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Nitrogen fertilization does not affect non-symbiotic N_2 fixation in northern forest soils despite its negative impacts on diazotroph communities

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ABSTRACT

Tree productivity in northern regions is limited by low soil nitrogen (N) availability, and biological N_2 fixation is a crucial N input to these forests. To enhance forest productivity, N fertilization has been proposed as a strategy although it may negatively affect N_2 fixation and the abundance of diazotrophic microorganisms. In contrast to N_2 fixation by the cyanobacteria-moss associations, there is limited understanding of non-symbiotic N_2 fixation in northern forest soils and the free-living diazotrophs involved. To assess the impact of N fertilization on non-symbiotic N_2 fixation and the diazotrophic community in soil, we sampled 15 forest sites along a latitudinal gradient in Sweden that are part of a fertilization experiment. Fertilization started between 41 and 55 years ago, using ammonium nitrate at 100–150 kg N ha^{-1} every 5th year for the first 25 years and thereafter every 7th year. We measured non-symbiotic N_2 fixation in the soil organic layer in laboratory incubations and analyzed the diazotrophic community. Both the abundance and diversity of diazotrophs decreased in response to N fertilization. However, this decline did not translate into significant changes in non-symbiotic N_2 fixation rates (22.4 $\pm$ 4.2 and 22.5 $\pm$ 5.7 ng N g^{-1} dry weight soil h^{-1} in the control and N treatments, respectively). Yet, N_2 fixation per area increased by 24 % in fertilized plots because of the increase in the organic layer stock caused by higher primary production. Additionally, we observed an influence of fertilization and mean annual temperature on diazotroph community composition across the gradient. Our findings indicate that N fertilization in northern forests strongly affects diazotrophs, the organic layer stock, and N_2 fixation. Although N fertilization positively affected the N_2 fixation rate per area in this experiment, its negative effect on diazotroph diversity might reduce N_2 fixation in the long run.

1. Introduction

Northern coniferous forests, located predominantly in the boreal zone, contribute considerably to carbon (C) storage while also providing wood, paper, and pulp (Gauthier et al., 2015). Primary production in these forests is considered to be nitrogen (N) limited, according to the positive response of tree growth to N addition (Tamm et al., 1999; Bergh et al., 2014; Sponseller et al., 2016; Högberg et al., 2017, 2021). Atmospheric N deposition is much smaller than the estimated N demand of growing boreal forest stands (15–50 kg N ha^{-1} yr^{-1}) (Sponseller et al., 2016; Högberg et al., 2017) as it ranges from 10 kg N ha^{-1} yr^{-1} at the southern limit of the boreal region in Sweden and Finland to $<$ 1 kg N ha^{-1} yr^{-1} in the northern regions (Korhonen et al., 2013; Ferm et al., 2019). Thus, N inputs via biological N_2 fixation are crucial for supplying N in these forests (Rousk et al., 2013; Sponseller et al., 2016; Högberg et al., 2017; Vázquez and Spohn, 2025). DeLuca et al. (2002) reported that N_2 fixation performed by cyanobacteria in close association with a common feather moss in boreal forest amounts to 1.5–2.0 kg N ha^{-1} yr^{-1} and after this landmark paper, these cyanobacterial-moss associations have been evaluated in many studies (e.g. Gundale et al., 2012; Rousk et al., 2013). Less attention has been paid to N_2 fixation by free-living diazotrophic microorganisms (non-symbiotic N_2 fixation hereafter) in soil, despite their potentially important contribution to N input in northern forests. The few existing studies indicate that non-symbiotic N_2

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fixation in boreal forest soils ranges between 0.4 and 1.4 kg N ha^{-1} during the growing season (May to October) (Nohrstedt, 1985; Cleveland et al., 1999) and could reach up to 10.0 kg N ha^{-1} yr^{-1} in the warmest areas of Sweden due to the positive effect of temperature on the soil organic layer stock (Vázquez and Spohn, 2025). However, there is limited understanding of the process controls of non-symbiotic N_2 fixation in northern forest soils and the microbial taxa involved.

Due to N limitations in northern forests, managed stands could be fertilized to increase biomass production of trees (Lindkvist et al., 2011; Bergh et al., 2014; Valinger et al., 2018; Boeraeve et al., 2025). Currently, this is restricted to limit the risk of N leaching which was aggravated in the 1990s due to increased atmospheric N deposition (Lundin and Nilsson, 2021). However, with the recent decrease in atmospheric N deposition and high demand of forest products, in the last years there is an increasing interest in N fertilization within the forest industry (Lundin and Nilsson, 2021; Boeraeve et al., 2025). Nevertheless, N fertilization of northern forest could impact the non-symbiotic N_2 fixation in soils because it typically decreases N_2 fixation and the abundance of diazotrophs (Reed et al., 2011; Wang et al., 2017; Fan et al., 2019; Zheng et al., 2019; Schleuss et al., 2020, 2021; Frey et al., 2023). Previous studies argued that N_2 fixation is a metabolically expensive process, and hence facultative N_2 fixers would downregulate their N_2 fixation in response to an increase in N availability, while obligate N_2 fixers might be replaced by non-N_2 fixers that are more competitive under high N availability (Fan et al., 2019; Schleuss et al., 2020, 2021). Fertilization typically also decreases the diazotroph diversity and changes the community composition in agricultural, forest and grassland soils (e.g. (Wang et al., 2018; Fan et al., 2019; Dietrich et al., 2024). In addition, continuous N fertilization in the form of urea or ammonium often leads to soil acidification, which has been shown to negatively impact the diazotrophic communities (Limmer and Drake, 1996; Fan et al., 2018; Yang et al., 2022). Therefore, N fertilization could decrease non-symbiotic N_2 fixation in northern forest, potentially making the fertilized stands more dependent on N fertilization in the long term. On the other hand, a positive effect of N addition on the organic layer stocks has been described (Gundale et al., 2014; Maaroufi et al., 2015; Sponseller et al., 2016; Jörgensen et al., 2021), which may counteract any negative effect of fertilization on N_2 fixation since N_2 fixation in northern forests largely depends on the stock of the soil organic layer (Vázquez and Spohn, 2025). Whether the expected changes in diazotroph size, diversity and community composition due to N fertilization would result in reduced non-symbiotic N_2 fixation in response to addition of reactive N in northern forests is therefore difficult to predict.

Mean annual temperature (MAT) plays a key role in northern forests, influencing primary production, organic layer stocks, and, consequently, non-symbiotic N_2 fixation per unit area (Jörgensen et al., 2021; Spohn and Stendahl, 2022; Vázquez and Spohn, 2025). In addition, previous studies in other regions have shown that MAT can shape diazotroph communities across temperature gradients (Zhao et al., 2020), thus relevant changes in the size and composition of diazotroph communities due to MAT can be expected in northern forest. A recent study analyzing data from commercial forestry across Sweden has shown that temperature also influence the response to fertilization of tree production (Boeraeve et al., 2025). However, it remains unclear if MAT also influences the response to N fertilization of non-symbiotic N_2 fixation and diazotroph communities in northern forests.

Here, we determined the long-term impact of N fertilization on the non-symbiotic N_2 fixation rates and the size, diversity, and composition of the diazotroph community using *nifH* as our marker gene, in soils of northern Scots Pine (*Pinus sylvestris*) forests located along a MAT gradient in Sweden. Our hypotheses are:

i) N fertilization decreases the size and diversity of the diazotroph community and modifies the composition of the diazotroph communities in northern forest soils
ii) N fertilization decreases the non-symbiotic N_2 fixation.
iii) Site MAT affects the composition of diazotroph communities and non-symbiotic N_2 fixation (expressed per unit area)

2. Material and methods

2.1. Study sites

We selected 15 forest stands dominated by *Pinus sylvestris* L. which are part of a series of long-term fertilization experiments distributed along a 1300 km latitudinal gradient in Sweden (56–67°N). The experiments were established between 1967 and 1981 to quantify the effect of thinning and fertilization on forest biomass production (Bergh et al., 2014, Fig. 1). The sites span a gradient of a mean annual temperature (MAT) between −0.05 and 7.23 °C, and a mean annual precipitation (MAP) between 588 and 801 mm for the period 1972–2022 (Swedish Meteorological and Hydrological Institute, https://www.smhi.se/data, last access: May 2023) (Table 1). At the time the experiments were set up, the forest stands were at the canopy closure stage, and the first operational thinning was done according to standard silvicultural practices in Sweden when the trees were between 12 and 18 m high and between 32 and 53 years old.

For the present study, we selected two treatments at each of the 15 sites: “control” (no fertilizer addition) and “nitrogen” (N fertilizer addition), making a total of 30 sampled plots. The size of each plot was approximately 0.1 ha (25 × 40 m), and the plots were surrounded by a buffer zone of at least 10 m. The first N fertilization was done in the N treatment using ammonium nitrate at a rate of 100–150 kg N ha^{-1} every 5th year during the first 25 years of the experiment and thereafter every 7th year. At the sampling in 2022, N addition started between 41 and 55

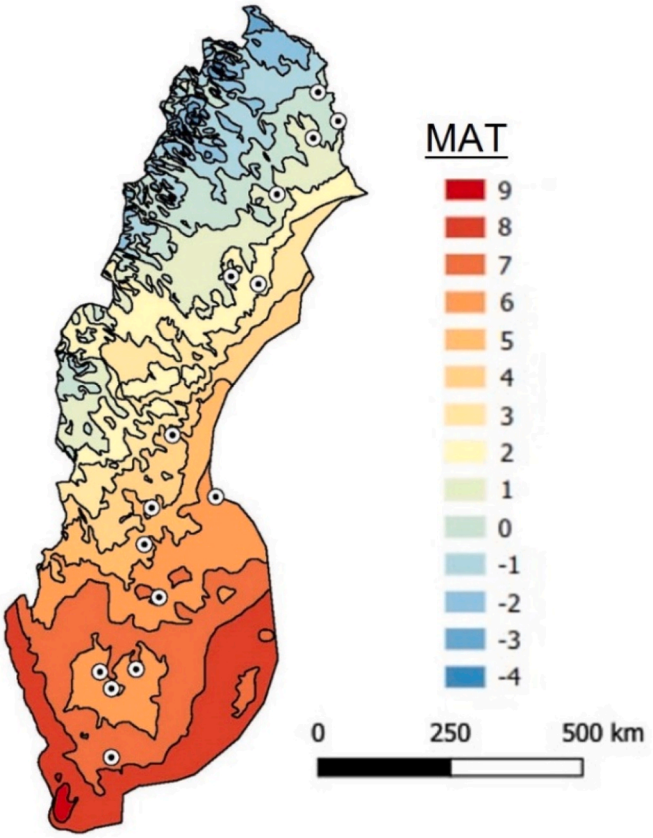

Fig. 1. Map of Sweden showing mean annual temperature (MAT) and the locations of the 15 *Pinus sylvestris* forest sites included in the study (indicated by dots).

Table 1
Characteristics of the 15 *Pinus sylvestris* forests distributed across Sweden.

Site ID	Experiment establishment	Latitude	Longitude	MAT	MAP	Last N fertilization	N fertilization dose	Total N load
	(yr)	(°N)	(°E)	(°C)	(mm)	(yr)	(kg N ha^{-1})	(kg N ha^{-1})
994	1978	67.48	23.1	−0.05	611	2016	100	700
951	1975	66.95	23.8	0.84	588	2020	100	800
952	1975	66.72	22.64	1.11	620	2020	100	800
1000	1981	65.84	20.87	1.12	648	2019	100	800
991	1976	64.35	19.82	1.88	681	2021	100	800
946	1976	64.51	18.77	1.96	630	2021	100	800
936	1974	61.86	16.33	3.93	653	2019	100	800
902	1969	60.01	15.3	4.94	807	2021	150	1350
918	1970	60.63	15.56	5.30	665	2015	150	1200
923	1973	57.84	13.87	6.02	821	2018	150	1200
787	1973	60.79	17.79	6.07	612	2018	150	1200
922	1967	57.89	15.02	6.20	699	2019	150	1350
931	1973	57.56	14.24	6.29	797	2018	150	1200
933	1973	59.11	15.78	6.53	647	2018	150	1200
940	1969	56.39	14.26	7.23	801	2021	150	1350

MAT, Mean annual temperature (period 1972–2022); MAP, Mean annual precipitation (period 1972–2022); Last N fertilization, year of the last fertilization event; N fertilization dose, dose of nitrogen applied at each fertilization event; total N load, total amount of nitrogen applied since the beginning of the experiment.

years ago and the stands were between 76 and 102 years old. Stand age or experiment duration do not correlate with the N_2 fixation rates and gene copies abundances studied, indicating that these factors did not confound the results. The N dose and total N load during the experiment is detailed for each site in Table 1, including additional details about the site characteristics. During this period, the experimental sites were visited periodically, and the stand growth and cumulative biomass volume evaluated as described in Bergh et al. (2014) using allometric functions based on plot average tree stem diameter and height.

2.2. Soil sampling, processing, and physical and chemical analyses

Soil sampling was performed between May and July 2022. The sampling campaign started in the southernmost and finished in the northernmost site, aiming to collect all samples at a similar vegetative stage (late spring). We sampled the soil organic layer by pooling 16 soil cores (3.5 cm diameter) collected every 2 m along two transects of 16 m within each plot. Living plants and litter (Oi horizon) were removed before sampling. Before pooling the soil cores sampled within each plot, the underlying mineral horizon and larger roots (>2 mm) were discarded and the thickness of the organic horizon was measured (Oe and Oa). The pooled samples were immediately stored in Styrofoam boxes and transported to the laboratory as fast as possible. All the stands were established on soils classified as Podzol with an organic horizon of a thickness between 2.8 and 7.6 cm and a pH ranging between 3.45 and 4.32 (Table S1).

In the laboratory, the samples were homogenized, and the gravimetric water content was determined to calculate the dry weight of the organic layer per ha based on the area of the 16 cores and a subsample was stored at −20 °C for microbial analysis and another was air-dried for chemical analysis. Soil water holding capacity (WHC) of each soil sample was estimated as the gravimetric water content upon 24 h of free drainage of saturated samples in the laboratory. Soil pH was measured in H_2O using a subsample of air-dried soil (1:5 w:v).

2.3. Non-symbiotic N_2 fixation

The non-symbiotic N_2 fixation rates in soils by free-living microorganisms was determined after incubating soil samples with 99.8 atom% $^{15}N_2$ according to the method described by Zechmeister-Boltenstern (1996). Briefly, four 30 mL serum flasks were filled with fresh soil (1.5 g dry-mass equivalent) adjusted to 80 % of WFC as four analytical replicates of each soil sample. The serum flasks were closed with a rubber stopper and pre-incubated in the dark at 12 °C for 24 h. Subsequently, each flask was closed with an aluminium crimp seal with PTFE/butyl septa (Product 854979, Supelco Inc., Bellefonte, PA, USA) and flushed with argon, evacuated, and finally filled with 25 mL of 99.8 atom% $^{15}N_2$ (Sigma Aldrich co., St. Louis, MO, USA) and 3.6 mL O_2 using a syringe. After filling with the $^{15}N_2$ enriched atmosphere, all flasks were incubated in the dark for 48 h at 12 °C (which equals approximately the air temperature at the sites in the month of sampling). Afterwards, the flasks were opened, ventilated, and the soils were freeze dried. The Certificate of Analysis of the $^{15}N_2$ gas lot that we used (MBBD1096 produced by Sigma Aldrich in 2021) shows that the N_2 gas is 99.9 % pure and contains <15 ppm N_2O (see the Certificate of Analysis of the gas lot in the Supplementary Material). This is a lower level of N_2O contamination than the level certificated for the low contamination gas lot of Sigma (MBBB0968V, produced by Sigma Aldrich in 2014) analyzed by Dabundo et al. (2014).

Soil samples exposed to the $^{15}N_2$ enriched atmosphere as well as non-exposed samples were milled and analyzed for ^{15}N using a continuous-flow isotope ratio mass Spectrometry of Flash EA 2000 via ConFlo IV open split interface to a Delta V isotope ratio mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) at the Stable Isotope Laboratory at The Swedish University of Agricultural Sciences. The non-symbiotic N_2 fixation was calculated using the isotope mixing model proposed by Zechmeister-Boltenstern (1996) as follows:

$$^{15}N_2 fixation\ rate\ \left(ng\ N\ g^{-1}\ DW\ h^{-1}\right) = TN\ \left(mg\ N\ g^{-1}\ \mathrm{DW}\right)\ x\ \frac{\left(\,^{15}Nlabeled\ (atom\%) - \,^{15}Ncontrol\ (atom\%)\right)}{100\ x\ t\ (h)}\ x\ 10^6$$

where TN is the total soil N content, $^{15}N_{labeled}$ is the percentage of ^{15}N atoms in the labeled samples, $^{15}N_{control}$ is the percentage of ^{15}N atoms in the control samples and t is the incubation time in hours (48 h). All the

non-symbiotic N_2 measurements including the 24 h of pre-incubation and 48 h of $^{15}N_2$ atmosphere incubation were performed within a week after the sampling to limit the alteration of the soil microbial communities due to storage. The C and N contents of the non-exposed samples, measured using the mass spectrometer described above, were considered the total C and N contents of the soil.

2.4. DNA extraction and quantitative PCR of nifH and 16S rRNA genes

DNA was extracted from 0.4 g of thawed soil using the NucleoSpin kit (Macherey-Nagel GmbH & Co, Düren, Germany) according to the manufacturer's protocol (using SL2 lysis buffer without the Enhancer solution). The quality of the extracted DNA was checked by gel electrophoresis, and the DNA concentration was measured using the Qubit fluorometer (Thermo Fisher Scientific, MA, USA). Quantitative PCR was used to estimate genetic potential for N_2 fixation by quantifying the *nifH* gene, a commonly used marker for diazotrophs as this gene encodes one of the subunits of the nitrogenase (Zehr et al., 2003; Angel et al., 2018). For *nifH*, the primers 19F (5′ GCIWTYTAYGGIAARGGIGG 3'; Ueda et al. (1995)) and R6 (5′ GCCATCATYTCICCIGA 3'; Marusina et al. (2001)) were used. In addition, the abundance of the 16S rRNA gene was quantified as a proxy for the size of the total bacterial community using the primers 515F (5′ GTGYCAGCMGCCGCGGTAA 3'; Parada et al. (2016)) and 926R (5′ CCGYCAATTYMTTTRAGTTT 3' (Quince et al., 2011);). Each 15 μL reaction contained 3.6 ng of extracted DNA, 1x iQ SYBR Green Supermix (BioRad, Hercules, CA, USA), 0.7 mg mL^{-1} Bovine Serum Albumine (New England Biolabs, Ipswich MA, USA), 2 μM (for *nifH*) or 0.5 μM (for the 16S rRNA gene) primers in a total volume of 15 μL. Two separate qPCR runs were performed for each sample and gene with the annealing temperatures at 52 °C and 50 °C for *nifH* and the 16S rRNA gene, respectively. Standard curves were obtained using serial dilutions of linearized plasmids containing the respective gene fragments. The qPCR efficiencies for the 16S rRNA and *nifH* genes were 90 % and 84 %, respectively. Abundances of *nifH* genes in the samples were corrected according to the proportions of amplicon sequence variants (ASVs) of *nifH* (see below). Prior to gene quantifications, potential PCR inhibition was tested for all samples by adding a known amount of the pCR4 TOPO plasmid (Thermo Fisher Scientific) to reactions with 3.6 ng soil DNA or water, followed by quantification the plasmid using plasmid specific M13 primers. The threshold values for plasmid quantification in controls with water were not significantly different from those with DNA extracts, indicating no inhibition.

2.5. nifH gene sequencing and bioinformatic analyses

To determine the diversity and composition of diazotrophic communities, *nifH* was sequenced using a two-step PCR protocol. The first step was performed in duplicate 25 μL reactions containing 3.6 ng extracted DNA, 2 μM of the abovementioned *nifH* gene-specific primers supplemented with Nextera adaptor sequences (Illumina Inc, San Diego, CA, USA), 0.7 mg mL^{-1} Bovine Serum Albumine and 1x DreamTaq Green PCR Mastermix (Thermo Fisher Scientific), followed by 28 cycles of 98 °C for 30 s, 52 °C for 30 s and 72 °C for 30 s, and a final extension step of 10 min at 72 °C. Duplicate amplicons were then pooled and purified using SeraMag magnetic beads (GE Healthcare, Chicago, IL, USA). The second amplification step was performed during eight cycles in duplicate under the same conditions as above using 10 % of the purified product from the first step and 0.2 μM primers with Nextera adapter- and barcoding regions for dual labelling of the fragments. Duplicates were then pooled and purified as above. Purified samples were quantified with Qubit fluorometer, pooled equimolarly, and sequenced by SciLifeLab in Uppsala on an Illumina MiSeq instrument using the 2x250 bp chemistry. The sequences are deposited in the European Nucleotide Archive (ENA), project accession number PRJEB95886, with sample accession numbers ERS25453047 to ERS25453076.

Sequence analyses were performed using the R software (v. 3.6.4 (R Core Team, 2019),). The *nifH* sequences were processed using the DADA2 package generating ASVs (v. 1.22.0; (Callahan et al., 2016). Since the *nifH* primer pairs co-amplify *nifH* homologs (Angel et al., 2018), the following procedure was used to manually exclude ASVs of *nifH*. First, the nucleotide sequences of ASVs were aligned against the *nifH* reference sequences from the Zehr Lab (v. June 2017 (Heller et al., 2014);) using HMM align (Eddy, 2011). The aligned amino acid sequences were inspected in ARB (v. 7.0 (Ludwig et al., 2004); followed by the construction of a phylogenetic tree using FastTree was visually checked in ARB to only retain the true *nifH* ASVs (10,025 out of initially 10,061 ASVs) for further analyses. The ASV abundance table was rarefied at 51,679 sequences per sample resulting in 9776 ASVs containing in total of 1,550,370 sequences across all samples.

To taxonomically assign the *nifH* ASVs, phylogenetic placement was used, with the database of *nifH* amino acid sequences from Koirala and Brözel (2021) as reference. These *nifH* amino acid sequences were aligned using MAFFT (Katoh et al., 2019) followed by the construction of reference phylogeny using FastTree. The placements of the *nifH* ASVs onto the reference phylogeny was performed using the EPA-NG (Barbera et al., 2019), and function 'accumulate' implemented in Gappa (v. 0.8.1; Czech et al., 2020) was used to identify the most likely location of the placement in the phylogeny (threshold 0.8). Phylogenetic edge correlations (function 'correlation') were calculated to investigate how *nifH* carrying taxa differ over the MAT gradient, and N_2 fixation rates. Results were visualized using iTOL (v.6 (Letunic and Bork, 2024), and ggplot2 (v.3.5.1, (Wickham et al., 2024).

2.6. Calculations and statistical analysis

The C and N stocks of the soil organic layer were calculated as the C and N contents multiplied by the dry weight of the organic layer per m^2. In addition, the non-symbiotic N_2 fixation was scaled up to mg N m^{-2} h^{-1} by multiplying the N_2 fixation rate with the organic layer stock (which is the dry weight of the organic layer per m^2). Similarly, the abundance of diazotrophs was expressed on an area basis by multiplying the *nifH* gene copies g^{-1} with the organic layer stock. Finally, the non-symbiotic N_2 fixation rate per *nifH* gene copy number was calculated by dividing the non-symbiotic N_2 fixation per g by the *nifH* gene copy number per g of soil.

The effect of N fertilization on the different variables was analyzed using a linear mixed model including fertilization (control or nitrogen addition) as fixed factor and site as a random effect. This model structure accounts for the paired experimental design, in which the two treatments were established at each of the 15 sites. Data were checked for normal distribution (Shapiro-Wilk-test, $p > 0.05$) and log-transformed when needed prior to further analyses. Additionally, we independently assessed the role of MAT on the different variables by determining bivariate correlations between MAT and each response variable within each treatment using Pearson's correlation test ($p < 0.05$). The potential influence of other site-level covariates, such as MAP, stand age, and experiment duration, was also preliminarily considered (Table S2). All these analyses were performed with SPSS 27 software (IBM SPSS, Inc., Chicago, USA).

Non-metric multidimensional scaling (NMDS) based on the Bray-Curtis distances of Hellinger transformed rarefied *nifH* ASV abundances was performed to visualize differences in diazotroph communities between samples using the vegan package (v. 2.6–4 (Oksanen et al., 2022), in R (v. 3.6.4, R Core Team, 2019)). Correlations between NMDS ordination axis and metadata variables were explored using 'envfit', and the effects of treatment and MAT on community composition was tested with PERMANOVA using 'adonis2'. Alpha-diversity indices were calculated using the function 'diversity' in the vegan package for the Shannon index, and 'pd' in picante package (v. 1.8.2, Kembel et al., 2010) for Faith's phylogenetic diversity (PD). Pielou's evenness was determined by dividing the Shannon index with the

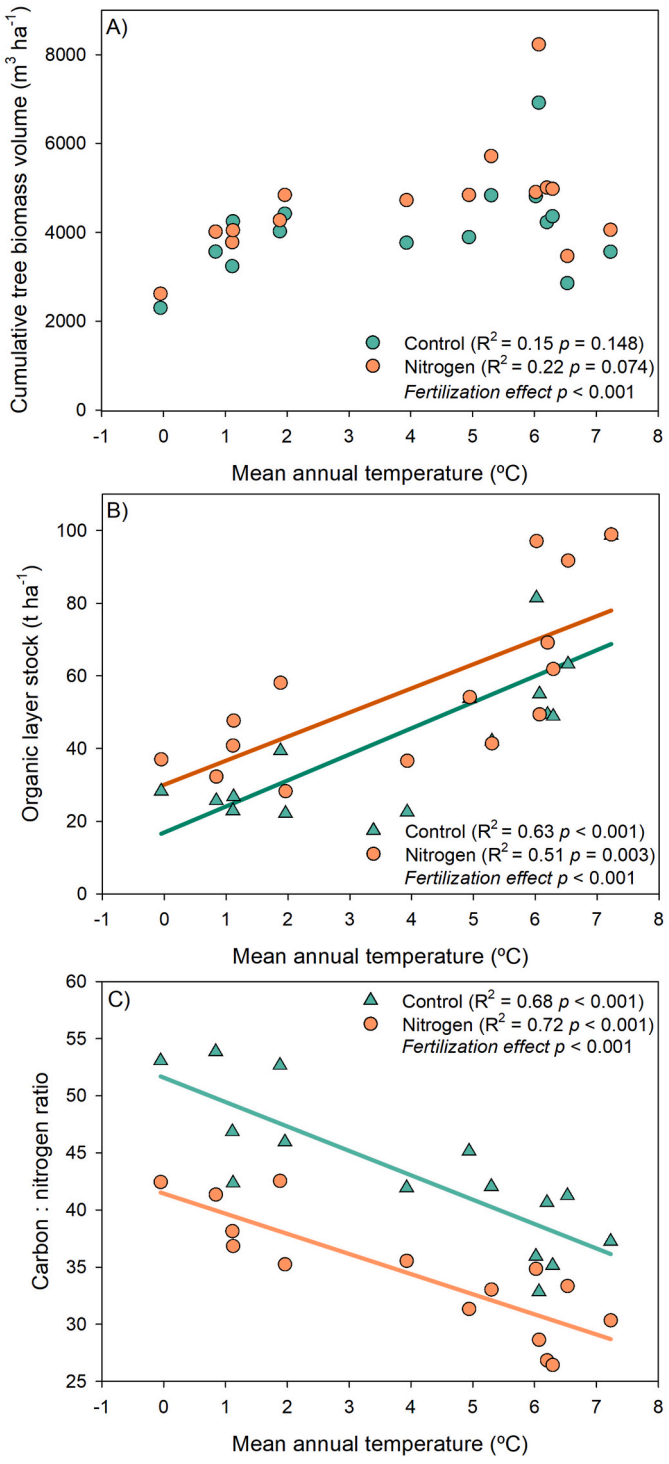

Fig. 2. Cumulative tree biomass volume in the control and the nitrogen treatment (A), the soil organic layer stock (B) and carbon-to-nitrogen ratio of the soil organic layer (C) as a function of the mean annual temperature (MAT) in 15 *Pinus sylvestris* forests distributed across Sweden. R^2 and p-value of the Pearson's correlation test for the control and the nitrogen fertilization treatment, and the statistical significance of the effect of fertilization are indicated.

logarithm of the number of ASVs.

3. Results

3.1. Impact of N on forest production and organic layer properties

The volume of tree biomass significantly increased due to N fertilization in the 15 *Pinus sylvestris* forests (Fig. 2A). The mean standing tree biomass volume across the 15 forest sites was 4075 m^3 ha^{-1} in the control and 4640 m^3 ha^{-1} in the N treatment (Fig. 2A–Table S1).

The organic layer stocks increased due to N fertilization from 4.54 kg m^{-2} in the control to 5.64 kg m^{-2} in the N treatment (Fig. 2B). Similarly, the C and N stocks of the organic layer increased upon N fertilization, largely due to the increase in the organic layer stock (Table S1). In addition, the organic layer stocks in both treatments were positively correlated with the MAT ($R^2 = 0.63$, $p < 0.001$ and $R^2 = 0.51$, $p = 0.003$ for control and N fertilization, respectively) (Fig. 2B–Table S1). Nitrogen fertilization significantly decreased the C:N ratio of the organic layer, from 43.2 in the control to 34.5 in the N treatment (Fig. 2C). This decrease was caused by an increase in the N content by 25 %, while the C content was unaffected by N addition (Table S1). The C:N ratio in the organic layer was negatively correlated with MAT in both treatments ($R^2 = 0.68$, $p < 0.001$ and $R^2 = 0.72$, $p < 0.001$ for control and N fertilization, respectively) (Fig. 2C–Table S1). The soil pH significantly increased due to N fertilization from 3.70 in the control to 3.88 in the N fertilization treatment (Table S1).

3.2. Non-symbiotic N_2 fixation in the organic soil layer

Long-term N fertilization did not affect the non-symbiotic N_2 fixation rates per g of soil across the 15 *Pinus sylvestris* forests (Fig. 3A). The mean non-symbiotic N_2 fixation rate of the control treatment was 22.4 ng N g^{-1} dw soil h^{-1}, while the mean in the N fertilized treatments was 22.5 ng N g^{-1} DW soil h^{-1}. No significant correlation between MAT and N_2 fixation rates per g soil were observed (see Table S2).The N_2 fixation rate per area (m^2 of forest floor) was affected by N addition, and the non-symbiotic N_2 fixation was 98.0 μg N m^{-2} h^{-1} in the control treatment and 121.7 μg N m^{-2} h^{-1} in the N fertilization treatment (Fig. 3B). This is due to the significantly positive effect of N addition on the organic layer stock (see section 3.1). Non-symbiotic N_2 fixation per area was positively correlated with MAT in both treatments ($R^2 = 0.58$, $p < 0.001$ and $R^2 = 0.31$, $p = 0.030$ for control and N fertilization, respectively) (Fig. 3B). However, we acknowledge that our non-symbiotic N_2 fixation measurements were conducted in an incubation experiment under controlled temperature and soil moisture conditions rather than in the field. Therefore, our rates should be interpreted as potential non-symbiotic N_2 fixation rates.

3.3. Diazotroph community abundance, diversity and composition

The abundance of diazotrophs per g of soil was significantly affected by N fertilization across all 15 sites, with a 24 % decrease in the mean *nifH* gene copy number in the N-fertilized plots compared to the control plots (Fig. 4A). Similarly, the total abundance of bacteria (as determined by the abundance of 16S rRNA gene copies) was 33 % lower in the N fertilization treatment than in the control (Fig. 4A). No significant correlation between MAT of the sites and abundance of diazotrophs per g soil was observed (Fig. S1A).

Non-symbiotic N_2 fixation per *nifH* gene copy number increased significantly due N fertilization (Fig. 4B). In addition, we observed a

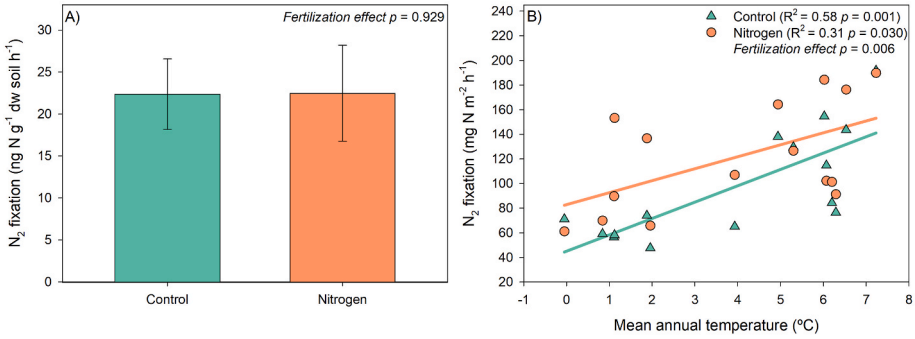

Fig. 3. The non-symbiotic N_2 fixation rate per dry mass soil in the control and the nitrogen treatment (A) and the non-symbiotic N_2 fixation rate per area unit (B) of the organic layer in the control and the nitrogen treatment as a function of mean annual temperature (MAT) in 15 *Pinus sylvestris* forests across Sweden. Statistical significance of the effect of fertilization is indicated (A, B) as well as the R^2 and p-value of the Pearson's correlation test for the control and the nitrogen fertilization treatment (B).

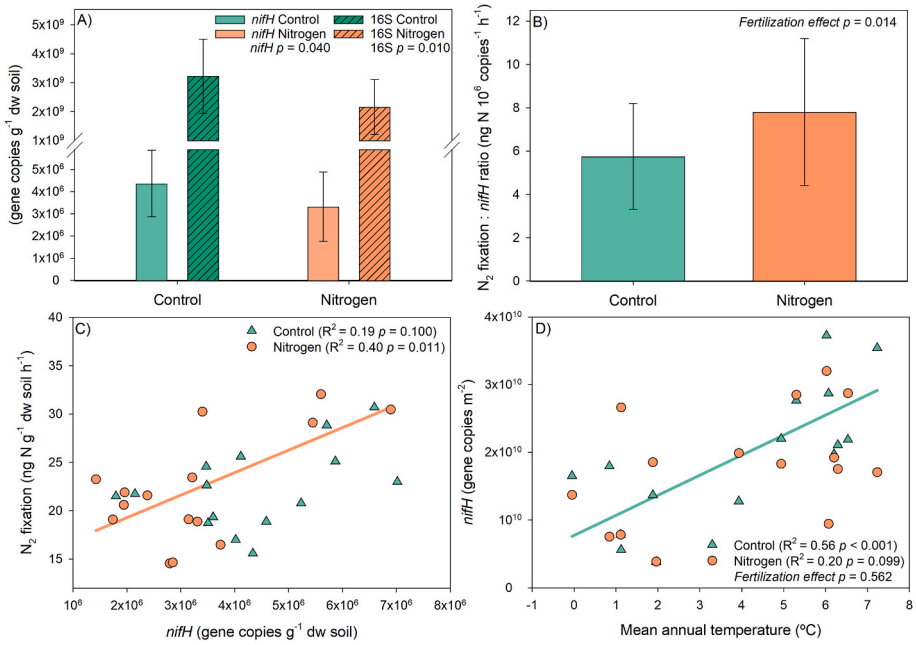

Fig. 4. Abundance of *nifH* gene copies and 16S rRNA gene copies per g of dry weight soil (A), the non-symbiotic N_2 fixation rate to *nifH* gene copies ratio in the control and the nitrogen treatment in the soil organic layer of 15 *Pinus sylvestris* forests across Sweden (B), and the non-symbiotic N_2 fixation rate as a function of the number of *nifH* gene copies per g of dry weight soil in the control and the nitrogen treatments (C) and the abundance of *nifH* gene copies per m^2 in control and nitrogen treatments as affected by the mean annual temperature of the sites (D). Statistical significance of the effect of fertilization is indicated (A, B and D) as well as R^2 and p-value of the Pearson's correlation test for the control and the nitrogen fertilization treatment (C and D).

Table 2
Effects of fertilization on diazotroph diversity in terms of Shannon index, Pielou's evenness and Phylogenetic diversity of the *nifH* gene in the soil organic layer in 15 *Pinus sylvestris* forests distributed across Sweden.

	Shannon Index	Pielou	Phylogenetic Diversity
Control	5.40 ± 0.15	0.885 ± 0.021	14.2 ± 0.7
Nitrogen	5.24 ± 0.23	0.874 ± 0.029	13.1 ± 1.1
Effect (p value)			
Fertilization	0.022	0.153	0.001

positive correlation between the *nifH* gene abundance and the non-symbiotic N_2 fixation rates in the N treatment ($R^2 = 0.40$, $p = 0.011$) but this was only marginally significant in the control ($R^2 = 0.19$, $p = 0.100$) (Fig. 4C). The abundance of diazotrophs on an area basis was not significantly affected by N fertilization ($F = 0.353$, $p = 0.562$) (Fig. 4D). The abundance of diazotrophs per m^2 was positively correlated with MAT in the control ($R^2 = 0.56$, $p < 0.001$) but not in the N fertilization treatment ($R^2 = 0.20$, $p = 0.099$) (Fig. 4D).

Nitrogen addition decreased the diversity of diazotrophs (Table 2) and for phylogenetic diversity, this was particularly noticeable at the sites where the last N fertilization event was more recent (Fig. S1B). In contrast to both phylogenetic diversity and the Shannon Index, Pielou's evenness index was not affected by N addition (Table 2).

Community composition of the diazotrophs was affected by both MAT (Fig. 5; PERMANOVA $R^2 = 0.054$, $p < 0.001$) and N fertilization (Fig. 5; PERMANOVA $R^2 = 0.04$, $p < 0.001$). The C, N, and organic layer stocks as well as N_2 fixation rates per area correlated significantly with communities in fertilized plots at sites with higher MAT.

The phylogenetic placements of the ASVs (Fig. 6) showed that the diazotrophic communities were largely dominated by ASVs similar to *nifH* in the class Alphaproteobacteria (order Hypomicrobiales) comprising 40 % of the *nifH* sequences (ca 3800 ASVs) across the dataset. More specifically, alphaproteobacterial *nifH* ASVs were similar to *nifH* in the families Nitrobacteraceae (*Bradyrhizobium,*

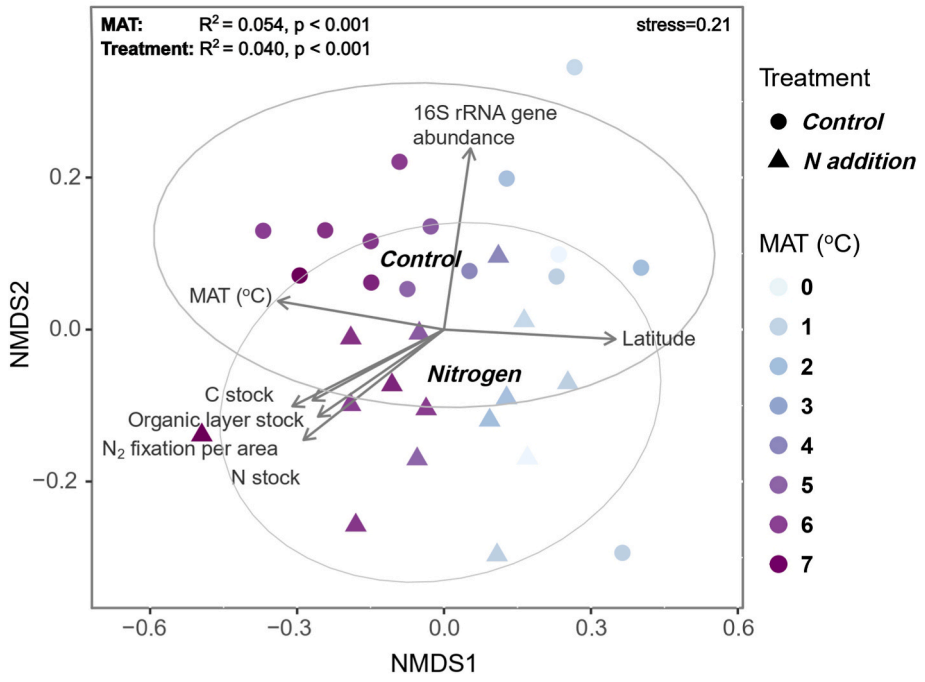

Fig. 5. Non-metric multidimensional scaling (NMDS) of diazotrophic communities based on the Bray-Curtis distance matrix of rarefied and Hellinger transformed ASV abundances based on *nifH* gene sequences. The colors of the symbols represent mean annual temperature (MAT, °C) of the sampling sites, while shapes designate the control (circle) or N treatment (triangle). Ellipses represent grouping of the samples into "Control" and "Nitrogen" with 95 % confidence intervals. The PERMANOVA results testing the effect of the treatment or MAT on *nifH* community composition are indicated in the graph. Significant ($p < 0.05$) correlations between ordination axes and metadata variables are shown as vectors, with length proportional to the strength of the correlations. Stocks are in tonnes per hectare, N_2 fixation per area designate N_2 fixation in μg N m^{-2} h^{-1}, 16S rRNA gene abundance designate 16S rRNA gene copies per g dry weight soil. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Rhodopseudomonas), Methylocystaceaea (*Methylocystis, Methylosinus*), Azospirillaceae (*Azospirillum, Nitrospirillum*), Rhizobiaceae (Rhizobium) and Phyllobacteriaceae (Mesorhizobium). The second largest group was photosynthetic cyanobacteria (class Cyanophyceae, order Nostocales) with ca 2800 ASVs comprising 31 % of the *nifH* sequences (Fig. 6). Ca 2000 ASVs (17.5 % of the sequences) were similar to the *nifH* in the classes Desulfobacteriota, Spirochaetota and Bacteroidota, and the phyla Thermodesulfobacteriota and Verrucumicrobiota, whereas the rest of the ASVs comprising ca 11 % of the sequences were similar to *nifH* in Beta- and Gammaproteobacteria, and the phyla Firmicutes, Actinobacteriota, Euryarchaeota (Fig. 6).

The abundances of the ASV placements (across the phylogeny) shows contrasting but weak correlations with the N_2 fixation rates per g of soil, and the strongest positive correlations were with *nifH* ASVs similar to class Cyanophyceae and the Alphaproteobacterial genera (Fig. 6A). Correlations between ASV placements and MAT was more pronounced (Fig. 6B). Negative correlations were found between MAT and the abundances of *nifH* ASVs similar to class Cyanophyceae, while positive correlations were found with ASVs similar to the *nifH* associated with classes Beta- and Gammaproteobacteria (Fig. 6B). Among *nifH* ASVs similar to Alphaproteobacteria constrasting patterns were observed, as *nifH* ASVs similar to the genera *Bradyrhizobium and Nitrospirillum,* and the family Methylocystaceaea were positively correlated to MAT, whereas *nifH* ASVs similar to the genera *Rhodopseudomonas* and *Azospirillum* were negatively correlated with MAT (Fig. 6B).

4. Discussion

4.1. Nitrogen fertilization decreases the abundance and diversity of diazotrophs in northern forests

The abundance of diazotrophs in the organic layer of northern forests decreased significantly due to N fertilization across the 15 sites (Fig. 4A), confirming the first hypothesis of a negative effect of N fertilization on diazotrophs. A negative effect of N fertilization on the total abundance of bacteria was also observed (Fig. 4A). The negative effect of N addition on the abundance of diazotrophs has been observed previously in other ecosystems (Wang et al., 2017, 2018; Fan et al., 2019; Shi et al., 2021; Zhong et al., 2023). However, to our knowledge, this is the first study to show this effect in northern forest soils. N fertilization can reduce the abundance of diazotrophs primarily through two mechanisms: (i) by increasing N availability in soils, which allows other microorganisms to outcompete obligate N_2-fixers due to the high energy cost of N_2 fixation, or (ii) by lowering soil pH as a result of acidification caused by continuous N fertilization in the form of urea or ammonium (Wang et al., 2017; Fan et al., 2019; Zhong et al., 2023). In our case, N fertilization increased soil pH significantly from 3.70 in control to 3.88 in the N fertilization treatment (Table S1) as the fertilization was performed using ammonium nitrate instead of other acidifying N sources (urea or ammonium sulfate). Thus, it seems likely that in the forest soils studied here, the reduction in the abundance of diazotrophs is caused by N enrichment (C:N ratio decreased from 43.2 in the control plots to 34.5 in the fertilized (Fig. 2C)), as the increased N availability may have led to some diazotrophs being outcompeted by non-N_2 fixing microbes.

The diversity of the bacterial community carrying *nifH* was significantly reduced by N addition, although the magnitude of this reduction was small (Table 2). This confirms our first hypothesis of a negative effect of N fertilization on diazotrophic diversity and is in accordance with previous results (Wang et al., 2017, 2018; Fan et al., 2019; Shi et al., 2021; Frey et al., 2023). The observed decrease in diversity caused by N fertilization suggests that the reduction in the abundance of diazotrophs particularly affected some specific groups of diazotrophs, likely those obligate N_2 fixers that cannot downregulate their N_2 fixation activity and hence are outcompeted by other taxa under N enrichment (Fan et al., 2019). A similar result was observed by Fan et al. (2019), who described 'winners' and 'losers' of long-term N fertilization in

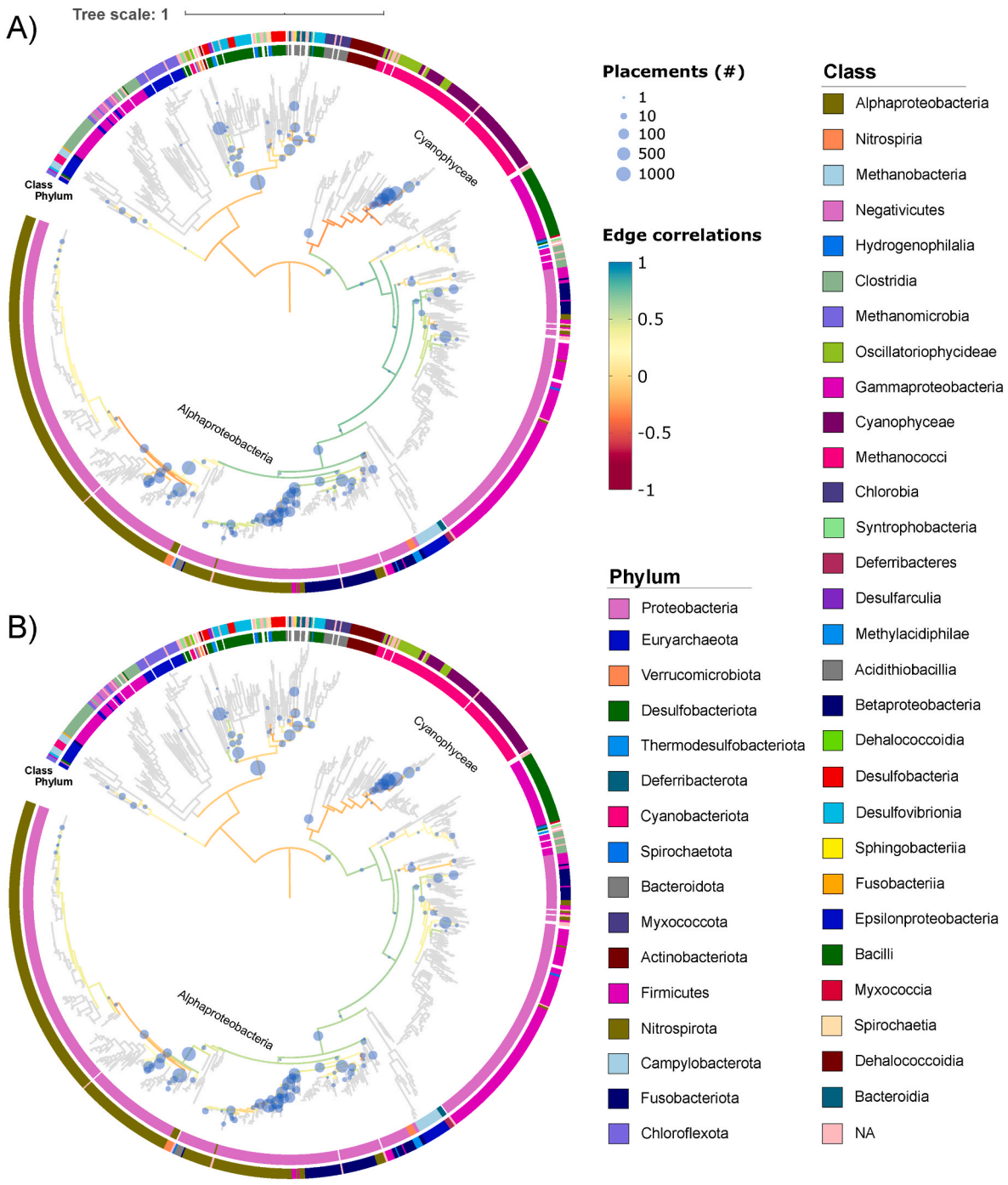

Fig. 6. Phylogenetic placements of the *nifH* ASVs on the *nifH* reference phylogeny, and correlations of the edges of the tree with the N_2 fixation rates per g of dry soil (A) or mean annual temperature (B) across all sites and treatments. The size of the circles is proportional to the number of ASVs placed in a specific location in the tree. Colors of the edges (branches) represent clades associated with positive (yellow to blue) or negative (yellow to red) shifts with either N_2 fixation (A) or mean annual temperature (B). Taxonomic classification at the phylum and class level are indicated by the color in the outer rings. The approximate locations of the two most abundant classes (Alphaproteobacteria and Cyanophyceae) are indicated with text labels. The scale bar denotes the amino acid exchange rate (WAG + R10). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

croplands. While fixers might benefit from N fertilization as they could be able to utilize the added fertilizer (the 'winners'), oligotrophic taxa were outcompeted, leading to a decrease in their relative abundance (the 'losers'). Given that the reduction in diversity caused by N fertilization in our study was relatively small (Table 2), it is plausible that the proportion of obligate diazotrophs in the community was also small.

The reduction in diazotrophic diversity observed in our study, likely accompanied by changes in community composition due to N fertilization, significantly influenced diazotrophic community structure, as revealed by PERMANOVA (Fig. 5, $R^2 = 0.04$, $p < 0.001$). The decrease in diversity and the change of community composition suggest a potential loss of functional traits and reduced resilience of the community. In a scenario of ceasing N fertilization, the absence of specific diazotrophs adapted to low-N environments, caused by previous fertilization, might reduce N_2 fixation (negative legacy effect). This could be the case during the period between the last fertilization and forest harvest. Yet,

the negative effect of N addition on phylogenetic diversity was stronger at the sites where the last N addition was conducted more recently (Fig. S1B), indicating that the phylogenetic diversity could recover from the negative effect of N addition in the medium-term (i.e., 5–7 years).

Our results revealed a dominance of *nifH* ASVs similar to the classes Alphaproteobacteria (40 %) and Cyanophyceae (31 %), while 17.5 % of the sequences were similar to *nifH* in the classes Desulfobacteriota, Thermodesulfobacteriota, Spirochaetota, Bacteroidota, Verrucumicrobiota (Fig. 6). Our results partially align with previous studies that reported the dominance of Alphaproteobacteria in forest ecosystems (Zhao et al., 2020; Hu et al., 2024; Masuda et al., 2024). However, those studies found Cyanophyceae to be in proportion much less relevant than in our study, highlighting the particularity of northern forests. Previous studies have emphasized significant N_2 fixation by cyanobacteria in association with mosses in northern forests (Gundale et al., 2012; Rousk et al., 2013), however, our results suggest that cyanobacteria may also play a key role in soil N_2 fixation, as previously observed in tundra soils (Pushkareva et al., 2017).

4.2. No effect of nitrogen fertilization on N_2 fixation rate per g of soil in northern forest

The non-symbiotic N_2 fixation rate per g of soil was unaffected by long-term N fertilization across the 15 sites (Fig. 3A). This result contrasts our second hypothesis of a negative effect of N addition on non-symbiotic N_2 fixation and previous studies reporting a decrease in non-symbiotic N_2 fixation upon long-term N addition in other ecosystems (Menge et al., 2009; Reed et al., 2011; Wang et al., 2018; Fan et al., 2019; Zheng et al., 2019; Shi et al., 2021). Previous studies argued that N_2 fixation is a metabolically expensive process, and hence facultative N_2 fixers would downregulate their N_2 fixation, while obligate N_2 fixers might be replaced by non-N_2 fixers under high N availability (Menge et al., 2009; Schleuss et al., 2020, 2021). Our results showed a reduction in the abundance, diversity and community composition of diazotrophs due to N fertilization, suggesting the disappearance of specific groups of diazotrophs (see Section 4.1 for further details). However, the remaining diazotrophs in the N fertilized plots maintained the N_2 fixation activity. This suggests either that the diazotrophs lost due to fertilization were not fixing N_2 or that the remaining community adjusted its activity to maintain ecosystem function, resulting in similar non-symbiotic N_2 fixation rates per g of soil as observed in the control plots. This increased N_2 fixation activity of the remaining community in N fertilized plots is evident from the non-symbiotic N_2 fixation normalized by the abundance of diazotrophs (number of *nifH* copies; Fig. 4B). Similar results were reported by Zheng et al. (2024), who found that diazotrophs could sustain N_2 fixation under long-term high N addition through community adjustment in temperate and subtropical forests. A similar community adjustment may also have occurred in our long-term experiment. However, further studies should examine whether short-term N fertilization has a stronger negative effect on N_2 fixation, as also reported Zheng et al. (2024). Our results support the perspective of Fan et al. (2019) regarding diazotroph 'winners' and 'losers' under long-term N fertilization as previously discussed. However, while their study reported a suppression of non-symbiotic N_2 fixation rates due to N fertilization, here we found that the decrease in abundance and diversity did not result in a reduction of non-symbiotic N_2 fixation rates due to the increased N_2 fixation activity of the remaining diazotrophs.

We observed a significant correlation between the abundance of diazotrophs and non-symbiotic N_2 fixation rates per gram of soil in the N fertilization treatment, but not in the control (Fig. 4C). This might suggest that some diazotrophs were not fixing N_2 in the control treatment. By contrast, most diazotrophs in the N fertilization treatment were likely active, resulting in a correlation between diazotroph abundance and N_2 fixation. In conclusion, the results suggest that the size of the diazotrophic community is not a good predictor of non-symbiotic N_2 fixation rates as found also in previous studies evaluating the impact of fertilization on non-symbiotic N_2 fixation (Tang et al., 2017). Finally, no correlation was observed between the Pielou, Shannon, and phylogenetic diversity indexes and non-symbiotic N_2 fixation rates per gram of soil, suggesting that there is no simple relationship between decreased diversity and lower non-symbiotic N_2 fixation.

The lack of effect of N addition on the N_2 fixation rate per g of soil observed might be explained by several particularities of northern forest ecosystems. First, northern forests have very low N availability due to the low background N inputs (Sponseller et al., 2016; Högberg et al., 2017). Therefore, N fertilization may not have sufficiently increased N availability to significantly affect N_2 fixation activity. N fertilization increased the soil N concentration on average by 2.5 g N kg^{-1} (a 25.5 % increase relative to the control) in the forest soils studied here, whereas Fan et al. (2019) reported an increase of only 0.24 g N kg^{-1} (+27.9 % relative to the control) but a 43 % reduction in N_2 fixation following long-term NPK addition in agricultural soils. Therefore, relative increases in soil N concentration of a similar magnitude (but much lower in absolute terms) in their study than those observed here appear sufficient to suppress N_2 fixation in their agricultural soils. This suggests that another characteristic of northern forest ecosystems must explain the lack of effect of N fertilization on the N_2 fixation rate per g of soil. Second, northern forest soils are characterized by low abundance of inorganic N forms due to the high and quick immobilization rate of inorganic N by ectomycorrhizal fungi (Högberg et al., 2017). This rapid immobilization of inorganic N can mitigate the negative effect of high inorganic N contents on nitrogenase activity, as generally described by Reed et al. (2011). Shortly after fertilization, inorganic N levels may return to their background levels, alleviating the limitation of N_2 fixation by inorganic N enrichment. Third, the high C content in the organic layers of northern forests (mean C:N ratio of 34.5 in the fertilized treatment, Fig. 2C) could offset the negative effects of N addition by providing sufficient energy to diazotrophs. Consequently, the remaining diazotrophs can continue active N_2 fixation, as observed in previous studies (Zheng et al., 2018, 2023; Schleuss et al., 2020). In addition, it has to be considered that the N application rate in the experiments studied here (100–150 kg N ha^{-1} every 5 or 7 years) is low compared to many studies about agricultural or grassland soil, in which the N application dose typically exceeds 100 kg N ha^{-1} yr^{-1} (Wang et al., 2018; Fan et al., 2019; Schleuss et al., 2020, 2021; Frey et al., 2023).

4.3. Nitrogen fertilization increases the N_2 fixation rate per area in northern forest

N_2 fixation per area (i.e., per m^2) increased due to N fertilization (Fig. 3B) because of the positive effect of N addition on the organic layer stock (Fig. 2B), which results from the positive response of tree growth to N (Fig. 2A). The positive effect of N addition on the organic layer stock has been previously described (Gundale et al., 2014; Maaroufi et al., 2015; Sponseller et al., 2016; Jörgensen et al., 2021). Primary production in northern forests is strongly N limited, thus increased N inputs can alleviate plant N limitation and enhance primary productivity and litter inputs to soil (Maaroufi et al., 2015; Sponseller et al., 2016; Jörgensen et al., 2021), which can sustain a larger diazotrophic community. Indeed, when the abundance of diazotrophs is expressed per area, no significant effect of N fertilization was observed ($p = 0.562$), as the increase in organic layer stock caused by fertilization compensates for the negative effect of fertilization on diazotroph abundance per gram of soil. This suggests that N fertilization might promote a positive feedback loop: increased N availability by fertilization leads to higher primary production, which increases soil organic matter stocks, which in turn leads to higher N_2 fixation, further supporting high primary production due to increased N availability.

The positive effect of N fertilization on N_2 fixation per area observed in our study contrasts with previous studies showing a negative impact of N addition on N_2 fixation by feather moss-cyanobacteria association (Gundale et al., 2011, 2013). These contrasting responses of N_2 fixation

processes by free living bacteria in soil and by cyanobacteria in association with mosses to N addition suggests that future studies on the N cycle in managed northern forest should consider them separately when evaluating the impact of forest N fertilization on N supply and potential N losses.

4.4. Impacts of MAT on diazotroph community and non-symbiotic N_2 fixation

No correlation between MAT and the abundance of diazotrophs per gram of soil in the organic layer was detected (Fig. S1A). Yet, the total diazotrophic community composition varied significantly along the MAT gradient (Fig. 5; PERMANOVA for MAT: $R^2 = 0.054$, $p < 0.001$). This suggests that along the MAT gradient, some diazotrophs may replace others better adapted to specific temperature conditions as MAT decreases, as previously described in studies performed across other temperature gradients (Zhao et al., 2020). This is supported by the strong correlations observed between certain diazotrophs and MAT (Fig. 6B). In particular, the abundances of *nifH* ASVs related to the class Cyanophyceae decreased with increasing MAT, supporting their relevance in cold ecosystems such as northern forests, as discussed in Section 4.1. Furthermore, no strong correlations were found between N_2 fixation per gram of soil and the abundances of specific *nifH* ASVs (Fig. 6A), suggesting that the active diazotrophs did not vary substantially among sites. Altogether, these results indicate that N_2 fixation activity of the community remains stable despite changes in diazotrophic community composition caused by MAT, likely because certain generalist *nifH* ASVs present at all sites were responsible for driving N_2 fixation. Further, the abundance of diazotrophs per m^2 was positively correlated with MAT in the control ($R^2 = 0.56$, $p < 0.001$) but not in the N fertilization treatment ($R^2 = 0.20$, $p = 0.099$) (Fig. 4D). This suggests an interaction between MAT and N fertilization, likely because the correlation between MAT and organic layer stocks is less strong in N treatment (Fig. 2B).

We observed a positive relationship between MAT and the non-symbiotic N_2 fixation rate per area (Fig. 3B), which is caused by the positive relation between MAT and the organic layer stock (Fig. 2B) rather than by an effect of MAT on N_2 fixation rate per g of soil as previously described (Vázquez and Spohn, 2025). In their study, they estimated an annual N input by non-symbiotic N_2 fixation ranging between 2 and 10 kg N ha^{-1} yr^{-1} for the control soils at the coldest and warmest end of the same temperature gradient, respectively. Here, we found no significant interaction between N fertilization and MAT on their impact on non-symbiotic N_2 fixation rate per area or per g of soil (Fig. 3B–Table S2), indicating that MAT does not modulate the response of non-symbiotic N_2 fixation to N addition. Thus, the effects of N fertilization on non-symbiotic N_2 fixation rates observed in our study can be assumed for other northern forest sites irrespective of MAT (in a range of −0.05 and 7.23 °C MAT). Finally, we also considered the potential influence of other factors, such as MAP, stand age, and experiment duration, on the N_2 fixation rates, *nifH* and 16S number of copies, tree biomass accumulation, organic layer stock and C:N ratio (Table S2). Experiment duration and stand age were not or only weakly correlated with any of the parameters, whereas MAP was significantly correlated with some of the studied variables. The correlations between the parameters and MAT were stronger than those with MAP, consistent with findings from a previous study analyzing the influence of environmental factors on soil properties in Swedish forests (Spohn and Stendahl, 2022).

5. Conclusion

Our results show that the non-symbiotic N_2 fixation rate per g of soil in northern forests is not affected by long-term N fertilization along a large latitudinal gradient in Sweden, despite the negative effects of fertilization on the abundance and diversity of diazotrophs. The remaining diazotrophs maintained their N_2 fixation activity upon N fertilization, possibly because the N concentration remained low, since inorganic N was rapidly immobilized, or since the C:N ratio was high enough to make N_2 fixation rewarding. However, the impact of decreased diazotroph diversity and shifts in community composition might suggest a potential loss of functional traits and reduced resilience in a hypothetical scenario, in which N fertilization ceases. Furthermore, we observed an increase in non-symbiotic N_2 fixation per area due to N addition, which was caused by the positive effect of N addition on the soil organic layer stocks resulting from the positive response of forest primary production to N addition. Taken together, this study shows that N fertilization in northern forests negatively affects the community of diazotrophs but positively influences the organic layer stock and the N_2 fixation rate per area.

CRediT authorship contribution statement

Eduardo Vázquez: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Jaanis Juhanson:** Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Sara Hallin:** Writing – review & editing, Validation, Supervision, Conceptualization. **Marie Spohn:** Writing – review & editing, Validation, Supervision, Resources, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

We have nothing to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2025.110037.

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