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**Brief Communication**

# Microorganisms in the phyllosphere of Norway spruce controlling nitrous oxide dynamics

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## Abstract

Current climate change assessments and greenhouse gas flux models often lack information on the microbiological processes that consume atmospheric nitrous oxide ($N_2O$), a potent greenhouse gas. There is limited understanding of phyllospheric microorganisms controlling $N_2O$ exchange. In this study, we determined the microbial potential for $N_2O$ consumption in aboveground vegetation in boreal forests. For this, we collected shoot samples from upland spruce forests in Finland and used a novel targeted metagenomics approach with a hybridization capture of gene-specific probes. Most of the samples contained *nos*Z genes, encoding the $N_2O$ reductase. Phylogenetic placement showed a significantly higher relative abundance ($P < .01$) of *nos*Z Clade I than *nos*Z Clade II. Bacterial members such as Comamonadaceae, *Hydrogenophaga*, and *Paracoccus*, which all harbor *nos*Z Clade I, were found in high relative abundance in the spruce shoots across the sites, suggesting they play a role in $N_2O$ consumption capabilities in the spruce phyllosphere. Anoxic incubations, utilizing gas chromatography for $N_2O$ analyses, showed potential $N_2O$ consumption activity across the spruce samples. The presence of *nir*K and *nir*S suggests potential for denitrification, possibly resulting in $N_2O$ production. Our finding provides evidence of microbial communities in spruce canopies with potential for $N_2O$ exchange. Given the vast coverage of boreal forests globally, understanding the role of phyllospheric microorganisms in $N_2O$ exchange is crucial for improving the accuracy of greenhouse gas models and enhancing climate prediction reliability.

**Keywords:** greenhouse gas (GHG); nitrous oxide; *nos*Z; phyllospheric; microbial community; probe captured metagenomics

## Background

Nitrous oxide ($N_2O$) is a major, long-lived greenhouse and stratospheric ozone layer-depleting gas, mainly produced by the microbial processes nitrification and denitrification [1, 2]. The only process known to reduce $N_2O$ to molecular nitrogen ($N_2$) in the biosphere is microbial reduction by both denitrifying and non-denitrifying microorganisms [3]. Nitrous oxide reduction is catalyzed by the enzyme $N_2O$ reductase, which is phylogenetically divided into two clades: Clade I and Clade II, and encoded by the *nos*Z gene [4, 5]. Several studies have investigated the role of $N_2O$-producing and consuming microorganisms for net $N_2O$ emissions from soil; less is known about aboveground, i.e. phyllospheric, compartments despite indications that plants or their associated microbiome contribute to $N_2O$ fluxes [6, 7]. A recent laboratory study demonstrated that the leaf microbiome in an urban greenery plant consumes $N_2O$ [8], and it has also been shown that canopy nitrification serves as a significant source of soil nitrate in forest ecosystems [9], indicating the important contribution of canopy communities to nitrogen cycling. The Earth's leaf surface area is around 400 million km$^2$ [8], emphasizing the critical role of the phyllosphere microbial community as a vital resource and its potentially significant role in greenhouse gas exchange. Therefore, identifying the microbial processes and specific microorganisms that are implicated in both the production and consumption of $N_2O$ in the phyllosphere of trees is essential for advancing our understanding of the role of forest canopies in global $N_2O$ budgets.

The aim of this study was to determine the prevalence of microorganisms with the capacity for $N_2O$ exchange in boreal spruce shoots and the $N_2O$ reduction activity in these shoots. However, it is challenging to isolate N-cycling genes from total DNA derived from plant tissue, primarily due to the overwhelming presence of plant DNA, but also because these functional genes are significantly less abundant compared to those involved in primary metabolism in the microbial community. To confront this issue, we employed metagenomics combined with a cutting-edge probe capture technique designed to effectively identify microbial N cycling genes within the spruce shoot samples (see detailed supplementary materials and methods). The probe capture method has distinct advantages over traditional amplicon sequencing, which can introduce PCR bias, and is

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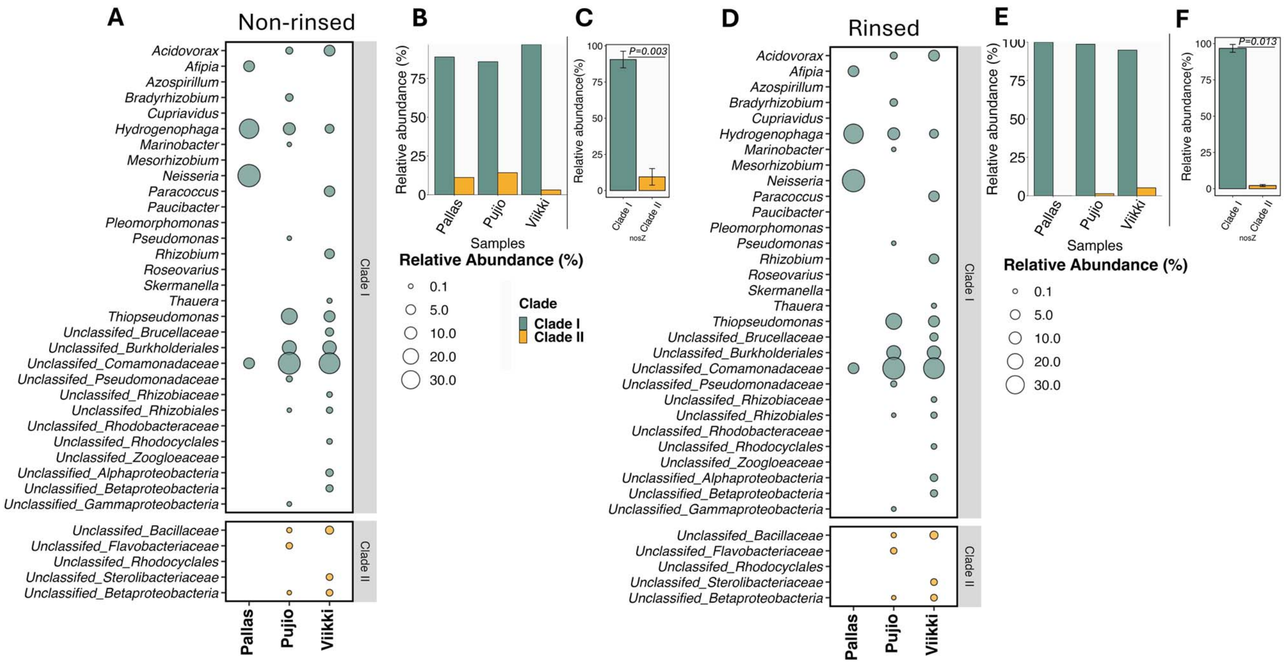

**Figure 1.** Prevalence of *nos*Z Clade I and Clade II in spruce shoots. The taxonomic distribution of *nos*Z Clade I and Clade II among the (A) non-rinsed samples (endophytes) and (D) rinsed samples (whole microbiome). The sequences that are not classified at the genus level but at the family level are designated as "unclassified" followed by the family name. The size of each circle represents the relative abundance of each taxon, while different colors (green and yellow) indicate Clade I and Clade II. Relative abundance of *nos*Z Clade I and Clade II across sites of (B) non-rinsed and (E) rinsed samples. Overall *nos*Z Clade I and Clade II distribution for (C) non-rinsed and (F) rinsed samples. All *P*-values are for the Wilcoxon test.

more effective than shotgun sequencing for studying specific microbial functions in host-microbe ecosystems dominated by DNA from the host [10, 11]. Using targeted probes to selectively sequence specific genes also allows for gene detection of rare microorganisms.

## Results and discussion

To evaluate the potential for the $N_2O$ exchange within the boreal forest phyllosphere, we collected samples from three locations across Finland: Pallas, Viikki, and Puijo (Details are provided in the Supplementary Material, Supplementary Fig. S1). Functional genes involved in $N_2O$ consumption and production were successfully identified from DNA extracted from the spruce shoots using probe-captured metagenomics (Supplementary Fig. S2). Notably, both *nos*Z clades were identified (Supplementary Fig. S3A), with *nos*Z Clade I being more abundant than Clade II in all shoot samples (Supplementary Fig. S3B). The family Comamonadaceae, along with the genera *Hydrogenophaga* and *Paracoccus*, which belong to the *nos*Z Clade I community, were present in all spruce shoot samples collected from the sites. These bacteria are previously reported as aerobic or facultatively aerobic and have the capability to reduce $N_2O$ [12–14], making them suitable for the phyllospheric region. The dominating *nos*Z Clade I taxa were consistently observed across both rinsed (targeting the endophytes) and non-rinsed (targeting the entire shoot microbiome) spruce shoots, with the exception of *Paracoccus* (Fig. 1A and D). *nos*Z Clade I organisms had a significantly ($P < .01$) higher relative abundance compared to *nos*Z Clade II in both rinsed and non-rinsed shoots (Fig. 1 and Supplementary Fig. S3). Additionally, phylogenetic analysis revealed that microorganisms belonging to *nos*Z Clade I and Clade II contain both distinct and phylogenetically comparable groups of organisms, depending on the collection site of the spruce samples (Supplementary Fig. S4).

The majority of previous research on $N_2O$ emission/consumption and the involvement of microorganisms in soil samples has shown either a dominance of *nos*Z Clade II or an equal abundance of the two clades [3, 15]. However, in root-associated communities, *nos*Z Clade I typically dominates over *nos*Z Clade II [9, 10]. *nos*Z Clade I is primarily found in Pseudomonadota [15], which may grow faster than organisms with *nos*Z Clade II, thereby outcompeting them [16]. Among Pseudomonadota with *nos*Z Clade I, there is a dominance of organisms with a complete denitrification pathway [17]. In addition to *nos*Z, functional genes involved in different steps of the denitrification pathway, i.e. *nir*S, *nir*K, *nor*B, *nar*G, were detected from the spruce shoot samples by captured metagenomics, both in rinsed and non-rinsed samples (Supplementary Fig. S2). This indicates that complete denitrification can occur, at least at the community level. Studies using pure cultures of denitrifying microorganisms have demonstrated that those with a complete pathway can be more competitive at low nitrate levels, as they can utilize all electron acceptors involved in the pathway [18]. Additionally, complete denitrifiers are often more metabolically flexible, allowing them to utilize a wider range of carbon substrates [19]. Altogether, this may help explain the prevalence of *nos*Z Clade I organisms in spruce shoots.

To confirm the role of microorganisms in spruce shoots concerning $N_2O$ dynamics, we conducted an anoxic incubation study to measure the $N_2O$ reduction potential (detailed experimental information is provided in the supplementary methods). Our findings demonstrated that $N_2O$ reduction is higher in samples with epiphytic microorganisms (rinsing media) compared to those with endophytic microorganisms (rinsed spruce shoots) (Fig. 2A). There was significant variation among the individual biological samples: four out of nine showed true $N_2O$ reduction, distinguishing them from the controls without shoots (Fig. 2A; Supplementary Fig. S5A). These results indicate that microorganisms in the spruce phyllosphere have potential for

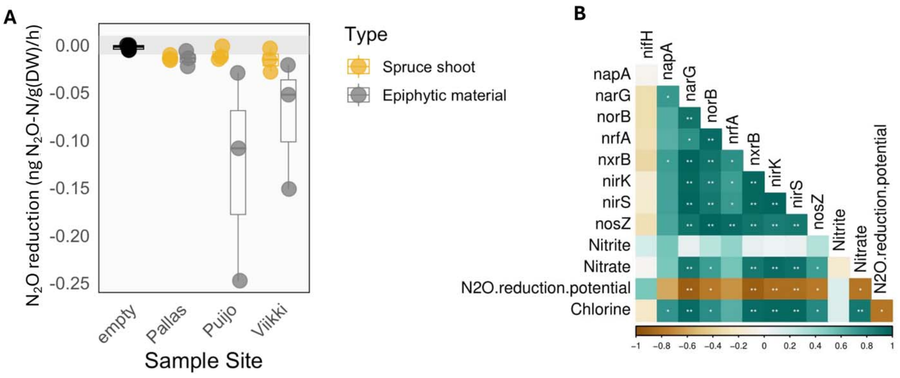

**Figure 2.** $N_2O$ reduction potential in spruce shoots. (A) $N_2O$ reduction potential measurements during incubation study of both the rinsed spruce shoots (endophytes) and rinsing media (epiphytes rinsed from the shoots, mean ± standard error, $n = 3$). The grey horizontal line indicates the zero line for the apparent $N_2O$ uptake. There were no significant differences between samples and control blanks at each site (Wilcoxon test). (B) Correlation analyses between nitrogen-cycling genes, tree chemical properties, and $N_2O$ reduction potential (* = $P < .01$, ** = $P < .05$; Spearman's correlation). Relative abundances of nitrogen cycling genes were obtained from captured metagenomics and chemical data from ion estimation conducted using ion chromatography of epiphytic water samples from the incubation experiment.

$N_2O$ reduction. Additionally, we observed variation in nitrite and nitrate content in shoot surfaces (Supplementary Table S1), which could be linked to nitrogen (N) deposition from the atmosphere, explained by the proximity to a city. Furthermore, nitrite and nitrate serve as nitrogen sources for plant cells, contributing to the variation in their content. Correlation analysis also revealed a significant positive correlation ($P < .05$) between denitrification genes, including *nos*Z abundance and nitrate and nitrite content, and a negative association with total $N_2O$ reduction potential. This suggests that the prevalence of denitrifiers may be important for $N_2O$ reduction in the boreal spruce forest ecosystem (Fig. 2B).

In conclusion, our findings, derived from a novel targeted metagenomics tool, provide new insights into the phyllospheric microbes associated with spruce trees, highlighting both $N_2O$ production and consumption genes. Notably, we observed a higher abundance of *nos*Z Clade I among $N_2O$-reducing organisms, underscoring the unique characteristics of plant-associated microbiomes. Our study centered on the reduction processes that may occur in anoxic or low-oxygen microhabitats within the phyllosphere, which can develop due to rain or dew accumulation [8]. Future studies should widen the scope to also include aerobic processes to reveal the scale of potential $N_2O$ production. Further, incorporating transcriptomics would advance our understanding of which $N_2O$-exchanging microorganisms and processes are active under different conditions. Finally, systematic studies incorporating a diverse array of tree species across broader spatial and temporal scales are essential for accurately assessing the ecological relevance of these microorganisms and their impact on the $N_2O$ balance.

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## Supplementary material

Supplementary material is available at *ISME Communications* online.

## Conflicts of interest

The authors declare no conflicts of interest for this manuscript.

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## Data availability

The data sets generated and analyzed during this study are available in the following repositories. Raw sequence data have been deposited in the NCBI SRA database under accession number SRR28227330-SRR28227352, with the Bioproject ID PRJNA1083928.

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