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Activity and Abundance of Nitrous Oxide Reducing Bacteria in *Platismatia glauca*: An Epiphytic Lichen in the Boreal Spruce Forest

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ABSTRACT

The nitrous oxide (N₂O) dynamics in boreal forests are better known at the ecosystem scale, with greater uncertainty associated with specific ecosystem compartments. We investigated the N₂O dynamics of the lichen *Platismatia glauca* in boreal forests near Kuopio, North Savo, Finland. At the study sites, *P. glauca* is the most abundant lichen colonising Norway spruce (*Picea abies*). Despite their abundance, the contribution of epiphytic lichens like *P. glauca* to N₂O dynamics in boreal forests has received little attention. By incubating *P. glauca*, we assessed the effects of moisture, temperature, and oxygen availability on its N₂O dynamics. We observed net N₂O consumption potential, particularly at +5°C at aerobic condition. Quantitative real-time PCR analysis targeting the N₂O reductase gene fragment (*nosZ*) revealed that it was present and active in both in situ and incubated lichens. *nosZ* transcription was higher at +5°C. Clade I *nosZ* was dominant, with most sequences affiliated with the order Rhizobiales. We confirmed the presence of *nosZ* gene with targeted metagenomics sequencing. Our results demonstrate that *P. glauca* acts as a net consumer of N₂O, with potential ranging between 0.1 and 0.4 ng N₂O–N g DW^{−1} h^{−1} under aerobic conditions.

1 | Introduction

Covering about 30% of the global forest area, boreal forests are important carbon (C) and nitrogen (N) reservoirs (Gauthier et al. 2015). In Finland, where this study was conducted, forests cover about 26 million hectares, with Norway spruce (*Picea abies* (L.) H.Karst., 1881) being one of the major tree species and providing canopy habitats for epiphytic lichens (Korhonen et al. 2021).

Nitrous oxide (N₂O) is a significant greenhouse gas and an ozone-depleting substance. It has global warming potential about 298 times greater than that of carbon dioxide (CO₂) over a 100-year period (Griffis et al. 2017; IPCC 2007). The N₂O fluxes from boreal forest are better known at the ecosystem

scale than the contribution of some system compartments (Pihlatie et al. 2005; Richardson et al. 2019; Tikkasalo et al. 2025). In Norway spruce forests, soils can act as sinks for atmospheric N₂O, particularly in spring and autumn, when anaerobic microsites form in silty soils and create favourable conditions for microbial activity to consume atmospheric N₂O (Siljanen et al. 2020). In cold, humid, and unfertilised soils, the balance between N₂O and N₂ exchange was close to zero, and even negative in some cases, suggesting that forest soils may absorb more N₂O than they emit (Machacova et al. 2019; Marchant et al. 2017; Moyes et al. 2016; Siljanen et al. 2020). Furthermore, forests with epiphytic moss and lichen cover showed greater N₂O uptake than those with less epiphytic coverage (Köster et al. 2018; Machacova et al. 2017). However, the limited available data suggest that soils and root

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systems remain the main drivers of N_2O uptake (Machacova et al. 2017; Moyes et al. 2016). The surfaces of epiphytic covers on plant tissues can exhibit either positive or negative fluxes of N_2O (Machacova et al. 2017; Moyes et al. 2016).

Lichens colonise diverse surfaces in boreal forests, including tree branches, stems, rocks, and soil, thereby expanding the active interface between the biosphere and the atmosphere (Machacova et al. 2017). Through their unique morphology and physiology, lichens can directly influence gas dynamics processes (Griffis et al. 2017; Machacova et al. 2017). Despite this ecological relevance, their contribution to N_2O dynamics remains largely unexplored although recent evidence from tree phyllospheres suggests that aboveground plant surfaces can host active N_2O -reducing communities (Zhang et al. 2025). As symbiotic organisms formed by fungi (mycobionts) and photosynthetic partners (photobionts, algae, or cyanobacteria), lichens contribute significantly to mineral cycling and energy flow at a global scale (Grimm et al. 2021; Pasqua et al. 2011). High-throughput genomic and proteomic analyses reveal that the bacterial community in lichens is abundant, structurally integrated, and potentially contributes to the host's health, growth, and fitness (Grimm et al. 2021). Lichens are abundant in boreal forests (Korhonen et al. 2013) and actively interact with atmospheric nitrogen compounds (Hauck 2010). As rootless organisms, they lack access to soil nutrient pools and depend on atmospheric deposition including precipitation, fog, and dry fall for nutrient acquisition (Fenn et al. 2007). In high-latitude regions such as the boreal zone, nitrogen deposition is generally low, well below critical load thresholds (Dentener et al. 2006), reinforcing the nitrogen-limited nature of their habitat. Moisture availability further modulates this limitation, fluctuating from near desiccation during cold periods to full saturation in warmer rainy days, with direct consequences for lichen physiology and microbial activity (Green et al. 2011, 2008; Kranner et al. 2008). Spruce tree surfaces are extensively colonised by diverse lichen species, with both abundance and diversity increasing markedly in old-growth forests (Marmor et al. 2011). Recent work has shown that microbial communities inhabiting the phyllosphere of Norway spruce (*Picea abies* (Linnaeus) H. Karsten, 1881) can actively control N_2O dynamics, highlighting the relevance of aboveground microbial habitats in boreal forests (Paul et al. 2025). In boreal coniferous ecosystems, lichen abundance is greater in Norway spruce than in Scots pine (*Pinus sylvestris* Linnaeus, 1753) stands, likely explained by more favourable conditions of shade, moisture, and the presence of dead branches (Liu et al. 2000). Lichens colonise well-lit surfaces in coniferous forests, with a relatively small but variable biomass ranging from 275 to 2155 g per tree (Liu et al. 2000). Despite their relatively small biomass, lichens represent an ecologically relevant forest component due to their large surface area in direct contact with the atmosphere. Microbial symbionts within lichens possess diverse metabolic capacities for adapting to environmental fluctuations (Grimm et al. 2021), with evidence suggesting the potential for bacterial reduction of N_2O to N_2 although this process remains poorly characterised.

Nitrous oxide reductase (N_2OR) catalyses the reduction of N_2O to N_2 , historically known as the terminal step of complete denitrification; recent knowledge points to be also important in partial denitrification. Either in the partial or in the complete

pathway, this enzyme is the only known enzyme that performs this reaction. The catalytic subunit Z of N_2OR , which contains the N_2O -binding site and is encoded by the *nosZ* gene, occurs in two major prokaryotic clades (Jones et al. 2013). Clade I organisms are typically canonical denitrifiers thriving in nitrate-rich, agriculturally impacted environments (Hallin et al. 2018; Jones et al. 2013; Sanford et al. 2012). In contrast, Clade II spans a broader phylogenetic spectrum across bacteria and archaea and displays greater ecological versatility, inhabiting nutrient-poor soils and ecosystems with fluctuating oxygen availability (Graf et al. 2016; Hallin et al. 2018). Clade II organisms, many of which lack the full denitrification pathway, reduce N_2O through non-canonical mechanisms and thereby function as important biological sinks in heterogeneous soil environments (Graf et al. 2016; Hallin et al. 2018). Across both clades, the N_2OR enzyme reduces N_2O to conserve energy via anaerobic respiration, while also contributing to cellular redox balance and mitigating N_2O -induced stress (Zumft 1997). More recent kinetic studies further demonstrate its activity across variable oxygen regimes, underlining its ecological importance (Suenaga et al. 2018). A detailed understanding of *nosZ* clades has clarified their evolutionary and ecological roles in N_2O reduction (Hallin et al. 2018). However, the abundance, diversity, and activity of N_2O -reducing microbes in epiphytic lichens are virtually unknown.

This study aimed to characterise the microbial community and functional genes involved in N cycling within *P. glauca* microbiome, and their activity for N_2O reduction. Particularly, the research questions of this study were: (I) what is the potential for N_2O dynamics in epiphytic lichens growing on *Picea abies*? (II) are *nosZ*-harbouring microbes present and transcriptionally active within these lichens? and (III) how do environmental drivers (moisture, temperature and oxygen) influence this potential? We hypothesised that: (1) the microbiome of epiphytic lichens acts as a net sink for N_2O under N-limited conditions; (2) moisture modulates this process by shaping microsites of reduced oxygen, and together with the increased nitrate stimulate the N_2O cycling microbes; and (3) low temperatures favour microbial communities adapted to N_2O reduction, whereas higher temperatures promote incomplete denitrification.

2 | Methods

2.1 | Sample Collection

Sample collection was conducted in May 2022 at three boreal forest sites in Kuopio, Northern Savo, Finland (Figure 1): Puijo Forest (62°54'30" N, 27°39'34" E), Jynkänvuori Forest (62°50'43" N, 27°41'39" E), and Kolmisoppi Forest (62°52'26" N, 27°35'53" E). All study sites were dominated by Norway spruce (*Picea abies* (Linnaeus) H. Karsten, 1881).

At each site, an undergrowth branch (~50 cm from eight spruce trees) was collected from representative forest areas and placed in airtight zip-lock bags. In the laboratory, branches were weighed and carefully stripped of lichens. The remaining wood was weighed separately, while lichens were identified to species level using specialised taxonomic keys (Moberg and Holmåsén 1990; Stenroos et al. 2021) and the iNaturalist recognition tool, and subsequently weighed. Lichen dry biomass was determined by

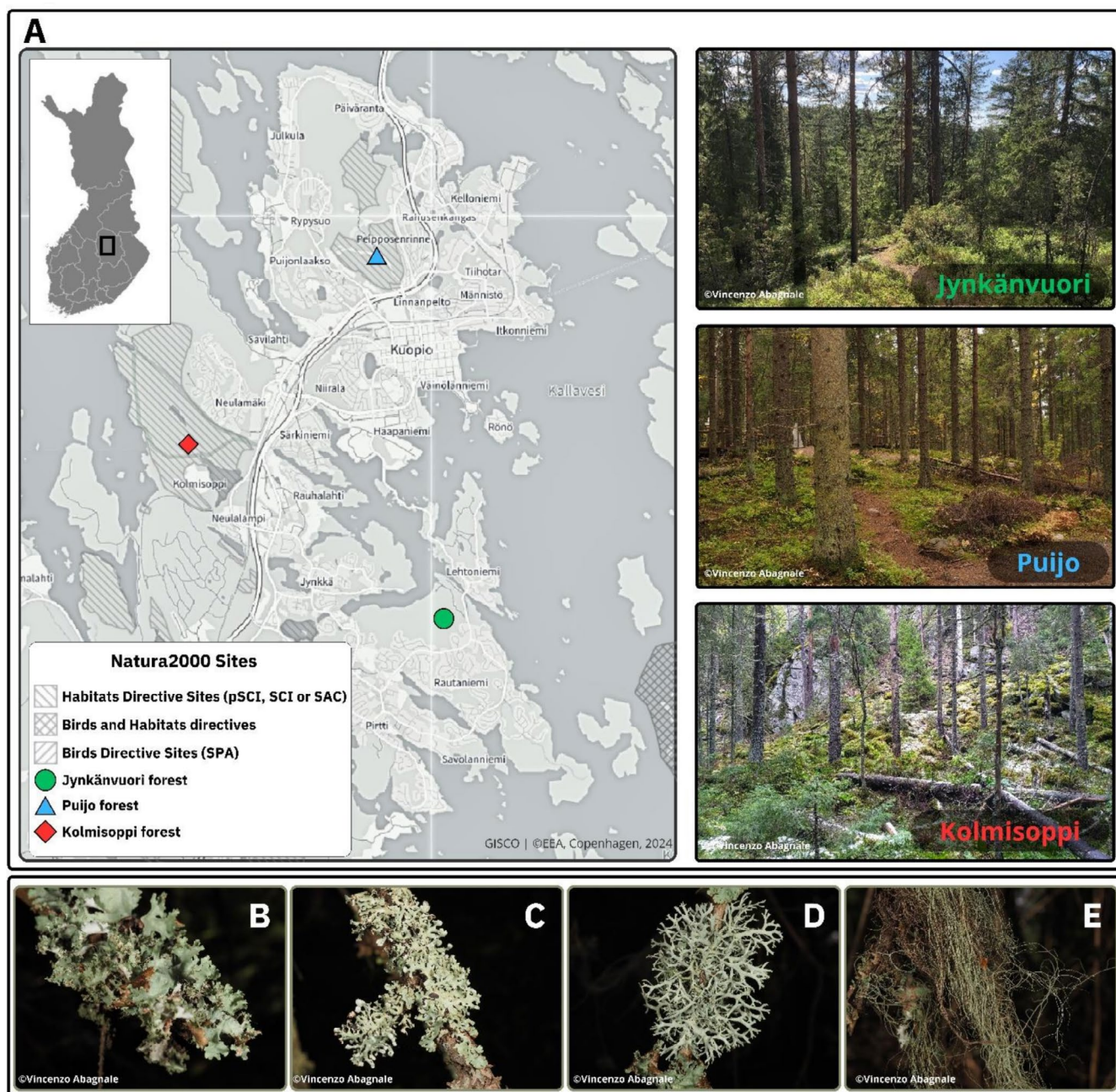


FIGURE 1 | (A) Right: Map of the areas classified according to the Natura 2000 regulation in Kuopio, North Savo, Finland. The symbols (circle, triangle, and diamond) indicate the sampled forest sites (left, view of these sites). The most abundant lichens in the forests of Kuopio: (B) *Platismatia glauca*, in (C) *Hypogymnia physodes*, in (D) *Pseudevernia furfuracea* and in (E) *Bryoria fuscescens* (photos made by Vincenzo Abagnale).

drying samples first overnight at room temperature ($\approx 20^{\circ}\text{C}$) and subsequently at 60°C for four days until constant weight was reached. For each lichen species (Figure 1), we determined relative biomass (%), water-holding capacity (WHC), dry weight fraction, and the lichen-to-wood biomass ratio (gDW/gDW), thereby quantifying both their abundance in the undergrowth and their capacity to retain water (Table 1).

For molecular analyses, *P. glauca* thalli were detached from branches on site and placed into sterile 50 mL polyethylene tubes (Thermo Fisher Scientific, Mexico). Samples were flash-frozen in liquid N (-196°C), transported to the laboratory in cryogenic containers, and stored at -80°C until analysis.

2.2 | Laboratory Incubations for N_2O Dynamics

The net N_2O dynamic potential of lichens was assessed in infusion-bottle incubations to compare the reduction capacity of different lichen species (see [Supporting Information](#) for further details). Prior to the incubations, the water holding capacity (WHC) and relative abundance of each lichen species at the study sites were determined (Table 1). Twigs that were rich in *P. glauca* were selected for the incubation study. This species was the most abundant across the study sites and had shown N_2O reduction capacity in preliminary assays (Table 1). Lichen thalli of *P. glauca* were carefully detached from twigs using sterile tweezers and air-dried overnight at room temperature. Incubation

TABLE 1 | Relative biomass, water-holding capacity (WHC), lichen-to-wood ratio, and Net N₂O exchange dynamics of epiphytic lichens on spruce branches in Kuopio forests.

Lichen species	Relative biomass (%)	WHC (%)	Lichen/Wood (gDW/gDW)	Consumption potential (ng N ₂ O-Ng ⁻¹ h ⁻¹)	
				100% WHC	0% WHC
<i>Platismatia glauca</i>	59.2 ± 8.7	72.4 ± 0.6	0.13 ± 0.12	−0.023 ± 0.008	−0.029 ± 0.007
<i>Hypogymnia physodes</i>^a	24.5 ± 6.4	75.5 ± 1.9	0.05 ± 0.03	−0.032 ± 0.010	−0.037 ± 0.008
<i>Bryoria fuscescens</i>^b	11.2 ± 5.7	70.6 ± 3.5	0.02 ± 0.03	−0.077 ± 0.058	−0.085 ± 0.063
<i>Pseudevernia furfuracea</i>	4.6 ± 4.0	74.7 ± 6.1	0.01 ± 0.03	−0.010 ± 0.007	−0.006 ± 0.005

Note: Values are mean ± SD (n = 3). Data for *Platismatia glauca* are shown in bold. Dry biomass fraction (%) can be obtained as 100 − WHC. Net N₂O dynamics was measured at 5°C and aerobic conditions.

^aSeparation of lichen from wood was difficult, which may have introduced bias in estimates of lichen biomass and lichen-to-wood ratio.

^bOther species of beard lichens were also present.

bottles (550 mL) were filled with 3.0 g of lichen material. The bottles for anoxic conditions were evacuated twice, at a 1-h interval, using helium (He) through the degassing and evacuation line. The bottles for oxygenated conditions (~21% O₂ in the headspace using laboratory air) were prepared in the same way. To increase the water content, a stock of melted snow collected from the study sites was sterile filtered (0.22 µm), degassed, and evacuated with helium; this water was then added to the incubation bottles. N₂O and CH₄ were injected at the start of incubation to yield final headspace concentrations of 2.8 times higher than atmosphere (~909 ppb) for N₂O and 1.4 times higher than atmosphere (~2.7 ppm) for CH₄. For anaerobic treatments, 60 mL of helium were added; for aerobic treatments, 60 mL of laboratory air were introduced. All bottles were incubated at 1.11 bar overpressures.

Headspace gas samples (20 mL) were withdrawn with a syringe and transferred into 12 mL pre-evacuated vials (−1 bar). Sampling was performed at 1, 20, 46, and 70 h after the start of incubation. Between samplings, bottles were maintained in complete darkness at constant temperatures (5°C or 15°C). After the final sampling, vials were analysed by gas chromatography, alternating sample vials with calibration standards. The gas chromatograph used was an Agilent Technologies 7890B with FID1A/ECD2B/TCD3C detector. Gas concentrations were converted to ppm, and N₂O dynamics were derived from the resulting concentration–time curves. N₂O dynamic potential rates were derived from changes in headspace concentration over time using a linear model. Data were normalised to lichen dry mass. For samples at 100% water holding capacity (WHC), corrections were applied to remove the solubility contribution of gases in water, ensuring that the calculated N₂O dynamic potential reflected lichen activity only. Furthermore, to quantify abiotic N₂O dynamics during incubations three control treatments were included: (i) empty bottle, (ii) empty bottle + filtered snow, and (iii) quiescent lichen (see further details in [Supporting Information](#)). Controls were sampled and analysed in parallel. Measured N₂O dynamic potential rate of controls was subtracted from lichen samples to correct background signals originating from bottle surfaces and water. To account for the detection limit of the gas chromatograph, reference standards were included at the beginning, middle, and end of each run. All incubations were performed in triplicate (three biological replicates per forest site) for each treatment.

2.3 | DNA/RNA Extraction and cDNA Synthesis

After gas sampling, lichen material of incubations was flash-frozen in liquid N (−196°C) to halt metabolic activity. Each bottle was opened and its contents transferred to a sterile mortar, where lichens were ground with a pestle under constant cooling by addition of liquid nitrogen. The resulting lichen powder was transferred into pre-labelled 15 mL tubes using sterile spatulas and stored at −80°C. The same procedure was applied to the three samples that had been flash-frozen directly in the field during sampling.

DNA and RNA were extracted from 0.5 g of lichen material using a CTAB buffer followed by phenol–chloroform–isoamyl alcohol extraction (Siljanen et al. 2019). Nucleic acid extracts were processed by PEG precipitation for DNA. For RNA, half of the DNA/RNA extracts were further purified using the RNeasy Plant Mini Kit (Qiagen). Extracted RNA was treated with RNase-free DNase I (Thermo Scientific) to remove residual genomic DNA without degrading RNA. The reaction was incubated at 37°C for 45 min, and the treated RNA was subsequently tested by PCR with *nosZ* primers to verify the absence of contaminating DNA. As no amplification was detected, the RNA was used as template for reverse transcription (cDNA synthesis).

cDNA synthesis was performed using Maxima MuLV H[−] reverse transcriptase (Thermo Fisher Scientific, Lithuania) and random hexamers. Random hexamers (200 ng/µL) were added to 7 µL of DNase-treated RNA and annealed at 65°C for 5 min. The reverse transcription buffer, 1 µL of RiboLock RNase inhibitor, and 1 µL of MuLV H[−] reverse transcriptase were then added. Reverse transcription reactions were incubated at 25°C for 10 min, followed by 30 min at 50°C.

2.4 | Q-PCR and RT-qPCR

DNA and cDNA extracts were amplified with primers targeting the *nosZ* gene from Clade I and Clade II (Table S2). These primers were chosen to quantify the relative abundance of Clade I and Clade II *nosZ* genes in the samples. PCR reaction mixtures and cycling conditions are provided in Table S3. Quantitative PCR was performed on a Bio-Rad MyCycler system, with duplicate reactions for each sample.

2.5 | Amplicon-Sequencing

For each sample, three technical replicates of PCR products were pooled prior to sequencing. PCR amplifications were performed with primers containing Illumina universal adapters. During library preparation, sequencing index adapters were added, and amplicon sequencing was performed on an Illumina MiSeq platform (2×250 bp) at Azena Genewiz Germany GmbH (Leipzig, Germany), generating approximately 80,000 paired-end reads.

Sequencing was carried out on triplicate DNA and cDNA samples derived from the +5°C and +15°C oxic incubations at the end of the experiment. The resulting FASTQ files were transferred to the Puhti server (CSC, Espoo, Finland). The FASTQ files were then converted to FASTA format using seqtk. *nosZ* fragments were identified from the FASTA files using the nhmmer algorithm with a *nosZ* HMMER profile. *nosZ* gene diversity was assessed by phylogenetic placement with RAXML on an IQ-TREE reference, following the pipeline described previously (Siljanen et al. 2025).

2.6 | Targeted Metagenomic Sequencing

We applied a probe-based capture metagenomics approach specifically designed to target key N cycle genes (Siljanen et al. 2025). This approach enabled the exploration of microbial contributions to N dynamics within the lichen-microbe symbiosis. Reference sequences for N fixation, nitrification, denitrification, nitrate reduction, and dissimilatory nitrate reduction to ammonium (DNRA) were compiled from public databases (NCBI, UniProt) and private repositories at CSC (Espoo, Finland). Conserved regions were identified from curated alignments using HMMER (Eddy 2011) and *tblastx* (Camacho et al. 2009). Using the MetCap pipeline (Kushwaha et al. 2015), over 263,000 probes were generated, with six unique 50-mer probes per gene cluster after dereplication at 80% similarity. DNA libraries, including adapter ligation and indexing, were prepared at Arbor Biosciences (USA) and pooled at equimolar concentrations. Hybridisation with probes was conducted at 47°C for 72 h, followed by sequencing on an Illumina NovaSeq platform (2×150 bp).

Raw paired-end reads were quality-checked with FastQC (Andrews 2010), and high-quality sequences ($Q > 30$) were retained using Trimmomatic (Bolger et al. 2014). High-quality forward and reverse reads were merged for downstream analysis. Functional genes were identified using HMMER profiles with an E-value threshold of $E < 0.0001$. Gene function and relatedness were confirmed by comparing sequences against UniProt-TrEMBL, Swiss-Prot, and NR databases using the *tblastx* method in the DIAMOND sequence search accelerator. *nosZ* Clade I and II sequences were further analysed using the GraftM database (Boyd et al. 2018) in combination with the Gappa tool (Czech et al. 2020), and taxonomy of the reads were analysed against *nosZ* database (Graf et al. 2022). The jplace files generated by GraftM were used to construct phylogenetic trees with IQ-TREE v1.6.12, providing deeper insights into genetic relationships. Final trees with phylogenetic

placements were illustrated in the iTol phylogenetic tree illustrator (Letunic and Bork 2021).

Sequencing data have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession numbers PRJNA1282212 (targeted metagenomics) and PRJNA1282427 (PCR amplicons).

2.7 | Statistical Analysis

Data, including N_2O dynamics rates, DNA and cDNA qPCR, and sequencing results, were tested for normality using the Shapiro-Wilk test. Treatment effects were evaluated with n-way ANOVA, and pairwise comparisons were assessed with Tukey's test. Unless otherwise specified, data are presented as mean ± standard deviation (SD). Statistical analyses were performed in Python, using the libraries Pandas (The pandas development team 2025), NumPy (Harris et al. 2020), SciPy (Virtanen et al. 2020), Statsmodels (Skipper and Josef 2010), and Matplotlib with Pylab tool (Hunter 2007). In R, analyses were conducted using the base *stats* package (R Core Team 2025), and the packages *tibble* (Müller and Wickham 2016) and *dplyr* (Wickham et al. 2026) for data manipulation, and *gt* (Iannone et al. 2020) for table generation.

3 | Results

3.1 | *Platismatia glauca* is the Predominant Lichen on Branches of Spruce

Four lichen species (*Bryoria fuscescens*, *Hypogymnia physodes*, *Platismatia glauca* and *Pseudevernia furfuracea*) were consistently observed and abundant on the sampled spruce branches in the study sites (Table 1, Figure 1). Among these, *Platismatia glauca* was predominant, representing 59% of the relative biomass. *P. glauca* covered extensive areas of branches, with thalli extending outward into the air and thereby increasing the surface area in contact with the atmosphere. It also showed the highest lichen-to-wood biomass ratio (Table 1). Accordingly, *P. glauca* was selected as the focal species for this study because of its predominance on spruce branches. The four lichen species exhibited similar water-holding capacities (WHC), ranging from 70% to 75% (Table 1).

3.2 | Net N_2O Dynamics of *P. glauca* at Different Moisture and Temperature Conditions

P. glauca was incubated under contrasting moisture (0% vs. 100% WHC), temperature (+5°C vs. +15°C), and oxygen (oxic vs. anoxic) conditions. Overall, *P. glauca* exhibited net N_2O consumption potential under oxic conditions, with the highest rate at +5°C and 100% WHC ($-0.409 \pm 0.055 \text{ ng } N_2O \text{ g}^{-1} \text{ h}^{-1}$, Figure 2). Under anoxic conditions, dynamics rates were close to zero in dry samples ($-0.003 \pm 0.038 \text{ ng } N_2O \text{ g}^{-1} \text{ h}^{-1}$, Figure 2) and shifted toward slight N_2O production in wet samples ($0.043 \pm 0.038 \text{ ng } N_2O \text{ g}^{-1} \text{ h}^{-1}$, Figure 2). The instrumental detection threshold (IDT) of the gas chromatographic analysis was $-0.015 \pm 0.031 \text{ ng } N_2O \text{ g}^{-1} \text{ h}^{-1}$; despite being close to this

limit, all measured N_2O consumption potential rates under oxic conditions were statistically below zero and different from IDT (Figure 2, $p < 0.05$). N_2O dynamics in the controls were close to zero and always below the instrument detection limits (Table S1). Measurements of other epiphytic lichens species in the forests showed that all act as net N_2O consumers in aerobic conditions (Table 1).

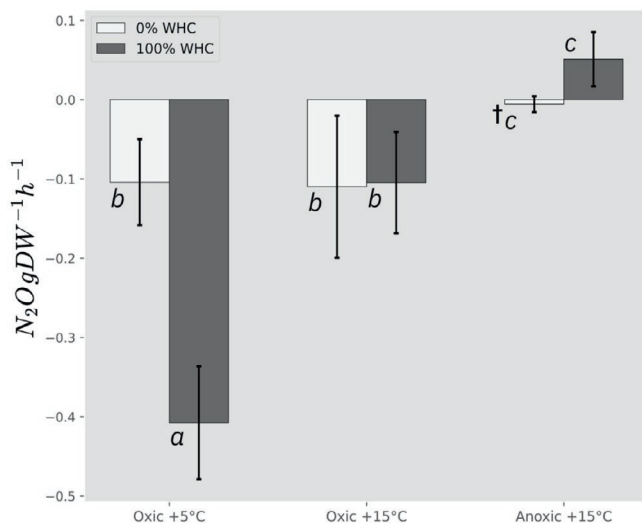


FIGURE 2 | Net N_2O dynamics of *P. glauca* under contrasting temperature, moisture, and oxygen conditions ($n = 3$). Positive values indicate net N_2O production, while negative values indicate net consumption. White bars represent dry samples (0% WHC) and black bars represent wet samples (100% WHC). Different letters (a, b, c) denote significant differences among treatments ($p < 0.05$). Bar marked with † is not statistically different ($p > 0.05$) from instrumental detection threshold derived from calibration standards analysed during the run (Table S1).

3.3 | Abundance and Transcription of *nosZ* Clade I Genes in *Platismatia glauca*

The *nosZ* Clade I gene copy numbers were substantially higher in incubated samples ($1.27 \times 10^4 \pm 2.80 \times 10^3$ copies g FW⁻¹) compared to in situ samples ($1.43 \times 10^2 \pm 1.17 \times 10^2$ copies g FW⁻¹, Figure 3). Gene copy numbers also differed between temperature treatments, with higher abundance at +5°C ($3.58 \times 10^4 \pm 7.40 \times 10^3$ copies g FW⁻¹) compared to +15°C ($1.32 \times 10^3 \pm 4.75 \times 10^2$ copies g FW⁻¹). Transcript levels showed the same trend, with the highest expression at +5°C ($8.69 \times 10^6 \pm 7.10 \times 10^6$ transcripts g FW⁻¹), lower expression at +15°C ($1.32 \times 10^3 \pm 4.75 \times 10^2$ transcripts g FW⁻¹), and the lowest levels in situ ($2.85 \times 10^2 \pm 2.33 \times 10^2$ transcripts g FW⁻¹). In situ transcription was detected at only two of the three sites (Puijo and Kolmisoppi). Samples incubated at 15°C under anoxic conditions showed a similar trend to those incubated at the same temperature under oxic conditions (Marchant et al. 2017). The *nosZ* Clade II gene copies remained below the detection limit.

3.4 | Taxonomy of *nosZ*-Harbouring Prokaryotes in *Platismatia glauca*

Amplicon sequencing of *nosZ* DNA and cDNA confirmed that Clade I dominated the community of *nosZ*-harbouring microbes in *P. glauca*, with the most abundant taxa belonging to the order Rhizobiales (syn. Hyphomicrobiales), particularly the genus *Bradyrhizobium*. The *nosZ* genes were detected in Puijo and Kolmisoppi, while *nosZ* transcripts were successfully detected in the Puijo study site. The *nosZ* gene community included Clade I organisms in class Alphaproteobacteria and order Rhizobiales, but also members in Clade II organism in phylum Bacteroidetes were detected at very low abundance with targeted metagenomics (Figure 4A,B).

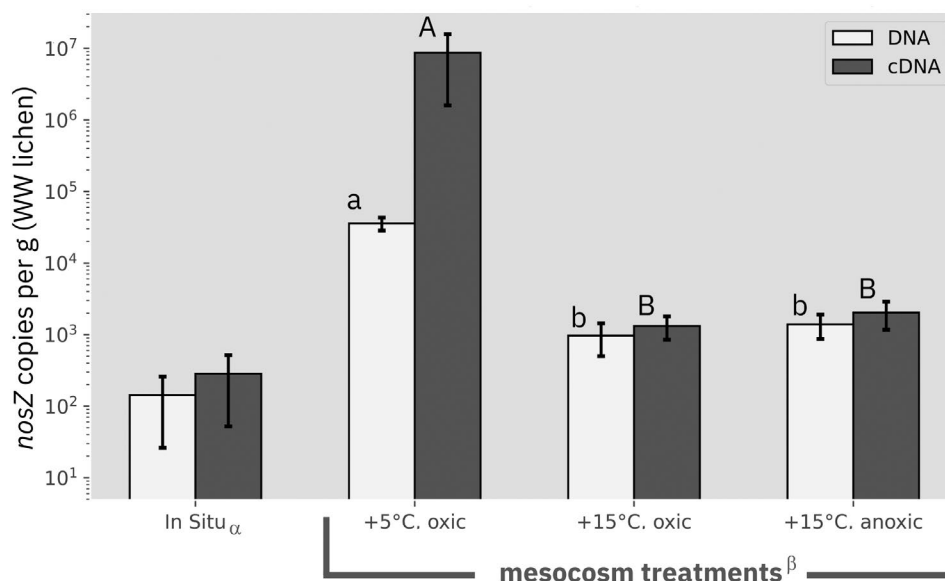


FIGURE 3 | Abundance of *nosZ* (Clade I) gene copies and transcription ($n = 3$) in the in situ and in the samples incubated at 0% WHC. The letters 'a' and 'b' and 'A' and 'B' indicate significant differences ($p < 0.05$) between treatments for gene and transcript copies, respectively. In the bar labels the letters in Greek indicate a significant difference ($p < 0.05$) between in situ and incubation samples. The Y axis is in log10 scale.

(A)

Clade	Taxa	RA
Clade I	Proteobacteria; Alphaproteobacteria	0.73%
Clade I	Proteobacteria; Alphaproteobacteria; Rhizobiales	2.19%
Clade I	Proteobacteria; Alphaproteobacteria; Rhizobiales; Bradyrhizobiaceae; Bradyrhizobium	84.67%
Clade I	Proteobacteria; Alphaproteobacteria; Rhizobiales; Hyphomicrobiaceae; Hyphomicrobium	10.22%
Clade I	Proteobacteria; Alphaproteobacteria; Rhizobiales; Rhizobiaceae; Rhizobium	1.46%
Clade II	Bacteroidetes	0.73%

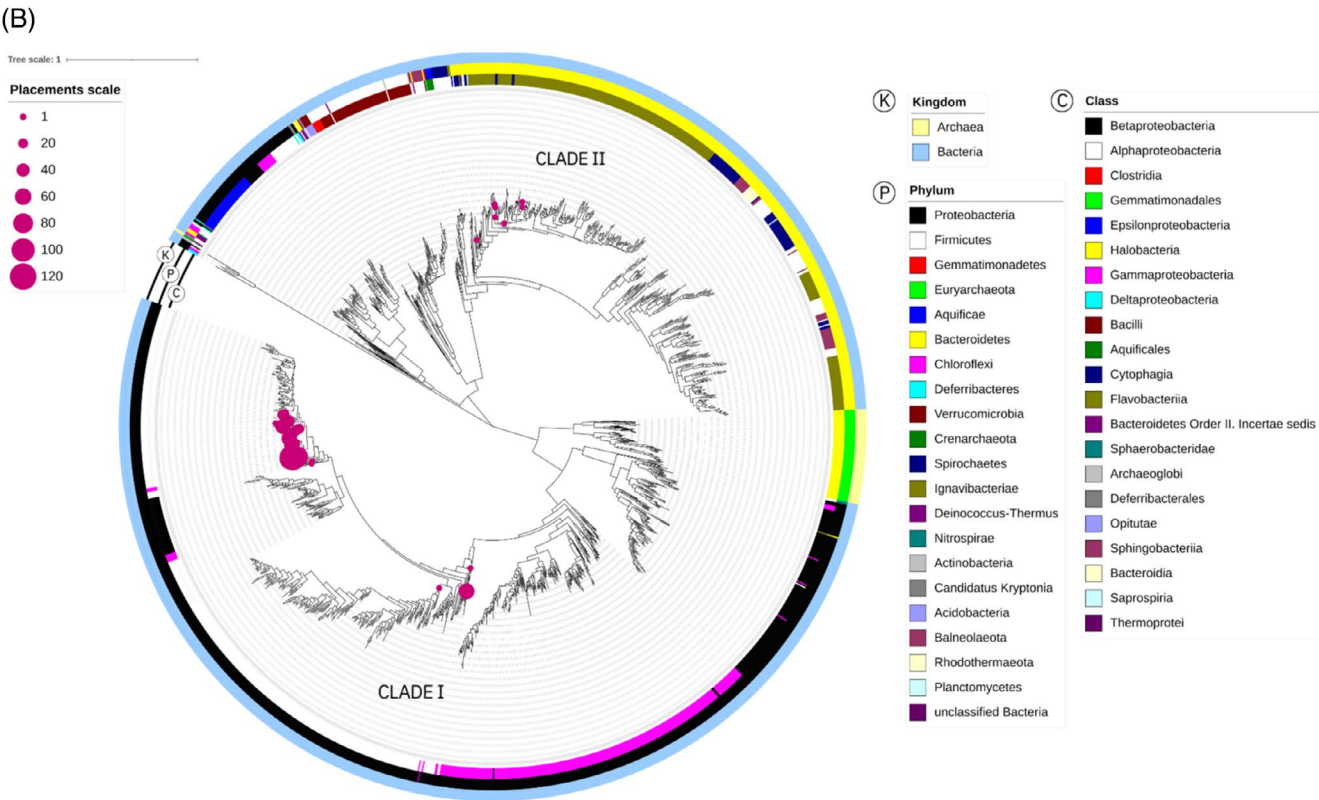


FIGURE 4 | Taxonomy and phylogeny of *nosZ*-harbouring prokaryotes in *Platismatia glauca*. (A) Relative abundance (%) of *nosZ* sequences by clade and taxonomic affiliation, based on amplicon sequencing. (B) Phylogenetic tree of *nosZ* sequences' phylogenetic placements of targeted metagenomics constructed with IQ-TREE (v1.6.12) using the GTR + G substitution model and 1000 ultrafast bootstrap replicates.

3.5 | Genetic Potential of N Functional Genes in *Platismatia glauca*

Targeted metagenomic sequencing of functional genes involved in mineral N transformations revealed that *nosZ* accounted for only 5.4% of the detected sequences (Figures 5,

S1). Higher proportions were associated with genes involved in nitrate reduction (*napA* 49.2% and *narG* 10.3%) and nitrogen fixation (*nifH*, 25.6%). Among the other denitrification genes, *nirK* was most abundant (7.1%), whereas *nirS* (0.8%) and *norB* (0.3%) occurred at lower proportions than *nosZ*. Genes associated with DNRA (*nrfA*) and nitrification (*amoA*) were

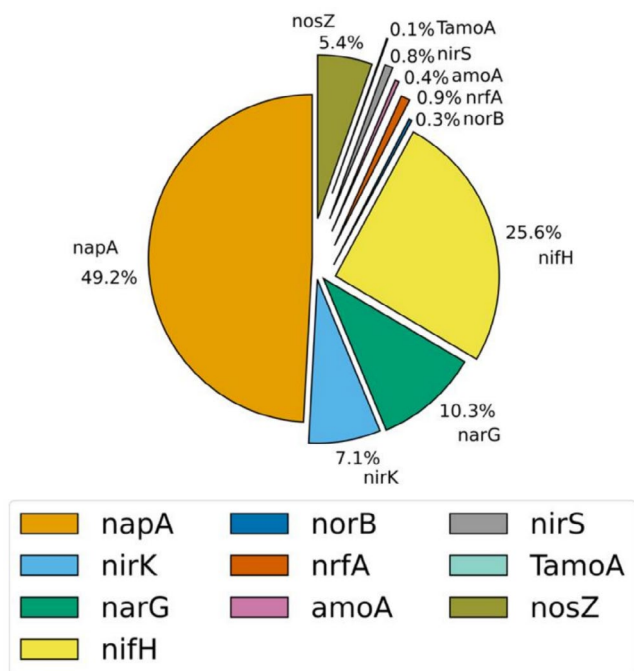


FIGURE 5 | Relative abundance of functional genes associated with nitrogen cycling in *Platismatia glauca* as revealed by targeted metagenomic sequencing. Genes include those involved in nitrate reduction (*napA*, *narG*), N_2 fixation (*nifH*), denitrification (*nirK*, *nirS*, *norB*, *nosZ*), DNRA (*nrfA*), and nitrification (*amoA*).

detected at low proportions. The *nosZ* gene was detected in two of the three DNA samples from Puijo and Kolmisoppi forest at 15°C (Table S5).

4 | Discussion

Our results demonstrate N_2O metabolism in epiphytic lichens of the boreal spruce forest. All lichen species acted as net N_2O consumers, while detailed incubations and molecular analyses confirmed this capacity for *Platismatia glauca*, supporting our first hypothesis. *P. glauca* exhibited net N_2O consumption potential under oxic conditions, with maximum uptake observed at 5°C and 100% WHC. This activity was associated primarily with Clade I *nosZ*-harbouring microbes, suggesting that oxygenated yet moist microenvironments can sustain N_2O reduction, as reported also for cryptogamic covers on trees and other aerobic–anaerobic interfaces (Machacova et al. 2017; Marchant et al. 2017). Incubation experiments demonstrated a clear net reduction of N_2O , which was paralleled by a significant increase in both *nosZ* Clade I gene copies and transcripts compared to in situ samples. The increase was most pronounced in samples incubated at 5°C, while it was markedly lower at 15°C. In contrast, *nosZ* Clade II appeared to play only a minor role, as gene copies and transcripts were below the qPCR detection limit in both incubated and in situ samples. However, targeted metagenomic sequencing still detected *nosZ*-harbouring microbes, suggesting that Clade II organisms were present at very low abundance and may have been underestimated by qPCR, while Clade I remained functionally dominant.

4.1 | An N_2O Consumption Optimum in Cold and Oxic Conditions

The net negative N_2O consumption potential observed at 5°C compared to 15°C suggests an adaptation of the lichen-associated microbiome to colder conditions in May, supporting our first hypothesis on the role of lichens as net N_2O consumers. Further studies spanning different seasons are required to confirm whether this apparent low-temperature optimum persists throughout the year, although this may change given the strong seasonality of the boreal forest. This low-temperature optimum aligns with seasonal patterns observed in boreal forests, where N_2O uptake has been reported during dormant periods, particularly in cold and humid conditions (Machacova et al. 2019, 2017). During these periods, reduced photosynthetic activity and C immobilisation in frozen soils may shift metabolism from aerobic photosynthesis toward alternative respiratory pathways, including denitrification, where N_2O dynamics becomes more relevant. In these conditions, nitrate is generally the main electron acceptor, but N_2O can also serve this role, particularly for microbes lacking the full denitrification pathway and therefore relying only on N_2O reduction (Graf et al. 2016). When temperatures rise to 15°C–20°C, decomposition and mineralisation of organic matter intensify, releasing ammonium, which can be nitrified to nitrite and nitrate. Elevated nitrate availability may alter the balance of denitrification by either inhibiting N_2O activity (Pauleta et al. 2013), or stimulating upstream reductases such as *nirK* and *nirS*, thereby shifting the system toward net positive N_2O dynamics (Hallin et al. 2018). As a result, net N_2O emissions can surpass the fraction removed via *nosZ*-mediated reduction (Hallin et al. 2018; Zumft 1997). Notably, Machacova et al. (2017) demonstrated that even under oxic conditions, tree stems and their cryptogamic coverings, including lichens and mosses, function as significant sinks for atmospheric N_2O . Using gas-tight chambers under natural conditions, they measured stem and cryptogam fluxes and showed that net negative N_2O dynamics was proportional to respiratory activity, particularly in cold and humid environments. Although denitrification is conventionally regarded as an anaerobic process, the simultaneous performance of aerobic and anaerobic respiration has been reported in coastal sediments (Marchant et al. 2017). Similarly, our results indicate that *P. glauca* exhibits net negative N_2O dynamics under oxic conditions, with this capacity being more evident at lower temperatures. Anoxic incubations were performed only at 15°C, reflecting a targeted experimental choice rather than a full factorial design. This limitation is acknowledged here and does not affect the main conclusions of the study, which focus on the presence and activity of N_2O -reducing microbes under oxic conditions.

Under wet conditions at 5°C, N_2O consumption was enhanced (Figure 2), which may indicate that water saturation creates microsites with reduced oxygen availability, thereby favoring N_2O reduction (Hypothesis 2). At 15°C under wet and anoxic conditions, the net N_2O dynamics shifted toward production (Figure 2), consistent with incomplete denitrification processes potentially fueled by nitrate and nitrite present in the snow (Table S4). This observation supports Hypothesis 3, which predicted net N_2O production under warmer, wetter,

and oxygen-limited conditions. N_2O likely contributed to the observed N_2O dynamics, helping to maintain a transient balance between net production and reduction. The near-zero net N_2O dynamics observed under dry, anoxic conditions at 15°C (Figure 2) likely reflect a combined effect of warmer temperatures, anoxia, and high moisture, which can favor N_2O production over reduction. Under such conditions, the electron transport chain may become saturated or redirected toward alternative acceptors, limiting the effective use of N_2O as a terminal electron acceptor (Pauleta et al. 2019). The effects of moisture, oxygen, and temperature on denitrification are complex; for example, a shift to anoxic conditions may transiently favor N_2O production over consumption, consistent with the bet-hedging strategies of denitrifying bacteria (Lycus et al. 2018). Nitrate availability can strongly modulate the temperature sensitivity of denitrification, altering the balance between N_2O production and reduction (Palacin-Lizarbe et al. 2018). At the same time, colder temperatures increase the solubility of N_2O , potentially facilitating its reduction, whereas the effect of temperature on nitrate solubility is likely negligible.

4.2 | Importance of *nosZ* Clade I Belonging to *Bradyrhizobium* Genus

Our results highlight the dominant role of *nosZ* Clade I bacteria, particularly those affiliated with the genus *Bradyrhizobium*, within the microbiome of *P. glauca*, suggesting their key contribution to the observed net negative N_2O dynamics. These bacteria may contribute to net N_2O dynamics under conditions of limited nitrate or oxygen availability, where microaerophilic pockets facilitate partial anaerobic metabolism (Brodo et al. 2002; Suenaga et al. 2018; Zumft 1997). In this context, cephalodia represent one of several specialised niches that can host microbial partners and provide favourable conditions for N cycling being morphologically distinct structures that emerge from the integration of cyanobacterial symbionts into cortical hyphae (Cornejo and Scheidegger 2013), but similar functions may also occur in the medullary matrix or in surface-associated biofilms (Grimm et al. 2021; Palmqvist and Dahlman 2006). Molecular surveys have further shown that lichens frequently harbour structured bacterial communities dominated by Rhizobiales, including *Bradyrhizobium*, supporting their potential functional role in N_2O reduction (Aschenbrenner et al. 2016; Bates et al. 2011). Importantly, complete anoxia does not appear to trigger cryptobiosis in *P. glauca*, a reversible ametabolic state documented in lichens and other poikilohydric organisms, where all metabolic processes, reproduction, and repair are suspended (Cannone et al. 2017; Crowe et al. 1992; Kranner et al. 2008). While the thallus can host anoxic microsites suitable for denitrification, our data indicate that *nosZ* expression does not increase under fully anaerobic conditions, suggesting that N_2O dynamics are favoured in more moderate, microaerophilic niches. This observation aligns with evidence that the lichen microbiome is structurally integrated and metabolically active (Grimm et al. 2021); reinforcing the view that N_2O reduction in lichens depends on a dynamic interplay between host morphology and microbial functionality. In contrast, *nosZ* expression was enhanced under low temperatures and oxic conditions, coinciding with increased net negative N_2O dynamics. During the

early phase of aerobic incubation, we observed pronounced net negative N_2O dynamics, which gradually shifted toward a stable balance between uptake and emission, suggesting a form of dynamic equilibrium among microbial processes. During the initial hours of aerobic incubation in darkness, *P. glauca* exhibited a phase of intense N_2O uptake, followed by a steady equilibrium between emission and uptake, indicating a “dynamic stability” of metabolic processes. Under dark conditions, photosynthesis was inactive, and at lower temperature (5°C) the reduced efficiency of aerobic respiration may have promoted alternative pathways where N_2O served as a terminal electron acceptor (Pauleta et al. 2019; Zumft 1997). Such a shift toward N_2O reduction in energy-limited states resembles a form of “forced metabolism” (Brodo et al. 2002), where microbes channel electrons to alternative acceptors to maintain redox balance. Members of the order Rhizobiales are also known to establish symbioses with algae, such as diatoms, where they contribute to N_2 fixation (Tschitschko et al. 2024). Similar symbiotic associations may occur in lichens, potentially supporting processes such as N_2O reduction or N_2 fixation.

4.3 | N Limitation in Lichens Indicates Potential for N_2O Dynamics and N_2 Fixation

Epiphytic lichens in the boreal spruce forest of Eastern Finland, like our study sites, live in a N-limited environment, as atmospheric deposition through precipitation is the main abiotic N source and remains very low. This N-limited environment may provide favourable conditions for net negative N_2O dynamics and for the genetic potential of N_2 fixation within the lichen microbiome, although the actual balance under natural conditions remains uncertain. Under nitrate limitation, atmospheric N_2O may serve as an alternative electron acceptor for lichen-associated microbes, potentially contributing to the net negative N_2O dynamics observed across epiphytic species. This effect is likely amplified under dry conditions, where limited moisture constrains nitrate input (Carter et al. 2017; Dentener et al. 2006). Under such stress, microbial repair processes may also be impaired, reinforcing reliance on atmospheric N_2O as an alternative electron acceptor (Pauleta et al. 2019). Filtering and degassing the incubation water to reduce the microbes and dissolved gases further enhance negative N_2O dynamics. Under fully anoxic conditions, *P. glauca* did not enter cryptobiosis, yet *nosZ* expression remained low and no enhancement of N_2O reduction was observed, indicating that net N_2O dynamics were not stimulated under these conditions. Taken together, these findings suggest that oxygen availability, low temperature, and adequate hydration may favour N_2O reductase activity, thereby enhancing net negative N_2O dynamics. Under N-limited conditions typical of boreal forests (Dentener et al. 2006), the microbiome may also rely on N_2 fixation, despite its high energetic cost, as an essential N source (Grimm et al. 2021; Palmqvist and Dahlman 2006). Such complementary strategies may illustrate how lichens and their associated microbes sustain metabolism under resource-limited environments (Hallin et al. 2018; Kranner et al. 2003). This interpretation is supported by the high proportion of *nifH* gene copies detected in the targeted metagenomic dataset of the *P. glauca* microbiome, indicating a strong potential for N_2 fixation alongside N_2O dynamics processes. Ammonium appears to be the preferred N source for *P. glauca*, with elevated availability

linked to higher growth rates of both the lichen and its photobiont algae (Palmqvist and Dahlgren 2006). This preference may shape microbial interactions and N_2O dynamics, as reliance on ammonium could reduce the importance of nitrate-driven denitrification pathways.

4.4 | Ecological Implications

This study provides the first direct evidence of *nosZ* expression within the microbiome of an epiphytic lichen. *Platismatia glauca* is widely distributed across Africa, Asia, Europe, and North America (Stenroos et al. 2021) and its genome has recently become available (Yahr 2024). Genome analyses may further clarify the contribution of the mycobiont to nitrogen transformations, such as nitrite reduction. Our results indicate that *P. glauca* and its associated bacteria can reduce N_2O to N_2 , particularly at 5°C under oxic conditions. This pattern is consistent with boreal forest dynamics, where N_2O uptake typically peaks during colder months alongside reduced photosynthetic activity (Brodo et al. 2002). The role of organic nitrogen within lichen tissues, and its subsequent mineralisation to ammonium and oxidation to nitrate available to lichens, remains uncertain but could help explain the reduced N_2O sink observed during warmer periods (Brodo et al. 2002). The pioneering nature of this work highlights the potential role of *P. glauca*, and epiphytic lichens more broadly, in mitigating atmospheric N_2O . Taken together, our findings provide experimental support for the three hypotheses tested in this study. First, the microbiome of epiphytic lichens acted as a net sink for N_2O under N-limited conditions. Second, moisture and nitrate modulate and increase the net N_2O dynamics. Third, low temperatures favoured microbial communities adapted to N_2O reduction, whereas higher temperatures promoted incomplete denitrification. These patterns underline the ecological potential of lichens and their microbial partners to influence N_2O dynamics in boreal ecosystems.

4.5 | Future Perspectives and Conclusions

Coming efforts should extend to additional lichen species, seasonal time points, a broader range of environmental conditions, helping to discern effects of light, moisture and reactive N. Future studies should explicitly disentangle the individual effects of moisture and nitrate availability on N_2O dynamics in lichens, as these factors co-occur under natural deposition but were not independently manipulated in the present study. Expanding metatranscriptomic and metagenomic datasets will also be crucial for clarifying regulatory mechanisms differentiating *nosZ* clades, and for defining the contribution of lichens to N cycling in cold ecosystems with fluctuating light and moisture regimes. Recent evidence for direct biological fixation of N_2O suggests a potentially novel, yet unexplored, link between N_2O dynamics and nitrogen fixation processes (Si et al. 2023). However, there is currently no direct evidence for such a mechanism in lichen-associated microbiomes, and its relevance should therefore be addressed in future studies.

Epiphytic lichens contribute to N_2O dynamics in boreal forests, as all four studied species showed a capacity for net N_2O uptake potential. Molecular analysis in *P. glauca*, the dominant

epiphytic lichen in the branches of *Picea abies*, provides evidence of a microbiome with complete dissimilatory denitrification capability. Furthermore, increased *nosZ* gene and transcript copy numbers were found in *P. glauca* samples incubated at close to the atmospheric content of N_2O (~909 ppb), at lower temperature, and under oxic and dark conditions, matching the highest N_2O consumption. Understanding how these microbial communities balance N_2O emission and consumption is therefore of high ecological relevance, particularly in the context of greenhouse gas mitigation under climate change.

Author Contributions

Henri M. P. Siljanen: conceptualisation, investigation, funding acquisition, methodology, validation, writing – review and editing, software, formal analysis, supervision, resources, project administration. **Carlos Palacin-Lizarbe:** writing – review and editing, supervision, conceptualisation, investigation, validation, methodology. **Jussi Ronkainen:** software, visualisation, methodology. **Dhiraj Paul:** writing – review and editing, software, methodology, visualization, data curation, formal analysis. **Johanna Kerttula:** methodology, formal analysis. **Vincenzo Abagnale:** conceptualisation, investigation, funding acquisition, writing – original draft, methodology, writing – review and editing, software, data curation, formal analysis, visualisation.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The sequencing data generated in this study have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession numbers PRJNA1282212 (targeted metagenomics) and PRJNA1282427 (PCR amplicon sequencing).

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Table S1:** Control dynamics of CO_2 and N_2O . Mean ($\pm$ SD, $n = 3$) CO_2 and N_2O dynamics measured in empty bottle and cryptobiosis (quiescent, dry lichen) control incubations. Statistical significance indicates one-sample tests against a theoretical zero slope (i.e., no net gas dynamics) and are reported only to assess whether a measurable slope was detected in each control. Pairwise p -values compare control treatments against the empty bottle reference.

Dynamics marked with † fall within the instrumental detection threshold (IDT) derived from calibration standards analysed during the run and are therefore indistinguishable from instrumental drift. **Table S2:** Clade 1 and 2 primers. **Table S3:** qPCR solutions and proportions. **Table S4:** Results of ionic chromatography analyses conducted on melted snow samples processed using microfiltration and a degassing system. **Table S5:** *nosZ*-targeted metagenomic sequencing reads for incubated samples at the indicated temperature. *Samples showing under the detection limit (UDL) reads at the respective temperature are the outcome of a very conservative bioinformatic analysis (including only protein functionally validated *nosZ* sequences in the UniProt and SwissProt databases), causing a higher detection limit for targeted metagenomic analysis compared to *nosZ* copies measured using qPCR (Figure 3). **Figure S1:** Relative abundance (mean + SD) N cycle-related genes in *P. glauca* under aerobic conditions.