper limit of the contribution of horizontal deposition to vertical deposition at BRU, UHL and MMJ could have been 30 %. If so, the total average N_r deposition was slightly higher than 18 kg ha^{-1} yr^{-1} at UHL and MMJ, and 16.5 kg ha^{-1} yr^{-1} at BRU (Tab. 1). Our study sites can thus be considered as highly or medium-polluted (Lamers et al., 2000). The overall N_r pollution decreased in the order UHL > MMJ > BRU.

We note that total atmospheric deposition may also contain measurable amounts of total organic N (TON; Violaki et al., 2010; Cornell, 2011). TON fluxes have not been considered as part of the N_r input in existing peatland BNF studies. Open-area precipitation at BRU, UHL and MMJ contained an additional 15, 45, and 13 % of total organic N, respectively, relative to the sum of the two inorganic N_r forms (Tab. S3; October 2018). More TON data in precipitation would be needed to realistically estimate annual deposition of organic N at our study sites.

4.2. Relationship between N_r pollution and N_2-fixation

In theory, chronic atmospheric deposition of pollutant N_r should suppress BNF in peatlands (Wieder et al., 2019, 2020). Saiz et al. (2021) quantified downregulation of BNF along a geographical pollution gradient. Relative to a practically unpolluted site receiving 2 kg N_r ha^{-1} yr^{-1} from the atmosphere, these authors reported a 54 % decrease in BNF rates under the atmospheric deposition of 6 kg N_r ha^{-1} yr^{-1}, a 69 % decrease under the deposition of 17 kg N_r ha^{-1} yr^{-1}, and a 74 % decrease under the deposition of 27 kg N_r ha^{-1} yr^{-1}. As seen in Fig. 3, our data did not confirm such an inverse correlation at Central European sites. Instead, the most and least polluted peat bog exhibited no instantaneous BNF, while MMJ, whose N_r inputs were lower than those at UHL and higher than those at BRU, showed a high mean BNF rate. Given that most previous studies of *Sphagnum* bogs reported non-zero BNF rates regardless of atmospheric N_r deposition level (*see* compilation in Tab. S5), non-detectable BNF rates at BRU and UHL were surprising. The mean instantaneous BNF rate at MMJ was lower than BNF rates in unpolluted high-latitude bogs in Canada (Vile et al., 2014) and Patagonia (Knorr et al., 2015). Among the studies listed in Tab. S5, the mean BNF rates at MMJ were the fourth highest. Our data from MMJ are consistent with a conclusion by Saiz et al. (2021) who suggested a development of diazotrophic microbes' tolerance to high rates of atmospheric N_r deposition in recent decades. Global assessments of the dependence of BNF on total N_r deposition are difficult to make for several reasons: (i) few studies consider horizontal N_r deposition which may be sizeable and depends not just on atmospheric pollution, but also on elevation; few studies have quantified atmospheric input of organic N (ii) there is a large within-site heterogeneity in BNF ($^{15}N_2$ incubations should be performed using a large number of replicates, *see* $\delta^{15}N$ differences between individual MMJ replicates in Tab. 2; *cf*., "BNF hotspots" in Stuart et al., 2021); and (iii) recalculation between two commonly used BNF units (µg N per 1 g of *Sphagnum* d^{-1}, g N m^{-2} yr^{-1}) in literature data requires information on additional site-specific parameters, such as peat density, seasonality in daily temperatures and snow cover duration. Additionally, it is often unclear to what maximum depth in peat bogs BNF proceeds and whether there is a gradient in BNF rates within this depth range (Vile et al., 2014; Knorr et al., 2015).

Since the differences in N_r deposition among sites were minor (Tab. 1; Fig. 2), we suggest that N_r deposition was not the primary control of the BNF rates in our study at the time of *Sphagnum* sampling.

4.3. Chemical and environmental parameters as possible BNF controls

4.3.1. The role of the NH_4^+-N/NO_3^--N ratio in atmospheric deposition

The impact of the two main N_r forms in deposition on BNF can be different. Because BNF generates NH_4^+, the need for BNF to complement metabolic demands of the moss may be lower if deposition of NH_4^+-N exceeds the deposition of NO_3^--N (van den Elzen et al., 2018; Saiz et al., 2021). At our study sites, the NH_4^+-N/NO_3^--N ratios were nearly identical (Tab. 1), slightly exceeding 1. It follows that this ratio was unlikely the driver of higher BNF potential at MMJ, compared to the remaining two sites.

4.3.2. The effect of temperature

MMJ is situated at a lower elevation, compared to UHL and BRU, and its mean annual temperature is higher than at the remaining two sites (Tab. 1). This could positively affect the rate of BNF (Basilier et al., 1978; Schwintzer et al., 1983; Urban and Eisenreich, 1988; Zivkovic et al., 2022; Yin et al., 2022). By contrast, Carrell et al. (2019) argued that BNF rates may decrease with an increasing temperature due to lower microbial diversity and greater mineralization rates leading to more N_r in bog water and hence lower demand for BNF. Under field conditions of the Czech sites and at the peatland scale, temperature likely is a key factor regulating BNF. In our ^{15}N assimilation study, however, the chosen temperature was identical for all three sites. Consequently, temperature was not the dominant control of the measured short-term BNF rates.

4.3.3. The effect of bog wetness

Fig. S2 shows monthly measurements of water table level below bog surface at BRU (Bohdalkova et al., 2013) and UHL (Tacheci, 2002). The mean annual water table depth was -5.2 ± 2.3 and -7.5 ± 1.1 cm at BRU at UHL, respectively. No water level monitoring data are available for MMJ, however, during our field sampling

https://doi.org/10.5194/egusphere-2023-827
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campaigns, numerous 10-to-20 cm deep water pools were observed near the bog center at MMJ, especially during the growing seasons of 2017 and 2019. Other high-elevation peat bogs on crystalline bedrock previously studied in the Czech Republic exhibited water table fluctuation at shallow depths of 5-8 cm, similar to BRU and MMJ (Novak and Pacherova, 2008). Based on visual inspection, somewhat drier conditions were typical of UHL, compared to the other two sites. Hydrological monitoring (GEOMON network database, Czech Geological Survey; Oulehle et al., 2021b) revealed significantly drier conditions at UHL in the water year 2018, compared to the long-term average given in Tab. 1. Precipitation totals at UHL were 1460 mm in 2016, 1370 mm in 2017, mere 892 mm in 2018, and 1230 mm in 2019. The ecosystem suffered from chronic drought in 2018 also at other GEOMON sites, JEZ (the nearest site to BRU) and UDL (the nearest site to MMJ; for location *see* Fig. 1). While *Sphagnum* for the $^{15}N_2$ incubation was collected at all three study sites at the same time (October 2018), site-specific moisture conditions could have affected microbial community structure and the BNF potential. In the laboratory experiment, however, similar wetness was ensured by the same volume of added bog water to *Sphagnum* from all three sites. Therefore, we suggest that water availability did not control the instantaneous BNF rates.

4.3.4. The effect of <u>Sphagnum</u> species

Stuart et al. (2021) showed that host identity is often the primary driver of BNF in peatlands. Under low N_r pollution, higher species-specific litter decomposability augments BNF by increasing nutrient turnover (van den Elzen et al., 2020). Saiz et al. (2021) observed higher BNF rates in *Sphagnum* species typical of hollows than those dominating hummocks. Specifically, *S. fallax* exhibited higher BNF rates than *S. capillifolium and S. papillosum.* The reason for such systematics appeared to be that the anoxic environment of wet hollows is more favorable for N_2 fixers (Leppanen et al., 2015; Zivkovic et al., 2022). By contrast, Vile et al. (2014) observed higher BNF rates in the hummock species *S. fuscum* than in the hollows species *S. angustifolium.* All moss samples for our ^{15}N assimilation experiment were collected in lawns. One exception was a subordinated number of plants of *S. cuspidatum* typical of hollows in the BRU incubation. While the moss species were identical in the UHL and MMJ incubation *(S. girgensohnii),* the BNF potential at these two sites was strikingly different (Fig. 3). Therefore, we suggest that *Sphagnum* species was not a key BNF control in our $^{15}N_2$ experiment.

4.3.5. Organic N availability

Wang et al. (2022) stressed the positive effect of organic N on BNF. Assimilation cost of amino acids was shown to be lower than that of NH_4^+ (Liu et al., 2013; Song et al., 2016). Organic N molecules can also serve as a C source for cyanobacteria, thus saving the cost of photosynthesis (Krausfeld et al., 2019). As seen in Tab. S3, concentrations of total organic N (TON) in bog water increased in the order: MMJ < BRU < UHL, and were thus probably unrelated to augmented BNF at MMJ *sensu* Wang et al. (2022).

4.3.6. Possible P limitation

Phosphorus is needed for the synthesis of ATP playing a key role in symbiotic BNF (Rousk et al., 2017; Wieder et al., 2022). In plant tissues, N:P ratios greater than 16 may indicate P limitation, while N:P ratios lower than 16 correspond to N limitation (Koerselman and Meuleman, 1996). Caution must be exercised in interpreting N:P ratios in atmospheric deposition as potential controls of P or N limitation. In addition to atmospheric input fluxes, bioavailable P and N in bog waters are strongly affected by a tight inner cycling with additional inputs from biomass decomposition (Walbridge and Navaratnam, 2006). Phosphorus input fluxes *via* atmospheric deposition into peat bogs may affect nutrient limitation in the long-run, depending on whether these input fluxes are large enough, compared to the frequently observed P leaching to deeper peat layers (Walbridge and Navaratnam, 2006, and references therein). According to Tab. S3, atmospheric deposition at all three study sites is consistent with P limitation that might limit BNF (high N:P ratios of 169, 60, and 112 at BRU, UHL, and MMJ, respectively). At the same time, N:P ratios in surface bog water were below 16 at two of the three sites, UHL (7.6), and MMJ (15). At BRU (N:P = 24), P limitation inferred from bog water chemistry would provide an explanation of non-detectable instantaneous BNF. At UHL, we found no indication of a relationship between P availability and zero BNF. The relatively P-rich bog water (165-490 μg P L^{-1}; Tab. S3) at all sites may contain, in addition to deposited P and mineralized P released during peat degradation, also, to some extent, geogenic P. Bedrock granite (BRU, UHL) contains P in accessory apatite and K-feldspar whose weathering was probably more efficient during the recent 40 years of acid rain. Phosphorus in phyllite (MMJ) is concentrated in apatite. Phosphorus concentrations in fresh bedrock were similar at BRU and MMJ (52-55 ppb), and twice lower at UHL (29 ppb; Gurtlerova et al., 1997; Pecina, 1999). The possible input of bioavailable geogenic P depended on local hydrology and could be site-specific.

Living *Sphagnum* had N:P ratios of 31, 12, and 7 at BRU, UHL, and MMJ, respectively (Tab. S4), indicating conditions favorable for BNF at the latter two sites. As seen in Fig. 7h, N:P < 16 marking N-limitation was characteristic of the entire vertical peat profile at UHL, and downcore to a depth of 15 cm at MMJ. In contrast, the N:P ratio was above 16 throughout the vertical peat profile at BRU. Phosphorus availability inferred from bog water and living *Sphagnum* gave consistent results with respect to possible BNF. As mentioned above, P likely limited BNF only at BRU.

Recently, measurements of regional P deposition started in headwater catchments of the GEOMON network (Oulehle et al., 2017). In the time period 2014-2018, UHL, a site directly included in the GEOMON network, exhibited lower P concentrations in the atmospheric input, compared to JEZ in the west (a proxy of BRU) and UDL in the east (proxy of MMJ; *see* Fig. 1 for catchment locations; the distance between JEZ and UDL, and between BRU and MMJ was approximately 70 km). Four-year average P concentrations at UHL were 72 and 36 μg L^{-1} in open-area precipitation and spruce throughfall, respectively. At JEZ, analogous P concentrations were 103 and 87 μg L^{-1}, At UDL, these sample types contained on average 110 and 91 μg P L^{-1}. The high P uptake by tree canopy resulting in low P concentration in throughfall might indicate P deficiency in UHL inputs. At the same time, the N:P ratio in total vertical atmospheric deposition was lower than 16 at all three sites (13.1 at JEZ, 15.5 at UHL, and 13.7 at UDL (GEOMON Hydrochemical Database, Czech Geological Survey).

4.3.7. Possible Mo limitation

https://doi.org/10.5194/egusphere-2023-827
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Nitrogenase requires molybdenum (Mo) in its active center to reduce N_2 to bioavailable NH_4^+ (Rousk et al., 2017; Bellenger et al., 2020). In principle, Mo limitation of BNF may have played a role in the contrasting BNF potentials observed at our sites. We do not have data on Mo concentrations in the studied ecosystems, except for trace metal analysis of the prevailing rock types (≤ 1 ppm; Gurtlerova et al., 1997). However, known Mo contents in coal massively mined/burnt in the Central European industrial region could shed some light on Mo availability *via* atmospheric deposition: North Bohemian soft coal (Sokolov basin close to BRU; Fig. 1) contains on average 18 ppm Mo, whereas Upper Silesian stone coal (Ostrava close to MMJ; Fig. 1) contains only ~0.6 ppm Mo, *i.e.*, 30 times less (Bouska et al., 1997). Since UHL is situated downwind of the North Bohemian cluster of coal-burning power plants, and very close to Turow (soft coal mining in the Polish part of the Lusatian basin; Fig. 1), atmospheric Mo inputs at UHL may be relatively high. Preliminarily, it appears to be unlikely that Mo significantly influences the contrasting BNF potentials at our study sites.

4.3.8. The role of SO_4^{2-} deposition

Large atmospheric inputs of acidifying sulfur forms (SO_2, H_2SO_4), characterizing northern Czech Republic since the 1950s (Hunova et al., 2022), can affect BNF in two ways: by suppressing methanogenesis, and by reducing the pH. Sulfate in peat bogs under high S deposition becomes an important electron acceptor (Pester et al., 2012) and bacterial sulfate reduction is thermodynamically favored relative to methanogenesis and fermentative processes (Vile et al., 2003). It not only decreases gross CH_4 production in peat, mitigating the flux of CH_4 to the atmosphere and minimizing climate warming, but also lowers the supply of CH_4 to methanotrophs that, at some sites, represent a major BNF pathway (Dise and Verry, 2001; Vile et al., 2014). Large SO_4^{2-} inputs may thus suppress BNF in peat bogs. In this context, is should also be mentioned that a $^{34}S/^{32}S$ isotope study has documented post-depositional vertical mobility of S in industrially polluted peat bogs (Novak et al., 2009). While long-term vertical S deposition, calculated according to Oulehle et al. (2016), was similarly high at UHL and MMJ (means of 18.6 and 17.0 kg ha^{-1} yr^{-1} for the 1900-2012 period), higher than at BRU (12.2 kg ha^{-1} yr^{-1}), UHL bog water at the time of this study was nearly 70 times richer in SO_4^{2-} than MMJ bog water, and eight times richer in SO_4^{2-} than BRU bog water (Tab. S3). Runoff at UHL was 4-5 times richer in SO_4^{2-} than runoff at MMJ and BRU. The zero instantaneous BNF at UHL in our $^{15}N_2$ incubation can be related to the highly elevated S deposition in the case that UHL primarily hosts methane oxidizing diazotrophs.

UHL waters were characterized by lower pH, compared to those at MMJ and BRU (Tab. S3). Runoff pH at UHL was 4.48, while runoff pH at MMJ was 7.40. Bog water pH at UHL was 4.02, while pH at MMJ was 4.88. Downregulation of BNF in more acidic environment has been reported, *e.g.*, by Basilier (1979) and van den Elzen et al. (2017). Accordingly, lack of BNF at UHL may be related to its lower pH, compared to the other two study sites.

4.4. Natural-abundance N isotope systematics

Sphagnum metabolizes bioavailable NH_4^+ approximately eight times faster than NO_3^- (Saiz et al., 2021). Because there were nonsignificant differences between $\delta^{15}N$ values of NH_4^+ and $NO_3^{-\,1}$ in rainfall at our study sites (Fig.

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S1), it is reasonable to use the entire $\delta^{15}N$ data set for a comparison with $\delta^{15}N$ values of living *Sphagnum* (Fig. 4). Slow lateral mixing of surface bog waters may bring throughfall N from the forested margins of each bog to the central unforested area and, therefore, we additionally included throughfall $\delta^{15}N$ data in Fig. 4 comparisons. The isotopically analyzed living *Sphagnum* plants represented on average a one-to-two-year increment (*cf.*, Wieder and Vitt, 2006). We found a statistically significant shift from isotopically light N of the deposition to isotopically heavier N of *Sphagnum* only at BRU ($p < 0.05$). This might indicate mixing with even heavier atmospheric N_2 taken up by diazotrophs. At BRU, BNF might have intermittently proceeded over the most recent growing seasons even though the $^{15}N_2$ experiment did not corroborate this process in October 2018.

A straightforward attribution of the N isotope pattern at BRU to BNF, however, is hampered by the fact that mineralization is a likely alternate source of dissolved N_r for assimilation by the moss (Zivkovic et al., 2022, and references therein). The often found high $\delta^{15}N$ values of mineralized N_r remaining in the bog ecosystem result from an isotope fractionation accompanying denitrification, a process known to occur especially in peat bogs that are not extremely acidic. Gaseous products of denitrification contain isotopically light N both in wetlands (Denk et al., 2017; for data from Czech peat bogs *see* Novak and al., 2015a, 2018), and aerated forest soils (Houlton and Bai, 2009; for data from Czech upland soils *see* Oulehle et al. 2021a). Nitrogen in surface bog water at BRU had a positive mean $\delta^{15}N$ value of 0.9 ‰ (Fig. 6). Isotope systematics at BRU are thus consistent with incorporation of mineralized N_r into moss biomass during assimilation instead of uptake of N resulting from BNF.

Advancing mineralization accompanying peat maturation with mobilization and export of gaseous low-$\delta^{15}N$ nitrogen is also responsible for the increasing $\delta^{15}N$ values of the residual peat substrate downcore (Fig. 7d).

Fig. S3 summarizes two general scenarios, under which a difference between N isotope composition of atmospheric input, *Sphagnum* and bog water indicates BNF: (1) the mean $\delta^{15}N$ values increase in the order: deposited N_r < bog water N_r < *Sphagnum* N_r < atmospheric N_2, or (2) the mean $\delta^{15}N$ values decrease in the order: deposited N_r > bog water N_r > *Sphagnum* N_r > atmospheric N_2. Whereas the $\delta^{15}N$ value of bulk atmospheric deposition in Central Europe is mostly negative, positive mean $\delta^{15}N$ values have been reported from other regions. One example is isotopically heavy N of dry-deposited HNO_3 in an industrial part of the U.S. (Elliott et al., 2009). Fig. S3 assumes that the magnitude of potential N isotope fractionations during uptake of inorganic N into plant biomass is relatively small and does not overprint the larger N isotope differences between the above discussed mixing endmembers.

It remains to be seen how to reconcile the relatively high instantaneous BNF rate at MMJ, measured in the laboratory, with the non-existence of a positive $\delta^{15}N$ shift from atmospheric deposition (mean of -4.3 ‰) to *Sphagnum* (mean of -4.4 ‰; Fig. 4; $p > 0.05$). Given that we explained the positive $\delta^{15}N$ shift from deposition to *Sphagnum* at BRU by mixing of low-$\delta^{15}N$ rainfall with high-$\delta^{15}N$ bog water, and that bog-water N at MMJ is isotopically heavy, a similar positive N isotope shift from rainfall to *Sphagnum* would be expected also at MMJ. Such was not the case. This observation is important because it might indicate that uptake of recently mineralized N_r from bog water at sites hydrologically similar to MMJ (and also BRU) may not control the N

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isotope signature of living *Sphagnum*. An input of isotopically light N_r for assimilation by the MMJ moss could, in principle, originate from shallow groundwater upwelling or lateral water inflow from other segments of the catchment possibly bringing legacy low-$\delta^{15}N$ nitrogen from the peak acid-rain period throughfall. Such within-site water inputs could affect the intermediate $\delta^{15}N$ value of *Sphagnum* at MMJ.

Conclusions

Based on hydrochemical monitoring data and statistical modelling, the three studied *Sphagnum* peat bogs located in the industrial northern Czech Republic received close to 18 kg N_r ha^{-1} yr^{-1} *via* atmospheric deposition. Since 1900, the atmospheric input of N_r affected the study sites in the order: UHL > MMJ > BRU. In the most recent years, the annual N_r inputs *via* vertical deposition between the sites differed by mere 1 to 2 kg ha^{-1} yr^{-1}. The sites can thus be classified as highly to medium-polluted. A 168-hour $^{15}N_2$ assimilation experiment revealed relatively high but variable rates of BNF at MMJ, and non-detectable BNF at the remaining two sites, characterized by slightly higher and slightly lower N_r depositions, respectively, compared to MMJ. We investigated in all 10 different parameters that might have served as controls of the presence or absence of instantaneous BNF in living moss. In addition to bulk N_r deposition fluxes, these parameters included: NH_4^+-N/NO_3^--N ratio in atmospheric input, temperature, wetness, *Sphagnum* species, organic-N availability, possible P limitation, possible Mo limitation, SO_4^{2-} deposition, and pH. Using the available data, we argue that P deficiency was the likely inhibitor of BNF at BRU. Assuming that methanotrophic bacteria represented a major type of diazotrophs, extremely high SO_4^{2-} inputs may have been the key control of the absence of BNF at UHL. While the long-term temperature and wetness at UHL were also lower, compared to the remaining two sites, they probably did not affect the results of the $^{15}N_2$ experiment since the incubation was performed under the same temperature and wetness for all sites. In general, higher concentrations of decomposition-inhibiting metabolites could be causally related to BNF rates. Such a control of BNF was unlikely since the same *Sphagnum* species from MMJ and UHL was used for the $^{15}N_2$ experiment that showed contrasting results for these two sites. Tthe large $\delta^{15}N$ differences between moss replicates that were collected from various segments of MMJ at the end of the $^{15}N_2$ incubation suggested an existence of BNF hotspots.

The use of natural-abundance N isotope ratios to corroborate the observed instantaneous BNF rates was hampered by isotopically heavy N of surface bog water. The bog water contained secondary N_r forms which could have resulted from partial *Sphagnum*/peat decomposition and removal of the complementary low-$\delta^{15}N$ products of denitrification. At BRU, we found statistically significant differences in $\delta^{15}N$ values in the order: deposited N_r < *Sphagnum* N_r < atmospheric N_2 < bog water N_r. Stable isotope ratios could not unambiguously distinguish between assimilation of bog-water N_r and atmospheric N_2 to form the observed N-isotope signature of *Sphagnum*. At UHL and MMJ, $\delta^{15}N$ differences between *Sphagnum* and the atmospheric input were statistically insignificant. The natural-abundance approach as a test of BNF presence may give more promising results at high-latitude sites often characterized by greater (30-40 cm) depth of the water table level below *Sphagnum* capitula than the Central European sites.

https://doi.org/10.5194/egusphere-2023-827
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Author contribution: M. Stepanova: conceptualization, data curation, visualization, writing – review and editing; M. Novak: conceptualization, data interpretation, writing – original draft; B. Cejkova: methodology, nitrogen fixation data acquisition, data interpretation; I. Jackova: methodology, concentration and isotope data acquisition; F. Buzek: methodology, data interpretation, validation; F. Veselovsky: field work; J. Curik: field work; E. Prechova: formal analysis, resources; A. Komarek: statistical analysis; L. Bohdalkova: data interpretation

Competing interests. The authors declare that they have no conflict of interest.

Acknowledgements. This is a contribution to the Strategic Research Plan of the Czech Geological Survey (DKRVO/CGS 2018-2022, grant. no. 310660 to MS). We thank Prof. Martin Sanda of the Czech Polytechnic, Prague, and Jan Knotek of the Jeseniky branch of the Czech Geological Survey for field work assistance. Dr. Filip Oulehle is thanked for modelling of long-term atmospheric N deposition at the study sites, and Oldrich Myska for providing monitoring data from the GEOMON database.

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