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BRIEF REPORT

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Biological nitrogen fixation, diversity and community structure of diazotrophs in two mosses in 25 temperate forests

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Abstract

Many moss species are associated with nitrogen (N)-fixing bacteria (diazotrophs) that support the N supply of mosses. Our knowledge relates primarily to pristine ecosystems with low atmospheric N input, but knowledge of biological N fixation (BNF) and diazotrophic communities in mosses in temperate forests with high N deposition is limited. We measured BNF rates using the direct stable isotope method and studied the total and potentially active diazotrophic communities in two abundant mosses, *Brachythecium rutabulum* and *Hypnum cupressiforme*, both growing on lying deadwood trunks in 25 temperate forest sites. BNF rates in both mosses were similar to those observed in moss species of pristine ecosystems. *H. cupressiforme* fixed three times more N_2 and exhibited lower diazotrophic richness than *B. rutabulum*. *Frankia* was the most prominent diazotroph followed by cyanobacteria *Nostoc.* Manganese, iron, and molybdenum contents in mosses were positively correlated with BNF and diazotrophic communities. *Frankia* maintained high BNF rates in *H. cupressiforme* and *B. rutabulum* even under high chronic N deposition in Central European forests. Moss N concentration and ^{15}N abundance indicate a rather minor contribution of BNF to the N nutrition of these mosses.

INTRODUCTION

Mosses are a species-rich clade found in almost all terrestrial ecosystems from the Arctic to the tropics (Geffert et al., 2013), with an often large impact on the carbon (C) and nitrogen (N) cycle (Elbert et al., 2012; Koranda & Michelsen, 2021). They live on soils, rocks and bark of living or dead trees under sometimes extreme climatic and nutrient-poor conditions (Dierssen, 2001; Müller et al., 2020). Especially in N-limited ecosystems and habitats, mosses have an important function due to their ability to host free-living diazotrophic bacteria which acquire ammonia by enzymatic conversion of atmospheric N_2 (Cornelissen et al., 2007). These diazotrophs not only supply mosses with reactive N (Jean et al., 2012) but drive N cycling and N enrichment in various ecosystems (Deluca et al., 2002; Gundale et al., 2011; Rousk et al., 2016). Large amounts of N stored in ecosystems worldwide originate from the diazotrophic activity in mosses (Elbert et al., 2012). Most of the studies on moss-associated biological N_2 fixation (BNF) have been conducted in N-poor environments such as arctic and boreal peatlands or forests (Knorr et al., 2015; Porada et al., 2014;

Christina Groß and Shakhawat Hossen share the first authorship.

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Rousk et al., 2014a) whereas little is known about BNF in mosses that are common in N-rich temperate forests.

Lying deadwood trunks (logs) are an important component of biodiversity in forest ecosystems (Stockland et al., 2012). Many logs are colonized by different moss species, often with a preference for a specific tree species, decay stage, size, and exposition of logs (Botting & DeLong, 2009; Żarnowiec et al., 2021). However, in Central European forests, some moss species are relatively unspecific and grow on logs of different tree species or even on the forest floor (Dittrich et al., 2014; Ódor et al., 2006). Fresh and slightly decomposed logs of common temperate tree species represent nutrient-poor habitats with extremely low N concentrations as indicated by C:N ratios of about 500–1000 (Kahl et al., 2017). Therefore, the decomposition of deadwood can hardly contribute to the N supply of inhabiting mosses.

Deadwood mosses can cover their N requirements by two pathways, BNF and uptake of reactive N (ammonium, nitrate, organic N compounds) from dry and wet deposition. Nitrogen deposition in the Northern Hemisphere and especially in Central European forests has been high for many decades (Borken & Matzner, 2004; Galloway et al., 2008; Schmitz et al., 2019). With such a high N supply, it is not at all clear whether substantial BNF occurs in mosses. To the best of our knowledge, there have been no studies of diazotrophs in mosses occurring in temperate forests with chronically high N deposition.

BNF in mosses was first attributed to cyanobacteria living on the surface or intracellularly in hyaline cells of *Sphagnum* (Basilier, 1974). Many subsequent studies have confirmed this finding and assign the photoautotrophic cyanobacteria a prominent role in BNF in mosses also in non-peatland mosses (Carrell et al., 2019; Klarenberg et al., 2022; Rajandu et al., 2021; Renaudin et al., 2022a; van den Elzen et al., 2020). However, other studies reported that members of the phyla *Acidobacteria*, *Actinobacteria*, *Alphaproteobacteria*, *Bacteroidetes*, *Betaproteobacteria*, *Eremiobacterota* and *Verrucomicrobiota* can also contribute to BNF in mosses (Holland-Moritz et al., 2021; Vile et al., 2014). Diazotrophs are capable of fixing atmospheric N_2 by carrying the enzymes of the dinitrogenase reductase complex, encoded by the *nif* gene family, from which the *nifH* gene is the most widely used biomarker to study the phylogeny, diversity, and activity of diazotrophs (Gaby & Buckley, 2012, 2014; Poly et al., 2001). A majority of studies used exclusively total nucleic acid extracts including active, dormant, and dead cells as well as extracellular DNA of diazotrophs. The metabolically active cells of the microbial community represent only a small fraction of the total DNA extracts (Klein et al., 2016) and can thus differ from the total community structure (Purahong et al., 2022; Wahdan et al., 2021). Therefore, nucleic acid-based methods are required to distinguish between the sequence information of metabolically active cells and inactive or dormant cells (Purahong et al., 2022). To our best knowledge, BNF rates and the active fraction of diazotrophic diversity and composition of mosses on logs under high N deposition in Central European forests have yet not been investigated.

In this study, we determined BNF rates and the *nifH* gene-based diversity and community composition of the total and active fraction in two abundant mosses (*Brachythecium rutabulum* (Hedw.) Schimp., *Hypnum cupressiforme* (Hedw.) growing on logs of three common tree species (*Fagus sylvatica*, *Quercus* spp., *Pinus sylvestris*) after 12 years of decay in 25 forests. *B. rutabulum* and *H. cupressiforme* are among the most abundant moss species growing on logs in Central European forests (Müller et al., 2020; Ódor et al., 2005). Nutrient requirements, including N, are higher for *B. rutabulum* than for *H. cupressiforme* (Hill et al., 2007; Simmel et al., 2021). The 25 forest sites are located in three regions across Germany and differ in terms of climate, soil, and atmospheric deposition. This study was part of a long-term field experiment on log decomposition, biodiversity, and organismic interactions in logs. Based on the experimental design, we asked the following questions:

1. How do BNF rates, dominating diazotrophic genera, diazotrophic richness, and community composition differ between *B. rutabulum* and *H. cupressiforme?*
2. How do moss nutrient content, deadwood tree species, and region influence BNF rates, diazotrophic richness, and community composition?
3. Can differences in BNF rates be explained by different diazotrophic community composition in the mosses?

EXPERIMENTAL PROCEDURES

Study region and experimental design

This study was part of the long-term experiment BELongDead within the Biodiversity Exploratories project at three regions across Germany (Schwäbische Alb [AEW], Hainich-Dün [HEW] and Schorfheide-Chorin [SEW]) (Fischer et al., 2010). The regions differed in their mean annual temperature (MAT) and precipitation (MAP). AEW had the lowest MAT (6–7°C) and the highest MAP (700–1000 mm), compared to HEW with intermediate MAT (6.5–8°C) and MAP (500–800 mm) and SEW with the highest MAT (8–8.5°C) and lowest MAP (500–600 mm) (Kahl et al., 2017). Bulk deposition of total N was similar with 11.1, 10.1 and 11.8 kg ha^{-1} $year^{-1}$ in AEW, HEW, and SEW, respectively (Schwarz et al., 2014). Nitrogen concentrations of mineral soils (0–10 cm depth) varied between 0.1% and 0.8% across regions and sites (Minnich et al., 2021).

The BELongDead experiment was established in a total of 25 independent forest sites in the winter of 2008/2009 ($n = 9$ in AEW, $n = 12$ in HEW, $n = 9$ in SEW), comprising European beech (*Fagus sylvatica*), Norway spruce (*Picea abies*) and Scots pine (*Pinus sylvestris*) forests (Kipping et al., 2022). Freshly cut logs (4 m length, ~ 31 cm diameter) of 13 tree species were placed on the soil in random order within 1 m distance from each other. All logs originated from the state forest of the Federal State of Thuringia in Central Germany and were thus grown under similar climatic conditions. In this study, we selected logs of *F. sylvatica*, *Quercus* spp. (*Quercus robur*, *Quercus petraea*) and *P. sylvestris*, representing a diffuse-porous angiosperm, a ring-porous angiosperm, and a gymnosperm tree species, respectively. Further details on the experiment and log decay are given by Kahl et al. (2017) and Edelmann et al. (2023).

Sample collection

Bryophyte-dominated communities of the accessible surface of the logs (i.e., upside and flanks) were surveyed. Single-species abundance (i.e., percentage of log surface area covered) was estimated using a simplified percentage scale (Dittrich et al., 2014). Visible dead parts of bryophytes, lichens, or vascular plants (including litter) were not included in the abundance estimation of the respective species. Based on an initial analysis, moss species *B. rutabulum* and *H. cupressiforme* were found to be the most abundant and most frequent species across all surveyed forest sites and logs, respectively.

Therefore, the upper part (including shoots) of *B. rutabulum* and *H. cupressiforme* were taken from the logs of the three chosen tree species (*F. sylvatica*, *Quercus* spp., *P. sylvestris*) (each $n = 9$ in AEW and HEW, $n = 7$ in SEW) in September 2020. The lower dark and potentially dead parts of the mosses were excluded from further analyses. In SEW, logs were not covered by any mosses in two of nine plots. We expected a total of 150 moss samples (25 plots × 2 mosses × 3 deadwood trees = 150) from all three regions. Finally, we collected in total ($19 + 26 + 17 = 62$) samples for the moss *B. rutabulum* and ($27 + 26 + 21 = 74$) samples for *H. cupressiforme* across three regions (AEW, HEW and SEW). The mean fresh weight of each moss sample was 1.4 ± 0.5 g. We did not find any mosses on the remaining deadwood logs. One part of the moss sample was stored in a plastic bag in the field and kept cool (~5°C) until further analyses. A second part of the sample was transferred into a 15-mL falcon tube in the field for subsequent bromodeoxyuridine (BrdU) incubation for 48 h and thereafter shock frozen by liquid N_2 and transported under dry ice to the laboratory (for details see below).

BNF rate

At the laboratory, moss samples were gently cleaned by removal of litter or dead moss tissues and then divided into two fractions. One moss fraction, consisting of about 15 cm^2 area was inserted into a glass flask (~131 mL, Media Bottle Rasotherm ISO, GL45), saturated with water by addition of 5–10 mL deionized water and pre-incubated for 1 week before the determination of BNF rate. The second fraction was dried at 60°C and served as a control for the natural ^{15}N abundance (see below). After pre-incubation, excess water was removed from the glass jars. The mosses were pre-incubated and incubated at 20°C under a plant lamp with photosynthetic active radiation of 105 μmol photons m^{-2} s^{-1} for 12 h with light and 12 h in the dark per day, which represents approximately the length of day and night during the sampling time.

The lids of glass flasks were equipped with 6-mm-thick chlorobutyl septa (Glasgerätebau Ochs, Germany) to allow injection and sampling of gases during incubation. After closure, the glass flasks were flushed for 10 min with a prepared gas mixture, consisting of 8% oxygen (O_2), 30% N_2, and 62% helium (Riessner-Gase GmbH, Lichtenfels, Germany). Additional O_2 was added to generate a concentration of 21% O_2 in the glass flasks. To determine the BNF rate as explained earlier (Weaver & Danso, 1994), 25 mL of pre-treated $^{15}N_2$ (99.1%, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany, batch number MBBC7366) was added to each glass flask using a gas-tight syringe. The pre-treatment was necessary to remove contaminations (NH_3, NO_x) from the $^{15}N_2$ gas (see Groß et al., 2022 for further details). To determine the potential contamination of mosses by the pre-treated $^{15}N_2$ gas, four samples were autoclaved at 121°C and 1.2 bar for 20 min. The autoclaved samples were incubated for 48 h in the dark at 20°C. The enrichment in the $\delta^{15}N$ signature in the autoclaved samples by contamination was negligible (0.005 μg ^{15}N per ml $^{15}N_2$).

The ratio of $^{15}N_2$ to total N_2 in the headspace was calculated for each glass flask using the total amount of N_2 and the gas pressures before and after the addition of $^{15}N_2$. Due to the mixture with $^{14}N_2$ and the pre-treatment of the $^{15}N_2$ gas, the ^{15}N signature in the headspace was 25–30 atom%. The samples were incubated for 72 h under the conditions mentioned above. To terminate the incubation, the flasks were opened, and the samples were immediately dried at 60°C for at least 48 h until mass constancy. The dry weight and gravimetric water content of mosses were determined by weighing before and after drying. Dried samples were ground using a ball mill (MM400, Retsch GmbH, Haan, Germany). Both fractions, $^{15}N_2$ incubated and respective non-incubated subsamples were analysed for $\delta^{13}C$ and $\delta^{15}N$ signatures, C and N contents using an elemental analyser (EA 3000, Eurovector, Pavia,

Italy) coupled to an isotope ratio mass spectrometer (NU Horizon, Wrexham, UK and HEKAtech, Wegberg, Germany). An amount of 4–5 mg of finely ground material was weighed into tin capsules for analysis. C and N contents are expressed on a mass % basis. Values of $\delta^{13}C$ and $\delta^{15}N$ are reported versus Vienna-PeeDee Belemnite (V-PDB) and air, respectively, using certified reference materials and working standards of known isotopic composition. After the conversion of $\delta^{15}N$ signatures of incubated and non-incubated (natural abundance) samples into ^{15}N atom%, BNF rates (μg N g^{-1} d^{-1}) referring to the dry weight of moss were calculated as follows:

$$\mathrm{BNF} = \frac{N \times \left({}^{15}\mathrm{N}_{\mathrm{enriched}} - {}^{15}\mathrm{N}_{\mathrm{NA}}\right)}{\mathrm{DW} \times t \times \mathrm{HS} \times 100}, \quad (1)$$

where N is the amount of N in the moss sample (μg), $^{15}N_{enriched}$ is ^{15}N atom% in the incubated moss sample, $^{15}N_{NA}$ is the ^{15}N atom% in the non-incubated moss sample (natural ^{15}N abundance), DW is the dry weight of the moss sample (g), t is the incubation time (d), and HS is the ratio of $^{15}N_2$ to total N_2 in the headspace of the glass flask.

Elemental analyses

Total concentrations of phosphor (P), sulfur (S), manganese (Mn), iron (Fe), and molybdenum (Mo) were determined in both moss species of all samples from each plot after pressure digestion using reverse aqua regia. For this purpose, 0.06 g of dried moss sample was filled in vessels, and 8 mL of nitric acid (HNO_3, 65%, Chemsolute, Th. Geyer GmbH & Co.KG, Renningen, Germany) and 0.25 mL of hydrochloric acid (HCl, 37%, Fisher Scientific, Loughborough, UK) were added. The mixture was microwaved (MARS6, CEM Corporation, Matthews, NC, USA) and filtered using sterile, cellulose acetate syringe filters (0.45 μm). Afterward, the supernatant was analysed at the Analytical Chemistry Lab, University Bayreuth, using an ICP-MS (Agilent 7500ce, Cetac ASX-510, Santa Clara, CA, USA) for Mo and an ICP-OES (Varian, Vista-Pro radial, Palo Alto, CA, USA) for P, S, Mn, and Fe according to manufacturer's instructions.

BrdU labelling, DNA extraction and molecular biological analyses

To identify metabolically active cells of diazotrophs, a labelling approach using BrdU was performed as described by Purahong et al. (2022). After sampling, each moss sample (with a mean weight of 1 g) was treated with 1 mL of 10 mM BrdU solution (Sigma-Aldrich Chemie GmbH). Thereafter, the moss samples were placed in sterile 50-mL tubes covered with aluminium with a loose cap and incubated at room temperature for 48 h. The added BrdU was only incorporated into the genomic DNA of replicating cells during DNA synthesis, whereas dormant, inactive, or dead cells were unable to incorporate BrdU. The Quick-DNA Fecal/Soil Microbe Miniprep Kit from (Zymo Research Europe GmbH, Freiburg, Germany) was used according to the manufacturer's instructions to extract all types of genomic DNA, which was referred to as the total fraction. Subsequently, an immunocapture approach was used to separate the BrdU-labelled DNA from the 'total' DNA as outlined earlier (Groß et al., 2022; McMahon et al., 2009; Wahdan et al., 2021). In short, for each sample, 2 μL of monoclonal BrdU antibodies (1 mg μL^{-1}, mouse anti-BrdU, clone BU-33, Sigma-Aldrich GmbH) was mixed with 18 mL of denatured herring sperm DNA (1.25 mg mL^{-1} in phosphatebuffered saline [PBS] from Promega, Walldorf, Germany) and incubated for 45 min at 30°C to create an antibody-herring sperm DNA complex. Denatured DNA (25 μL; approximately 200 ng DNA + 10 μL PBS) was mixed with an antibody-herring sperm DNA complex and incubated for 30 min at 30°C to capture BrdU-labelled DNA. Next, 6.26 μL of washed Dynabeads™ Goat Anti-Mouse IgG (Invitrogen, Life Technologies GmbH, Darmstadt, Germany) was added to the incubated solution. The mixture was then rotated (66 rpm) for 30 min at room temperature on an RM-2 roll mixer (Carl Roth GmbH, Karlsruhe, Germany) to form a Dynabead complex, which consisted of Dynabeads, BrdU antibodies, and BrdU-labelled DNA. Subsequently, the Dynabead complex was treated eight rounds with 100 μL of a PBS–BSA solution (consisting of 0.05 g bovine serum albumin in 50 mL PBS) and was washed using a DynaMagTM-2 (Invitrogen, Life Technologies GmbH). The Dynabead complex (Dynabead-BrdU antibodies-BrdU-labelled DNA) was washed with 100 mL PBS–BSA eight times by adding the wash solution, and trapping the complex with a magnetic particle concentrator (Dynal). BrdU-labelled DNA was released from the washed Dynabeads by adding 20 μL BrdU solution (1.7 mM in PBS–BSA) and then incubated under slow rotation for 35 min. The resulting BrdU-labelled DNA was hereafter termed as active fraction.

NifH gene-based amplicon sequencing and bioinformatics analysis pipeline

Nucleic acid extracts from the total and active fraction of each sample were sent to LGC Genomics Berlin, Germany for high-throughput amplicon sequencing using the *nifH* gene as a marker. Three independent *nifH* gene-based PCR reactions using the primer set *PolF* and *PolR* (Poly et al., 2001) were carried out for

each sample, and respective amplicons were pooled for each sample of both fractions separately. Thereafter, Illumina Nextera XT indices were ligated to both ends and sequenced on an Illumina MiSeq platform according to the manufacturer's instructions.

The *nifH* gene sequences corresponding to the forward and reverse primer were filtered from the demultiplexed raw reads using cutadapt (Martin, 2011). Paired-end sequences were quality-trimmed, filtered for chimeras, and merged using the DADA2 package (Version 1.24.0) (Callahan et al., 2016) pipeline implemented in R (v4.2.1) (R Core Team, 2022). The parameters for the DADA2 pipeline were: truncLen = *c* (20, 160), max*N* = 0, maxEE = *c* (2, 5), trunc*Q* = 2, m. phix = TRUE). The forward reads were trimmed to a length of 240 bp and the reverse reads to 160 bp. The forward and reverse reads were then truncated beyond the first instance of quality scores below 3 ('truncQ = 2'). The maximum expected error during denoising ('maxEE') was 2 and 5 for forward and reverse reads, respectively. Then, denoised reads were merged, and chimeric contigs discarded using the functions 'mergePairs' and 'removeBimeraDenovo' (Callahan et al., 2016). Too short (<250 bp) and too long sequence reads (>400 bp), which could potentially be introduced through non-specific priming, were removed from the sequence table. A DADA2 formatted *nifH* gene database customized from the taxadiva script (https://github.com/lavanyarishishwar/taxadiva) was used for taxonomic assignment. Non-chimeric sequences were assigned into amplicon sequence variants (ASV) using the DADA2 function 'assignTaxonomy'. All numbers of processed sequence read through the analysis pipeline can be found in Table S1. From the total and active fraction, 60 and 32 diazotrophic genera, respectively, were acquired after rare taxa filtering, an approach to avoid sequencing errors that lead to misclassifying the taxa of DNA fragment reads as introduced earlier (Cao et al., 2020). The dataset was rarefied using the sample with the smallest number of sequences through the rarefy_even_depth function in the phyloseq package (McMurdie & Holmes, 2013). One sample from the active fraction was excluded due to low sampling depth (<1000 reads), and all subsequent analyses were performed on the rarefied dataset.

Statistical analyses

Statistical analyses were performed using R (v4.2.1) (R Core Team, 2022) and PAST Version 2.17 (Hammer et al., 2001). The data were tested for normality using the Shapiro–Wilk test and for the equality of group variances using the *F*-test (for two datasets) and Levene's test (for more than two datasets). The influence of moss species and region as well as their interaction on BNF and diazotrophic richness of active fraction were tested by two-way analysis of variance (ANOVA). The Kruskal–Wallis test and the Wilcoxon rank-sum test, both with Bonferroni correction ($p < 0.05$) at a 95% confidence interval, were employed for pairwise comparisons. Further, two-way ANOVA was performed to explore any potential effect of moss species and region on nutrient concentration (Mo, Mn, P, S, Fe, N and C: N ratio), isotopic signatures, and cover by mosses. Multivariate statistical analyses of the diazotrophic community structure in the active fraction related to moss species and region were initially assessed using permutational multivariate analysis of variance (three-way permutational multivariate ANOVA [PERMANOVA]) with the adonis2 function from the Vegan package (Oksanen, 2022). This analysis revealed a significant difference between both moss species (*B. rutabulum* vs. *H. cupressiforme*). Hence, further multivariate analyses were performed separately for *B. rutabulum* and *H. cupressiforme*. This was carried out by two-dimensional nonmetric multidimensional scaling (NMDS) based on Bray–Curtis distance using the functions metaMDS also from the Vegan package. The functions ordispider and ordiellipse were employed to visualize the distance matrix of centroids and to add standard error (SE) ellipses of groups (AEW, HEW and SEW). To assess correlations between the *nifH* gene-based community structure of the active fraction and moss-associated nutrient concentrations (Mo, Mn, P, S, Fe, N and C:N ratio), variables were fitted as vectors onto the NMDS plots using the function envfit of the package Vegan (Oksanen, 2022) with *p* values over 999 permutations. Spearman correlations were conducted to characterize the relationships between moss-associated parameters, and BNF and diazotrophic richness of the active fraction. In addition, the relation between BNF and the richness of diazotrophs in the active fraction was tested separately for both moss species using Spearman correlations.

RESULTS

Moss cover of logs

After almost 12 years of log decay, *H. cupressiforme* was the dominant moss species in AEW and SEW, covering on average 38% and 33% of the habitable log surface, respectively (Table 1). The degree of coverage was similar for *H. cupressiforme* (20%) and *B. rutabulum* (21%) in HEW. The cover was significantly different between both moss species ($p = 0.005$) while region and the interaction between moss species and region had no effect. Log cover of both moss species varied between 0 and 95% across the three regions, and logs of two plots in SEW were not covered

TABLE 1 Mean (±SE) element concentrations and isotopic signatures of *Brachythecium rutabulum* and *Hypnum cupressiforme* covering logs in forest plots across three regions (Schwäbische Alb [AEW], Hainich-Dün [HEW], and Schorfheide-Chorin [SEW]).

	B. rutabulum			*H. cupressiforme*			Moss species		Region		Moss × region	
	AEW	HEW	SEW	AEW	HEW	SEW	*F*	*p*	*F*	*p*	*F*	*p*
Moss cover (%)	17.7 ± 6.0	20.5 ± 5.5	12.8 ± 4.7	37.9 ± 5.	20.0 ± 5.0	33.4 ± 6.5	8.2	**0.005**	0.7	0.481	2.5	0.086
C (%)	42.8 ± 0.1	42.2 ± 0.1	42.0 ± 0.1	43.0 ± 0.1	42.5 ± 0.1	42.5 ± 0.1	13.5	**<0.001**	14.9	**<0.001**	0.4	0.669
N (%)	1.8 ± 0.1	2.0 ± 0.1	1.8 ± 0.1	1.8 ± 0.1	1.7 ± 0.1	1.6 ± 0.1	4.4	**0.039**	1.5	0.221	1.7	0.193
C:N ratio	25.3 ± 2.0	21.5 ± 1.2	25.1 ± 1.5	25.1 ± 1.3	27.2 ± 1.5	27.5 ± 1.6	5.2	**0.024**	0.9	0.422	2.0	0.146
$\delta^{13}C$ (‰)	−30.5 ± 0.2	−30.6 ± 0.2	−31.3 ± 0.2	−30.7 ± 0.1	−30.8 ± 0.2	−30.8 ± 0.2	0.0	0.998	2.1	0.130	2.2	0.110
$\delta^{15}N$ (‰)	−5.3 ± 0.3	−3.4 ± 0.5	−5.9 ± 1.0	−5.6 ± 0.2	−4.2 ± 0.3	−6.9 ± 0.4	6.1	**0.015**	17.2	**<0.001**	0.3	0.778
P (mg g^{-1})	2.0 ± 0.2	2.1 ± 0.2	1.7 ± 0.2	1.5 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	15.7	**<0.001**	0.5	0.606	0.7	0.508
S (mg g^{-1})	1.2 ± 0.1	1.3 ± 0.1	1.1 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	16.1	**<0.001**	1.8	0.167	1.3	0.283
Mn (mg g^{-1})	0.2 ± 0.0	0.3 ± 0.1	0.4 ± 0.1	0.2 ± 0.0	0.3 ± 0.0	0.4 ± 0.1	2.0	0.160	8.8	**<0.001**	0.1	0.890
Fe (mg g^{-1})	0.4 ± 0.0	0.6 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	1.1	0.306	6.6	**0.002**	6.3	**0.003**
Mo (µg g^{-1})	0.3 ± 0.0	0.6 ± 0.1	0.5 ± 0.1	0.2 ± 0.0	0.4 ± 0.0	0.4 ± 0.1	4.8	**0.033**	5.2	**0.008**	1.0	0.365

Note: Significant differences ($p < 0.05$), based on two-way ANOVA, between the moss species and among the regions are indicated in bold.

by any moss species. *B. rutabulum* had significantly higher cover on logs of *F. sylvatica* and *Quercus* spp. ($p = 0.001$) than on logs of *P. sylvestris* while the cover of *H. cupressiforme* was not affected by tree species (not shown). Other, less dominant bryophyte species (e.g., *Brachythecium salebrosum*, *Dicranum* spp., *Herzogiella seligeri*, *Lophocolea heterophylla*) together covered the logs at 8.65% (AEW), 2.21% (HEW) and 3.75% (SEW) (Table S2).

Element concentrations and isotopic signatures of mosses

Element concentrations partly differed between both moss species and among the regions (Table 1). *B. rutabulum* had higher N ($p = 0.039$), P ($p < 0.001$), S ($p < 0.001$) and Mo ($p = 0.033$) concentrations than *H. cupressiforme* where the higher N level coincided with an enrichment in the ^{15}N abundance ($p = 0.015$) and lower C:N ratio ($p = 0.024$). Concentrations of Fe and Mn and ^{13}C signatures were not different between both moss species. Regional differences in chemical moss properties were found for C ($p < 0.001$), $\delta^{15}N$ ($p < 0.001$), Mn ($p < 0.001$), Fe ($p = 0.002$) and Mo ($p = 0.008$). For both moss species, the $\delta^{15}N$ was most depleted in SEW (−5.9‰ and −6.9‰), followed by AEW (−5.3‰ and −5.6‰) and HEW (−3.4‰ and −4.2‰). A significant interaction ($p < 0.01$) of moss species and region was only found for Fe concentration, indicating that Fe concentrations in *B. rutabulum* and *H. cupressiforme* differed among the three regions (Table 1).

BNF, diazotrophic richness and community composition

Moss-associated BNF rates ranged from <1 to 95 µg N g^{-1} day^{-1} and differed between moss species and region (Figure 1A). In all regions, *H. cupressiforme* showed consistently higher BNF rates ($p < 0.01$) than *B. rutabulum*. The highest BNF rates were found for both mosses from SEW, followed by HEW and AEW, however, the difference was not significant for *B. rutabulum* between AEW and HEW. Log tree species did not affect BNF rates in *H. cupressiforme* and *B. rutabulum* (not shown).

Like BNF, significant differences ($p < 0.05$) were observed for diazotrophic richness (ranged from 77 to 277 ASVs) between moss species and region (Figure 1B). Richness in *B. rutabulum* was significantly higher ($p < 0.05$) in SEW than in HEW and AEW while no differences occurred in *H. cupressiforme* among the regions. When comparing diazotrophic richness between the moss species within the regions, significant differences ($p < 0.05$) were observed for AEW and

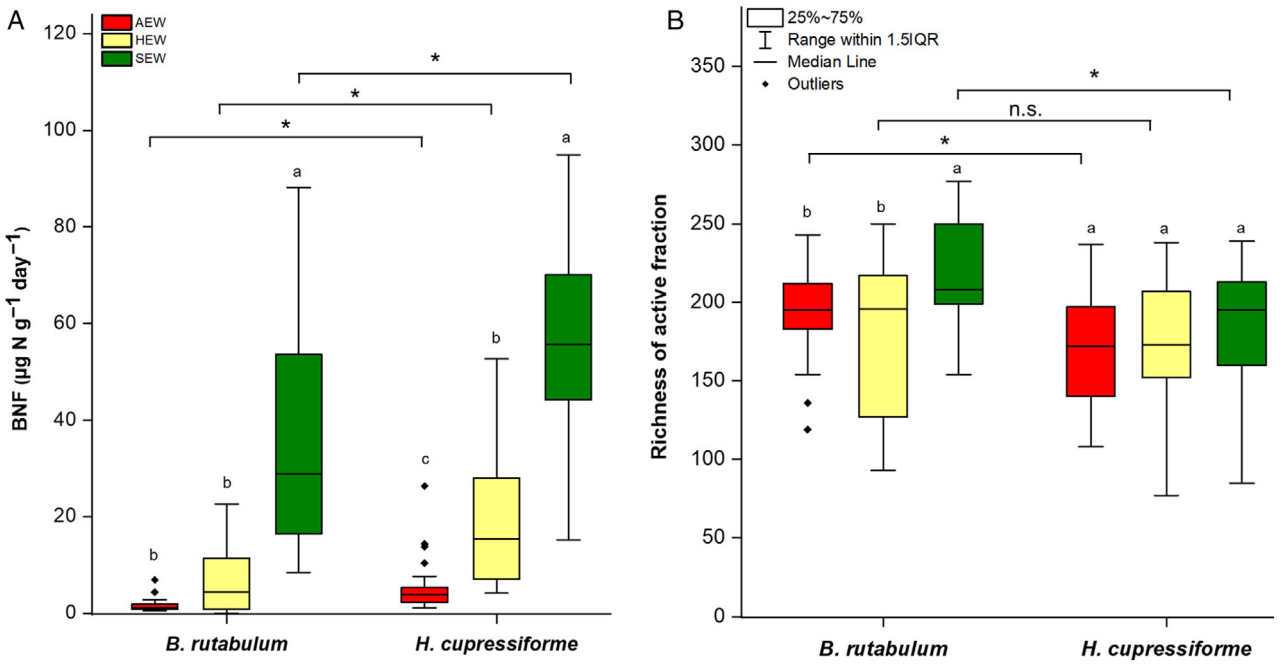

FIGURE 1 Biological N fixation (BNF) (A) and diazotrophic richness of the active fraction (B) in both moss species (*Brachythecium rutabulum*, $n = 62$ and *Hypnum cupressiforme*, $n = 74$) across three different regions (Schwäbische Alb [AEW], Hainich-Dün [HEW] and Schorfheide-Chorin [SEW]). Significant differences ($p < 0.05$) in the BNF rates and diazotrophic richness are indicated by different letters for differences between regions within the moss species and by an asterisk for differences between moss species within the region, based on Kruskal–Wallis and pairwise Wilcoxon rank-sum test.

SEW. An interaction effect (moss species and region) has been found on BNF but not on the diazotrophic richness of the active fraction (Table S3). The genus *Frankia*, accounting for up to 70% of the relative sequence read abundance of the active diazotrophic community composition, and dominated the diazotrophic community across moss species and regions (Figure 2). Members of the genera *Nostoc*, *Gluconacetobacter* and *Ruminiclostridium* were the next most abundant diazotrophs from total of 32 genera. In contrast, the total fraction of the diazotrophic community was dominated by *Bradyrhizobium* and *Frankia* (accounting for 36% relative sequence read abundance of total diazotrophic community composition) in both moss species and all regions (Figure S1).

Diazotrophic community composition of the active fraction was significantly different between *B. rutabulum* and *H. cupressiforme* and between the regions, however, explaining only a low part of the variation (Table 2). NMDS ordinations separately performed for both moss species showed that this difference was more pronounced for the diazotrophic community composition of *B. rutabulum* than of *H. cupressiforme* (compare Figure 3A,B). Element concentrations, specifically, Mn and Fe contributed significantly to the variation in the diazotrophic community compositions of the active fraction, while other elements had no effect (Table 3). Diazotrophic communities of *B. rutabulum* in HEW were distinctly separated from AEW and SEW on the first NMDS axis (Figure 3A) whereas communities detected in *H. cupressiforme* clustered between HEW and SEW on the second NMDS axis (Figure 3B).

Drivers of BNF and diazotrophic richness in mosses

A positive relationship between BNF rate and diazotrophic richness of the active fraction ($p = 0.014$) was found in *B. rutabulum* (Figure 4A), whereas the correlation was not significant for *H. cupressiforme* (Figure 4B). Spearman correlations revealed positive relationships between BNF rates and concentration of Mn ($R = 0.41$, $p = 0.01$) and Mo ($R = 0.39$, $p = 0.02$) in *B. rutabulum* (Table 3). For the same moss species, diazotrophic richness was positively correlated with moss cover ($R = 0.28$, $p = 0.03$) and negatively correlated with Fe concentration ($R = -0.38$, $p = 0.02$). In contrast, Fe concentration in *H. cupressiforme* was positively correlated with both BNF ($R = 0.34$, $p = 0.03$) and diazotrophic richness ($R = 0.53$, $p < 0.001$). The concentration of C was negatively correlated with BNF ($R = -0.39$, $p = 0.001$), and the concentration of Mn was positively correlated with richness ($R = 0.42$, $p = 0.008$).

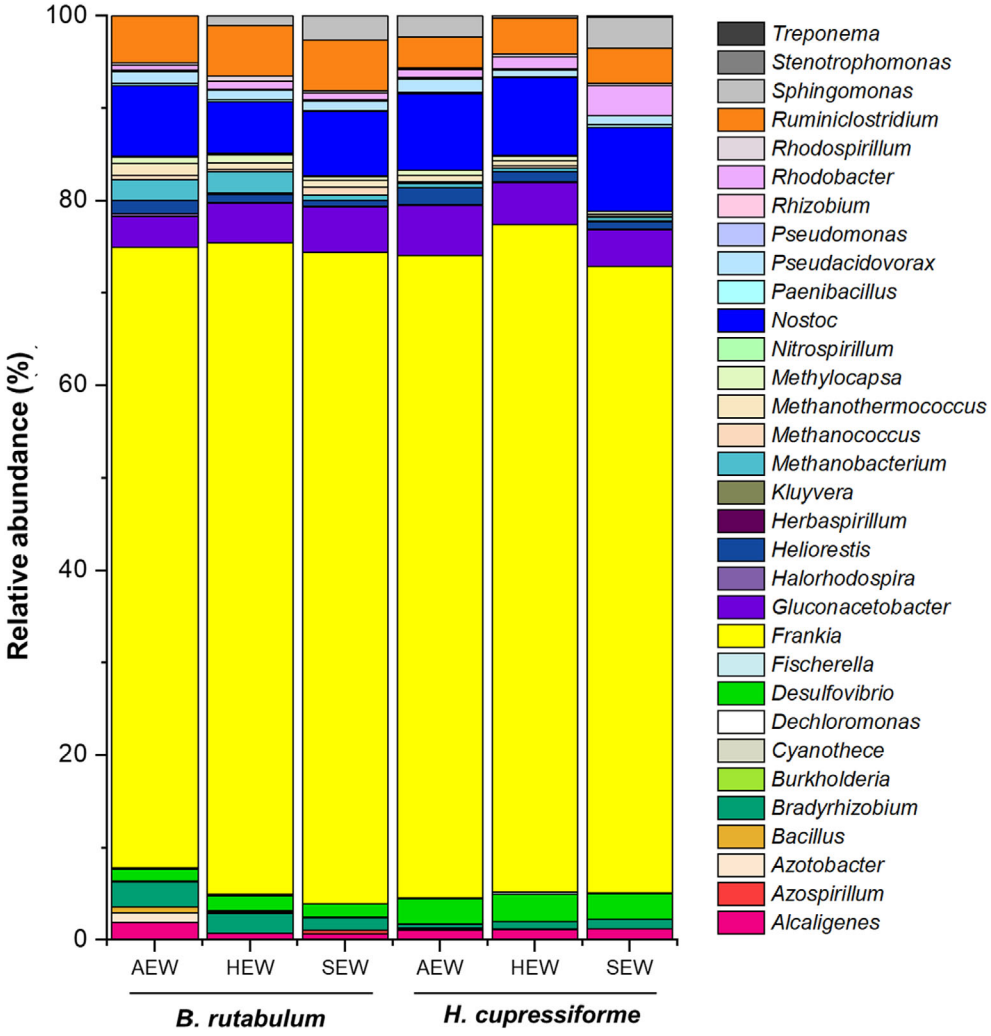

FIGURE 2 Diazotrophic community composition of the active fraction at genus level retrieved from moss species *Brachythecium rutabulum* ($n = 62$) and *Hypnum cupressiforme* ($n = 74$) in relative sequence read abundances and their distribution between each region (Schwäbische Alb [AEW], Hainich-Dün [HEW] and Schorfheide-Chorin [SEW]). The diazotrophic community composition of the total fraction at the genus level can be found in Figure S1.

DISCUSSION

High BNF rates in *B. rutabulum* and *H. cupressiforme*

The average BNF rate in *H. cupressiforme* was almost three times higher than in *B. rutabulum* across all regions and logs (Figure 1A). This finding is surprising because concentrations of N, P, S and Mo showed an opposite pattern for these moss species. Low levels of Mo and P can severely limit the biomass or BNF rate of free-living diazotrophs (Renaudin et al., 2022a; Wurzburger et al., 2012). The addition of Mo and P to two boreal moss species was shown to increase BNF immediately, although the fertilization effects strongly declined in the long term (Rousk et al., 2017). Concentrations of Mo (0.2–0.6 μg g^{-1}) in *B. rutabulum* and *H. cupressiforme* were remarkably higher than in the feather mosses *Pleurozium schreberi* and *Ptilium crista-castrensis* (<0.1 μg Mo g^{-1}) from Canadian boreal forests (Renaudin et al., 2022a). It suggests no severe Mo limitation of BNF at our study sites, even though a weak positive correlation was found for Mo and BNF rate in *B. rutabulum* (Table 3). Likewise, P concentration did not appear to have a limiting effect on BNF rates in both mosses, although their P concentrations were 50–70% smaller than in *P. schreberi* and *P. crista-castrensis* (Renaudin et al., 2022a). A weak limitation of BNF by P was reported for several *Sphagnum* species (van den Elzen et al., 2020), but the P levels were consistently lower as compared to our study. It should be noted that nutrient concentrations of mosses only provide limited information about the nutrient availability of associated diazotrophs. The nutrient requirements of mosses can differ, and the high BNF rates in *H. cupressiforme* could indicate a sufficient Mo

TABLE 2 Effect of moss species (*Brachythecium rutabulum* and *Hypnum cupressiforme*), tree species (*Fagus sylvatica*, *Pinus sylvestris*, *Quercus* spp.) and three regions (Schwäbische Alb [AEW], Hainich-Dün [HEW] and Schorfheide-Chorin [SEW]) on moss associated diazotrophic community composition of the active fraction based on three-way permutational multivariate ANOVA (PERMANOVA) and Bray–Curtis dissimilarities.

	df	Sum of squares	Pseudo *F*	R^2	*p*
Moss species	1	0.87	1.92	0.01	**0.001**
Tree species	2	1.58	1.75	0.02	**0.001**
Region	2	2.17	2.41	0.03	**0.001**
Moss species × tree species	2	0.92	1.02	0.01	0.277
Moss species × region	2	1.04	1.15	0.02	**0.015**
Tree species × region	4	2.09	1.16	0.03	**0.001**
Moss species × tree species × region	4	1.65	0.92	0.03	0.987
Residual	117	52.71		0.84	
Total	134	63.03		1.00	

Note: Significant effects ($p < 0.05$) are indicated in bold.
Abbreviation: df, degree of freedom.

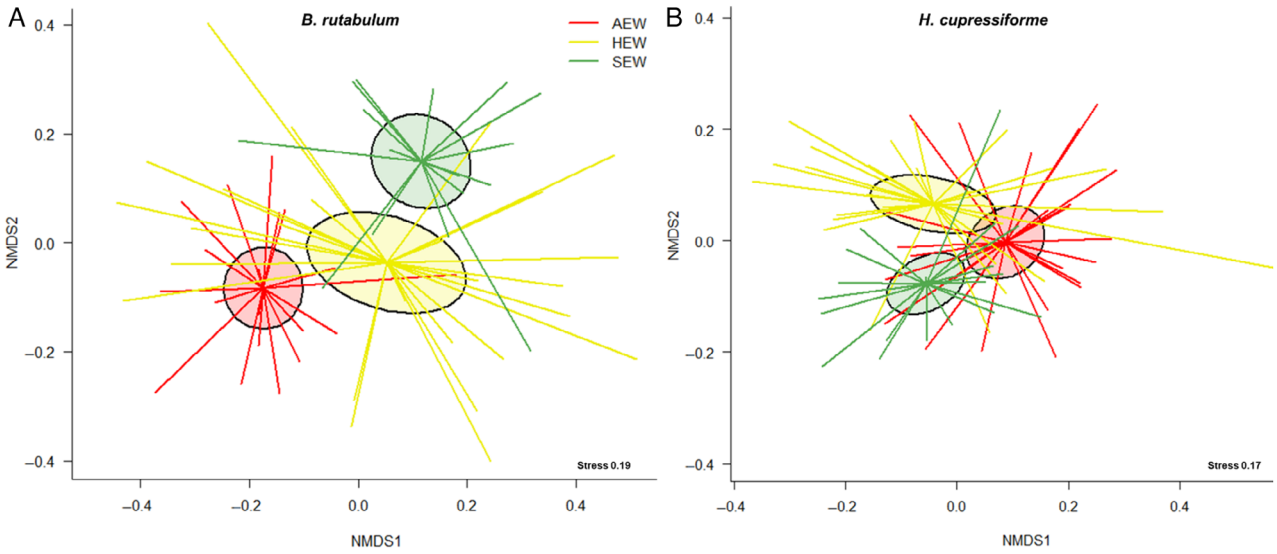

FIGURE 3 Non-metric multidimensional scaling (NMDS) ordination displaying diazotrophic community composition of the active fraction of *Brachythecium rutabulum* (A, $n = 62$) and *Hypnum cupressiforme* (B, $n = 74$) in relation to respective region (Schwäbische Alb [AEW], Hainich-Dün [HEW] and Schorfheide-Chorin [SEW]). The ellipses indicate standard error (SE) around the centroids in each regional group, and the lines connect each data point assigned by each group to its respective centroid.

and P supply of diazotrophs despite lower bulk concentrations in moss.

We expected relatively low BNF rates in both moss species compared to rates reported for pristine ecosystems because of ongoing high atmospheric N deposition across the study regions (Schwarz et al., 2014). Diazotrophs may switch their energy-intense BNF activity when sufficient ammonium or nitrate is available (Dynarski & Houlton, 2018; Norman & Friesen, 2017). Most N-fertilization experiments showed decreasing BNF rates in several moss species (Gundale et al., 2011; Kox et al., 2016; Wang et al., 2022), but some studies also reported no effect on BNF following moderate N additions to mosses (Rousk et al., 2014a; van den Elzen et al., 2018). A decrease in BNF due to the addition of reactive N was yet not reported for mosses from temperate forests, which may have higher N need or greater tolerance against high N availability than boreal mosses. Both mosses exhibited relatively high N contents (Table 1) compared to boreal mosses (e.g., (Gundale et al., 2011). Of the two studied mosses, their N contents support an earlier finding that *H. cupressiforme* requires less N to sustain its N demand than *B. rutabulum* (Hill et al., 2007). The $\delta^{15}N$ signatures also revealed different N acquisition strategies, where ^{15}N signatures ranging from −3‰ to −7‰ could be due to different BNF rates or moss-specific preferences for ammonium and nitrate (Weldon et al., 2022). However, based on mean BNF rates and assuming a growth period of 150 days per year, it

TABLE 3 Spearman correlations between BNF and diazotrophic richness of the active fraction, and moss-associated parameters measured in *Brachythecium rutabulum* and in *Hypnum cupressiforme* and goodness-of-fit statistics (R^2), based on the 'envfit' function, for moss coverage, nutrients, and isotopic signatures fitted to the two-dimensional non-metric multidimensional scaling (NMDS) ordination of the diazotrophic community structure of the active fraction.

	Spearman		Correlation			Goodness-of-fit statistics (R^2)		
	B. rutabulum		*H. Cupressiforme*			*B. rutabulum*	*H. Cupressiforme*	
	BNF	Richness	BNF	Richness	R^2	*P*	R^2	*P*
Moss cover (%)	0.18	**0.28**	−0.04	−0.02	0.05	0.46	0.17	**0.04**
C (%)	−0.20	−0.04	−**0.39**	−0.23	0.03	0.64	0.02	0.75
N (%)	0.06	0.09	−0.06	0.16	0.05	0.47	0.12	0.12
C:N ratio	−0.02	−0.07	0.03	−0.17	0.07	0.34	0.12	0.12
$\delta^{13}C$ (‰)	−0.24	−0.05	−0.05	−0.07	0.19	**0.03**	0.05	0.41
$\delta^{15}N$ (‰)	−0.17	−0.15	−0.16	−0.06	0.02	0.74	0.03	0.63
P (mg g^{-1})	−0.22	0.04	0.02	0.13	0.07	0.34	0.05	0.45
S (mg g^{-1})	−0.14	−0.07	0.14	0.31	0.07	0.32	0.14	0.08
Mn (mg g^{-1})	**0.41**	0.02	0.30	**0.42**	0.21	**0.02**	0.15	0.06
Fe (mg g^{-1})	−0.27	−**0.38**	**0.34**	**0.53**	0.17	**0.04**	0.22	**0.01**
Mo (µg g^{-1})	**0.39**	−0.20	0.30	0.02	0.11	0.15	0.11	0.15

Note: Significant effects ($p < 0.05$) are indicated in bold.

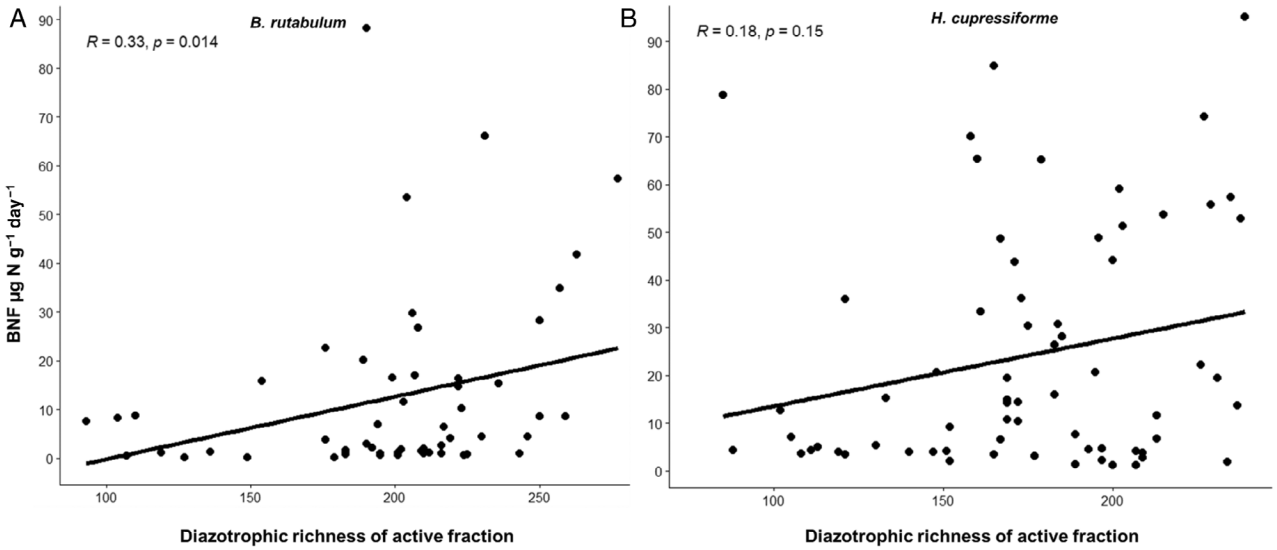

FIGURE 4 Spearman correlations between Biological N fixation (BNF) and diazotrophic richness of the active fraction in mosses *Brachythecium rutabulum* (A, $n = 62$) and *Hypnum cupressiforme* (B, $n = 74$) that were collected across three regions (Schwäbische Alb [AEW], Hainich-Dün [HEW], and Schorfheide-Chorin [SEW]). Black dots indicate observations, and the solid black line indicates the best-fitting linear regression.

would take at least 3 years (*H. cupressiforme*) or 6 years (*B. rutabulum*) to achieve the N content per unit mass of moss solely from BNF. Since our measured BNF rates are already at the upper end of published values (see below), atmospheric deposition is the dominating N source of mosses growing on deadwood in our study. Nevertheless, the ability to fix substantial amounts of N provides an advantage over other plants, especially during phases of intensive growth. Different from vascular plants, several bryophyte species reach maximum growth in autumn and winter, or spring like *B. rutabulum* (Rincon & Grime, 1989). Since we sampled in September, the lower BNF rates in *B. rutabulum* could be due to less moss growth, which would be verified by measurements at other times of the year.

Regional differences in BNF rates, being highest in SEW and lowest in AEW, were not related to nutrient concentrations in mosses or N deposition. Higher

amounts of precipitation and higher water contents in mosses should have had a positive effect on BNF rates in AEW as hypothesized for observed differences in arctic environments (Gavazov et al., 2010). The water content in mosses controls indirectly the activity of the oxygen-sensitive nitrogenase due to low oxygen diffusion in water. Moreover, high water contents promote the growth of mosses and associated microorganisms. Different sampling times in the three regions and water saturation of mosses prior to incubation could have led to regional differences in BNF rates. We also found no effect of the moss of the three log tree species on BNF rates, suggesting that logs of deciduous and coniferous tree species create similar conditions for moss growth and activity of diazotrophic bacteria. According to Herrmann and Bauhus (2018), logs may serve as a nutrient sink for a long time, before nutrients are significantly released with strongly advanced deadwood decay. The majority of the sampled logs were effectively at a medium decay stage (Edelmann et al., 2023). Therefore, nutrient uptake by deadwood-inhabiting vegetation out of the wood, particularly in non-vascular plants, may be of lower relevance. Furthermore, most deadwood-inhabiting bryophytes, including the two selected moss species, are not specifically bound to the wood or tree species, respectively (Dierssen, 2001). Therefore, the microbial communities associated with both mosses and especially the diazotrophs, potentially contribute to the available nutrients for the mosses. Mean BNF rates of 13 μg N g^{-1} d^{-1} in *B. rutabulum* and 37 μg N g^{-1} d^{-1} in *H. cupressiforme* were in the upper range compared to literature values for mosses growing in forest ecosystems. Most studies reported BNF rates <10 μg N g^{-1} d^{-1} (Gundale et al., 2011; Rosen & Lindberg, 1980; Stuart et al., 2021; Wang et al., 2019), but also BNF rates of up to 120 μg N g^{-1} d^{-1} were found in forest mosses (Jean et al., 2018). However, most of the studies focused on boreal (Lindo & Whiteley, 2011; Stuart et al., 2021) or subtropical-tropical ecosystems (Fan et al., 2022; Markham & Fernández Otárola, 2021; Matzek & Vitousek, 2003; Vitousek, 1994) while BNF in mosses of temperate forests has yet not been studied to our best knowledge. Our BNF rates from a single sampling could differ from in-situ rates considering favourable incubation conditions. We also cannot exclude the possibility that the composition of active diazotrophs changed due to pre-incubation and whether this resulted in altered BNF rates. Overestimation of BNF rates by the application of contaminated $^{15}N_2$ gas, as reported by (Dabundo et al., 2014), was avoided by the removal of reactive NO_x and NH_3 from the original $^{15}N_2$ gas. Taken together, diazotrophic bacteria can effectively contribute to the N nutrition of *B. rutabulum* and *H. cupressiforme* despite or because of high N deposition in Central Europe.

Frankia dominates the diazotrophic community

The mosses in our study were inhabited by a diverse pool of potential N_2-fixing microorganisms. Members of the genus *Frankia* generally dominated in both moss species sampled. Our results revealed that *Frankia* (*Actinobacteria*) contributed a significant portion (up to 70% sequence read abundances) of the *nifH* gene pool of moss-associated diazotrophs in the active fraction (Figure 2), whereas only 8% belonged to *Nostoc* (Cyanobacteria). It is the first time that *Frankia* was detected as dominating free-living diazotrophic bacteria in two moss species. *Frankia* is often present in soil (Santi et al., 2013) and forms symbiosis in root nodules of a few plant species (Ben Tekaya et al., 2018). According to our study, there may be various types of metabolically active diazotrophs, beyond the phylum Cyanobacteria that can perform BNF in mosses (Figure 2). This contradicts the common assumption that Cyanobacteria are the primary N_2-fixers in mosses (Holland-Moritz et al., 2021; Ininbergs et al., 2011; Leppänen et al., 2013; Renaudin et al., 2022b; Warshan et al., 2016). It is important to note that although the abundance of N_2-fixers and BNF rates is not necessarily linked, even a low abundance of N_2-fixers can contribute significantly to high BNF rates (Warshan et al., 2016). Our finding suggests that non-cyanobacterial diazotrophs can dominate in temperate mosses, which is in line with previous studies on boreal mosses (Holland-Moritz et al., 2018). They showed that *Cyanobacteria* accounted for <10% of the bacterial communities while *Proteobacteria* followed by *Acidobacteria*, and *Actinobacteria* were the most abundant bacterial phyla in boreal moss species. Our findings are in contrast with recent studies that have shown sequence read dominance of the members of the phyla *Cyanobacteria* in the diazotrophic bacteriome of moss in Canadian boreal forests (Renaudin et al., 2022b). These disparities in diazotrophic diversity are most likely attributed to the use of different primer pairs and the primer degeneracy of the PolF/PolR primers, as previously addressed (Gaby & Buckley, 2012). Whether the different diazotrophic communities in boreal and temperate mosses are associated with climatic factors or whether this is caused by environmental changes such as high N deposition is not clear.

Kox and colleagues found that N deposition had a detrimental effect on the activity of diazotrophic microorganisms, that is, mainly *Cyanobacteria* (Kox et al., 2016). In addition, there is a possibility of variation in the response of moss-associated cyanobacterial species to N deposition in temperate ecosystems (Calabria et al., 2020) as the community composition of diazotrophs underwent a shift with increasing N deposition. Elevated levels of atmospheric N deposition,

estimated to be around 15–20 kg N ha^{-1} $year^{-1}$ (Schwarz et al., 2014) in our study regions, could have triggered a shift in the dominant group of diazotrophs from *Cyanobacteria* to *Actinobacteria* (*Frankia*). However, further systematic studies on diazotrophic communities are needed, especially with regard to their response to N deposition, to gain a general understanding of diazotrophs in temperate mosses.

The ability of *Frankia* to thrive in harsh environments and form symbiotic relationships with simple plants like mosses has allowed it to dominate in several ecosystems (Pawlowski & Bergman, 2007). *Frankia* is known to be effective in fixing atmospheric N_2 and relies on substrates from mosses for its growth and survival, thus forming a mutually beneficial symbiotic relationship with these plants. Mosses produce a variety of organic compounds such as simple sugars and amino acids (Carrell et al., 2022; Santi et al., 2013) that can be metabolized by *Frankia*. However, there is not enough evidence available about the mutual relationship between moss and *Frankia*. While there could be direct competition among different clades of diazotrophs, there is also the possibility that they occupy distinct ecological niches within the moss host. Accordingly, *Frankia* can compete with other diazotrophs and occupy a wide range of niches.

The richness and structure of the metabolically active diazotrophic communities differed significantly between moss species across different geographical regions investigated (Figures 1B and 3 and Tables 2 and S3) while both moss species were colonized by similar dominant diazotrophic genera. This is in agreement with the results reported for the forest moss *P. schreberi* (Ininbergs et al., 2011) and with a recent study including 34 moss species from Alaska (Stuart et al., 2021). ASV richness was higher in *B. rutabulum* than in *H. cupressiforme*. Indeed, the structure of diazotrophic communities is known to vary among moss species (Holland-Moritz et al., 2018) which in turn may affect BNF rates. Moss species vary with different morphological and physiological features such as specific surface area, the density of shoots in the carpet, nutrient concentrations and water retention capacity (Renaudin et al., 2022a) that create specific microhabitats conditions, potentially affecting bacteria including diazotrophic community composition and abundance. Moreover, it has been reported that several moss traits, especially moss hydration rate control cyanobacterial colonization on moss (Liu & Rousk, 2022). Interestingly, the differences between geographical regions were more pronounced in the communities of *B. rutabulum* than in the corresponding communities of *H. cupressiforme* (Figures 1B and 3A). This suggests that *B. rutabulum* may serve as a favourable environment, providing a suitable habitat for a greater number of well adapted diazotrophs. The increased coverage of mosses on logs can be attributed to the level of moisture in the log substrate. When the humidity of logs is high, it creates a favourable environment for the colonization of mosses (Błońska et al., 2017). This, in turn, increases the species richness. Our study found similar results, as we observed a significant positive correlation between the coverage of mosses and the richness and community composition of metabolically active diazotrophs (Table 3). We have also found a significant effect of log species on the community composition of metabolically active diazotrophs, which has been considered an important habitat factor explaining the variation of microbial community composition in bryophytes on fallen logs (Jansová & Soldan, 2006).

Relationship between moss nutrients, diazotrophic richness and community composition

Nutrient concentrations (P, S, Mn, and Fe) in *B. rutabulum* and in *H. cupressiforme* were similar to other moss species (Malmer, 1988; Culicov & Yurukova, 2006; Parzych, 2014), whereas Mo was slightly higher in our samples compared to the literature (Chaligava et al., 2021; Renaudin et al., 2022a). Our results indicate that micro-nutrients such as Mn and Fe have a significant effect on the moss diazotrophic richness and community composition of the active fraction (Table 3). We assume that diazotrophic richness peaks when Mn and Fe in mosses occur in sufficient contents for diazotrophic proliferation. Moreover, positive effects of Mo addition have been reported previously on diazotrophic richness in soil (Ma et al., 2019) and cyanobacterial colonization in mosses (Rousk et al., 2017). Although Mo was not correlated with diazotrophic richness, a positive relationship was found with the BNF rate in *B. rutabulum*.

Relationship between richness of active diazotrophic and BNF

Diazotrophic richness was found to be higher in *B. rutabulum* compared to *H. cupressiforme* and was positively correlated with BNF (Figure 4A). Our finding is in contrast with a previous study of *Sphagnum* mosses in Finland (Leppänen et al., 2015), but is in agreement with the results of several other studies (Renaudin et al., 2022a; Renaudin et al., 2020; Rousk et al., 2017; Arróniz-Crespo et al., 2014). The latter studies focused mainly on *Cyanobacteria* in boreal mosses, suggesting that BNF and active diazotrophs are linked. A high richness of active diazotrophs may allow mosses to maintain their N demands under changing environmental conditions. However, our experimental procedures for BNF rate measurement involved a longer pre-incubation period compared to

the BrdU approach, and therefore the metabolically active fraction during BNF rate measurement may be altered. The diazotrophic richness can thus fulfil an important function for the N supply of mosses or reflects different N demands of moss species (Leppänen et al., 2015; Renaudin et al., 2022a). Although high N availability following N addition may lower the richness of diazotrophs (Zackrisson et al., 2004; Rousk & Michelsen, 2016; Li et al., 2018), our study shows that high N deposition does not decrease diazotrophic richness.

CONCLUSIONS

The active diazotrophic community associated with two common moss species in temperate forests differed from those in boreal forests and peatlands. Members of the genus *Frankia* contribute effectively to N_2 fixation in *B. rutabulum* and *H. cupressiforme* while *Cyanobacteria* are less important for BNF in these mosses. The large diversity of diazotrophic bacteria in mosses suggests that other members of the community could become more active under different environmental conditions or at different times of the year. Relatively high BNF rates indicate no negative impact of high N deposition on diazotrophs in both mosses. However, *in situ* investigations with other moss species at different times of the year are required to understand the function of BNF and diazotrophs for mosses and N cycling in temperate forest ecosystems. Nutrient contents like phosphorous, sulfur, manganese, iron, or molybdenum in *B. rutabulum* and *H. cupressiforme* appear to have little or no consistent relevance to BNF and the diazotrophic community. The high variability in BNF rates could be related to the photosynthesis of mosses since *Frankia* relies on substrate supply and may also depend on moss metabolites. Overall, this study suggests a high potential of BNF in mosses in temperate forests even with strongly increased N deposition.

AUTHOR CONTRIBUTIONS

Matthias Noll: Conceptualization; investigation; funding acquisition; writing – original draft; methodology; validation; visualization; formal analysis; project administration; supervision; resources; data curation. **Christina Groß:** Methodology; validation; investigation; writing – original draft; formal analysis; data curation. **Shakhawat Hossen:** Investigation; writing – original draft; methodology; validation; bioinformatics; formalanalysis; visualization; data curation. **Sebastian Dittrich:** Investigation; methodology; validation; visualization; writing – review and editing. **Klaus-Holger Knorr:** Methodology; validation; writing – review and editing. **Werner Borken:** Project administration; validation; investigation; conceptualization; funding acquisition; writing – review and editing; supervision; resources.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data will become publicly available at https://www.bexis.uni-jena.de/ddm/publicsearch under IDs 31330, 31419, 31420 and 31422.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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