**Sphagnum capillifolium** holobiont from a subarctic palsa bog aggravates the potential of nitrous oxide emissions

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Melting permafrost mounds in subarctic palsa mires are thawing under climate warming and have become a substantial source of \(N_2O\) emissions. However, mechanistic insights into the permafrost thaw-induced \(N_2O\) emissions in these unique habitats remain elusive. We demonstrated that \(N_2O\) emission potential in palsa bogs was driven by the bacterial residents of two dominant *Sphagnum* mosses especially of *Sphagnum capillifolium* (SC) in the subarctic palsa bog, which responded to endogenous and exogenous *Sphagnum* factors such as secondary metabolites, nitrogen and carbon sources, temperature, and pH. SC’s high \(N_2O\) emission activity was linked with two classes of distinctive hyperactive \(N_2O\) emitters, including *Pseudomonas* sp. and *Enterobacteriaceae* bacteria, whose hyperactive \(N_2O\) emitting capability was characterized to be dominantly pH-responsive. As the *nosZ* gene-harboring emitter, *Pseudomonas* sp. SC-H2 reached a high level of \(N_2O\) emissions that increased significantly with increasing pH. For emitters lacking the *nosZ* gene, an *Enterobacteriaceae* bacterium SC-L1 was more adaptive to natural acidic conditions, and \(N_2O\) emissions also increased with pH. Our study revealed previously unknown hyperactive \(N_2O\) emitters in *Sphagnum capillifolium* found in melting palsa mound environments, and provided novel insights into SC-associated \(N_2O\) emissions.

**KEYWORDS**

*Sphagnum* moss, bacteria, \(N_2O\) emitters, \(N_2O\)-related genes, pH, permafrost peat

**Introduction**

Arctic permafrost soils store ample nitrogen (N) reservoirs that may be subject to remobilization due to climate warming (Christensen et al., 2013), that leads to permafrost degradation and thawing (Borge et al., 2017). After permafrost thaws, increased nitrous oxide (\(N_2O\)) emissions are observed in arctic permafrost
peatlands (Voigt et al., 2017a,b). \( \text{N}_2\text{O} \) is a potent greenhouse gas and contributes to the disruption of the ozone layer (IPCC, 2007; Ravishankara et al., 2009). Therefore, urgency to understand the primary source of \( \text{N}_2\text{O} \) emissions in this arctic environment is crucial.

Peatlands store one-third of global soil carbon, and boreal peatlands account for 83% of the global peatland area (Eurola et al., 1984; Savolainen et al., 1994). Bare peat in permafrost peatlands has been identified as a hot spot for \( \text{N}_2\text{O} \) emissions due to low availability nitrogen (N) competition in subarctic tundra (Repo et al., 2009; Marushchak et al., 2011). \emph{Sphagnum}-dominated bogs have low nutrient content, low primary production, low-quality plant litter, low litter decomposition rates, and low mineral content combined with a low pH (4.5) environment, which is vital for carbon (C) sequestration (Chronáková et al., 2019). Mineral N deposition to \emph{Sphagnum} bogs has progressed, with ammonification, ammonia oxidation, and denitrification playing a critical role in the emission of \( \text{N}_2\text{O} \) (Van Geem, 1998; Francis et al., 2007). In addition, the water table level also affects \( \text{N}_2\text{O} \) emissions in northern peatland, as lowering the water table leads to increased \( \text{N}_2\text{O} \) production (Regina et al., 1996). Once the peatlands are drained, \emph{Sphagnum} vegetation and surface peat layers are exposed to the atmosphere, activating nitrification due to ammonium (NH\(_4\)) release in aerobic peat degradation, followed by denitrifier stimulation in N-enriched conditions to emit \( \text{N}_2\text{O} \) (Martikainen et al., 1995; Regina et al., 1999; Minkinen et al., 2020). Palmer and Horn (2012) reported that palsa peatlands in the northwestern Finnish Lapland showed \( \text{N}_2\text{O} \) emissions \emph{in situ} from –0.02 to 0.01 \( \mu \text{mol} \text{N}_2\text{O} \text{ m}^{-2} \text{ h}^{-1} \). Emissions of \( \text{N}_2\text{O} \) may rise considerably during the thaw of permafrost, representing another ongoing change in northern peatlands. It was reported that a five-fold increase in \( \text{N}_2\text{O} \) flux from palsa mire peat in a permafrost thaw experiment (Voigt et al., 2017b) occurred. However, determining which active \( \text{N}_2\text{O} \) emitters in these northern ecosystems contribute to high emissions remains largely elusive.

\emph{Sphagnum} mosses (non-vascular plants) dominate the vegetation of many northern mire ecosystems and harbor a high diversity of nitrifiers and denitrifiers (Dedysh et al., 2006; Gilbert et al., 2006; Opelt et al., 2007). In these moss communities, \( \text{N}_2\text{O} \) gas is mainly produced \emph{via} nitrification, nitrifier denitrification, and denitrification pathways (Wragge et al., 2001). High hummocks in bogs and palsa mire permafrost mounds have relatively thick aerobic acrotelm layers and are the most potential microhabitats to \( \text{N}_2\text{O} \) emissions. These microhabitats are characteristically dominated by \emph{Sphagnum fuscum} (SF) and \emph{Sphagnum capillifolium} (SC) (Markham, 2009; Novak et al., 2015; Zhong et al., 2020), which are widely distributed throughout European and North American peat bogs. These keystone species develop climax-type, raised bog hummock vegetation. Upon exposure to high N inputs, polyphenol secondary metabolites produced by these \emph{Sphagnum} mosses, such as caffeic acid, are often reduced (Bragazza and Freeman, 2007; Montenegro et al., 2009). These secondary metabolites may impact the activity and community composition of the microbiota within the holobiont and the associated \( \text{N}_2\text{O} \) emission rates (Wang and Cernava, 2020).

Our previous work has demonstrated that the \( \text{N}_2\text{O} \) source in southeastern Finland was mainly from \emph{Sphagnum} moss rather than peat soil. However, this previous study only focused on the single keystone and dominant species of SF in Finnish temperate marine climate areas (Nie et al., 2015). The different contributions of \( \text{N}_2\text{O} \) emissions between several dominant \emph{Sphagnum} species, especially in a typical subarctic permafrost peatland [hot-spots of \( \text{N}_2\text{O} \) emission (Voigt et al., 2017b)] in Finland, is largely unknown. This study uses SF as the control plants and aim to answer three questions: (1) Are the \( \text{N}_2\text{O} \) emission potentials between the two dominant \emph{Sphagnum} species (SC and SF) similar or different in the subarctic palsa bog? (2) How does the culture-based \( \text{N}_2\text{O} \) assay for the bacterial community composition of the two \emph{Sphagnum} species influence the \( \text{N}_2\text{O} \) emission potential? (3) What is the dominant process of \( \text{N}_2\text{O} \) production by active \( \text{N}_2\text{O} \) emitters under aerobic conditions of peat bogs? By investigating \( \text{N}_2\text{O} \) emission potential in SF and SC grown in drained palsa peat bogs of northwestern Finland, we aim to characterize the dominant \( \text{N}_2\text{O} \) emitters hidden in the microbiota of SF and SC in association with their \( \text{N}_2\text{O} \) emission traits in response to major holobiont factors.

**Materials and methods**

**Sampling \emph{Sphagnum} mosses**

Composite samples of SF and SC (photos of them at one site are shown in Supplementary Figure S1) were collected from a plateau of a permafrost mound of a palsa mire near Kilpisjärvi (68°43'; 21°25'N), northwestern Finland (Figure 1A). Each sample of SC/SF was formed from three random sampling sites with three replicates in August–September, 2014. SC and SF were collected from the same patch (within 50–100 cm) and the sampling sites were 50 to 100 m away from each other. From each sampling site, random 533 to 565 individual plants of either SC or SF were collected and mixed for each sample in order to guarantee the sample's representation. Both SC and SF were collected from large homogeneous stands with a 40 cm thaw layer above the permafrost surface. The region has a low annual mean temperature (~2.3°C) and moderate mean annual precipitation (487 mm). The growing season is one of the shortest in continental Europe (~100 d when the mean daily temperature is ≥5°C). The \emph{Sphagnum} samples stored in Ziploc® bags at 4°C were used for further culture-based \( \text{N}_2\text{O} \) emission measurements.
Comparison of N$_2$O emission potentials in two Sphagnum mosses

To evaluate the potential for N$_2$O emission of the two *Sphagnum* mosses under an experimental nitrogen load, we took three *Sphagnum* mosses plants (~0.1 g in dry weight) randomly from the respectively, composite sample of SC and SF using sterilized tweezers. At the same time, we standardized the dry weight for the N$_2$O assay. Either 100 µL of *Sphagnum* moss leaf extract (100 mg/10 ml) or 3 plants were added to N$_2$O assay medium [10 ml of modified Winogradsky’s Gellan (MWG) medium containing 0.005% yeast extract and solidified with 3% gellan gum with 22.6 ml of headspace in each vial (30 ml gas-chromatographic vial with a butyl rubber plug) (Nichiden-Rika Glass Co., Kobe, Japan)] with 0.05% sucrose diluted with sterilized Milli-Q water (the solution was adjusted to pH = 5.0 with 2 M H$_2$SO$_4$) (three replicates in each case) (Hashidoko et al., 2008). After incubation at 15°C (according to the mean value of summer temperature of Finland) for 7 days in the dark, an N$_2$O assay was carried out by using an electron capture detector (ECD)-gas chromatograph (Shimadzu GC-14B, Kyoto, Japan) connected to a Porapak N column (1 m long, Waters, Milford, MS, USA). In another treatment, 0.1 g L$^{-1}$ of caffeic acid instead of 0.05% sucrose was added as the carbon source to the vials with three plants (~0.1 g in dry weight) randomly taken from the above composite samples (pH 5). A control for the assay, without any carbon source, was also performed simultaneously (three replicates in each case). After incubation at 15°C in the dark for 4, 8, and 15 days, an assay of N$_2$O was performed as mentioned above.

DGGE profiling of the bacterial communities in two *Sphagnum* species

Polymerase chain reaction-denatured gradient gel electrophoresis (PCR-DGGE) was performed to observe the culture-based bacterial communities on the leaves of the two *Sphagnum* mosses. First, genomic DNA was extracted from the medium after the N$_2$O assay using an Isoplant II DNA Extraction kit (Nippon Gene, Toyama, Japan). The PCR steps and conditions were as follows: PCR denaturation for 5 min at 95°C, and 30 cycles of amplification (15 s at 95°C, 30 s at 55°C, 30 s at 72°C), and 10 min elongation at 72°C. Then PCR products for DGGE were obtained by using the common 16S rRNA primers GC-341F (CGC CCG CCG CGC CCC GCG GGG GTC CCG CCG CCC CCG CCC GCC T AC GGG AGG CAG CAG) and 907R (CCG TCA ATT CCT TTR AGT TT) ([Ferris et al., 1996](#10.3389/fpls.2022.974251)) and run on a 30–70% denatured gradient gel (6% w/v). The sequences of DGGE-cutting bands were obtained.
using an ABI prism™ 310 Genetic Analyzer and retained in the NCBI (BioProject No. PRJNA681491).

Culture-dependent screening and identification of $N_2O$ emitters

100 µl of medium with three Sphagnum mosses (after incubation for 7 days) was diluted 1×10^4- and 10^6-fold and inoculated onto MWG plates to screen $N_2O$ emitters. After incubation for 5 days at 20°C in the dark, 13 distinguishable bacterial colonies characterized by colony characteristics were selected for streak cultivation on MWG plates and transferred to potato dextrose agar (PDA) plates until purified. Each Pure strain [a total of 108 isolates (13 bacterial colonies with 8 replicates), with 100 µl of each bacterial cell suspension (OD=600nm = 0.9–1.0)] was inoculated into an $N_2O$ assay vial with 10 ml of modified MWG medium to test their $N_2O$ emission ability. The three pure strains SC-K1, SC-L1, and SC-H2 (from SC) showed relatively higher $N_2O$ production and were active $N_2O$ emitters (Supplementary Table S2, data collected from six top active $N_2O$ emission-bacterial colonies). The genomic DNA of each strain was extracted, and the 16S rRNA gene was amplified through PCR by using a series of primers 27F, 338R, 341F, 907R, 1080R, 1380R, 1492R, 1112F, and 1525R. Sequencing was performed with an ABI Prism™ 310 Genetic Analyzer (Applied Biosystems, USA) (Nie et al., 2015). All the resulting 16S rRNA gene sequencing datasets were deposited in the NCBI database (accession nos. MW301596–MW301598) and compared with sequences in the nucleotide basic local alignment search tool (BLASTN) database program provided by NCBI (National Center of Biotechnology Information, Bethesda, MD, USA; http://Blast.Ncbi.nlm.nih.gov/Blast.cgi).

$N_2O$ emitters response to nitrogen sources, pH, and temperature

The pure isolates (SC-K1, SC-L1, and SC-H2) pre-cultured on PDA for 4 days at 15°C were separately scraped with a nichrome wire loop and suspended into 1.5 ml Milli-Q water (equal amounts of each pure strain was guaranteed). A 20 µl portion of the inoculant that showed an optical density of OD=600nm 0.9–1.0 was added to the $N_2O$ assay vial and then was thoroughly vortexed for 30s. 1 mM NH$_4$NO$_3$, KNO$_3$, and NH$_4$Cl were tested and incubated at 15°C for 5 days with 0.05% sucrose (pH = 5.0) to determine the optimal nitrogen substrates for pure $N_2O$ emitters. The pH was adjusted with 1 M H$_2$SO$_4$ and 1 M KOH solutions to 4.6, 5.0, 5.7, 6.8, and 7.3 before autoclaving and incubated at 15°C for 5 days with 0.05% sucrose to determine the optimal pH for $N_2O$ emitters. Different temperatures (4, 10, 15, 20, 25, and 30°C) were set in separate incubators and incubated for 5 days with 0.05% sucrose to find the appropriate temperature. All experiments were performed with three replicates.

Carbon source- and polyphenol-supplementation assays

Sucrose and E-caffeic acid were applied as carbon sources and secondary metabolites (polyphenols), respectively, for the microbiota inhabiting Sphagnum moss (Nie et al., 2015). The inoculants were prepared as described in Nie et al. (2015). To observe the responses of the $N_2O$ emitters (SC-K1, SC-L1, SC-H2) to sucrose, 0 (control), 0.05, and 0.5% sucrose were used for the separated/cultivated bacterial strains. To determine the optimal concentrations of E-caffeic acid for $N_2O$ emitters (SC-K1, SC-L1, SC-H2), 0 (control), 0.005, 0.01, 0.05, 0.1, 0.5, and 1 g L$^{-1}$ E-caffeic acid were used. Each treatment contained three analytical replicates incubated at 15°C for 5 days with inoculants for $N_2O$ emitters. Their $N_2O$ emissions were separately measured.

Analysis of denitrification rates of $N_2O$ emitters

We applied the acetylene inhibition assay, which is widely used to measure denitrification rates (Sørensen, 1978). The activity of $N_2O$ reductase was inhibited by adding acetylene (C$_2$H$_2$) at pH 5.0 and 7.0, and 10% C$_2$H$_2$ gas was injected into the headspace of vials inoculated with $N_2O$ emitters (the same with above inoculation method) (Bollmann and Conrad, 1997). At the same time, treatments without injected C$_2$H$_2$ gas were carried out as controls to compare the $N_2O$ reductase activity (three replicates in each case). Incubation conditions were the same as described above.

Detection of nitrogen cycling functional genes in $N_2O$ emitters

Functional genes of nitrogen cycling, including narG, nirK, nirS, and nosZ (Supplementary Figure S4), were detected by using the PCR method. The target genes were amplified by using the primers narGF (TGG GCC AAG GCC CAT GAG TAC) and narGR (TTT CCG ACC AAG TGG CCG TCG), nirS637Af (AAG GYS AAG GAR ACS GG) (Nie et al., 2015) and nirSR3cd (GAS TTC GGR TGS GTG CTG T) (Throbäck et al., 2004), nirK-1F (GGM ATG GTK CCS TGG CA) and nirK-5R (GCC TCG ATC AGR TTR TGG) (Braker et al., 1998), nosZ-1111F (STA CAA CWC GGA RAA SG), nosZ-661F (CGG CTG GGG GCT GAC CAA), nosZ-1527R (CTG RCT GTC GAD GAA CAG),
and nosZ-1773R (ATR TCG ATC ARC TGB TCG TT) (Scala and Kerkhof, 1998). The exact reaction conditions of the PCR amplifications are presented in Supplementary Table S1.

Statistical analysis

The data were expressed as mean with standard error (SE). The data were examined for normality and homoscedasticity using the Shapiro-Wilk’s and Levene’s tests, respectively (SPSS, version 23.0). All data was found to fit the normal distribution and homogeneity of variances. Comparisons were made using a one-way analysis of variance (ANOVA) among two or more groups. One-way ANOVA was used to compare differences in N₂O emission with different inoculants (Sphagnum mosses or their leaves washing), physicochemical factors [pH, temperature, sucrose, nitrogen types, and secondary metabolite (E-caffeic acid)], and C₂H₂ inhibition assay. Using the Fisher’s Least Significant Difference(LSD) method, multiple comparisons were carried out using IBM SPSS 23.0 software (Chicago, Illinois, USA).

Results

N₂O emission potential and microbial communities

After incubation for 7 days, we found that the average N₂O emissions of SF were 1.9 ng vial⁻¹ d⁻¹ in the leaf extract and 69.9 ng vial⁻¹ d⁻¹ in the leaf samples. The SC sample showed N₂O emissions of 9.1 in the leaf extract and 956.2 ng vial⁻¹ d⁻¹ in the leaf samples (Figures 1B,C).

The PCR-DGGE profile showed that the major culture-based bacterial communities in these Sphagnum mosses were similar. However, the SC sample harbored the family Enterobacteriaceae (Figure 1D, Supplementary Figure S2), while the SF sample contained the genus Dyella of Gammaproteobacteria (Supplementary Figure S2). N₂O production increased with 0.1 g L⁻¹ caffeic acid addition in both samples, and the effect was significantly larger in the SC sample than in the SF sample (p < 0.05) (Figures 2A,B).

Major N₂O emitters in Sphagnum mosses

Compared to PCR-DGGE, the culture-based approach revealed distinctive profiles of N₂O emitters (Supplementary Figure S2). Two Burkholderia spp. were isolated from the SF sample, while three Gammaproteobacteria (one Pseudomonas sp., one Serratia sp., and an unidentified Enterobacteriaceae bacterium) and one Burkholderia sp. were isolated from the SC sample. Among them, Serratia sp. SC-K1, Enterobacteriaceae bacterium SC-L1, and Pseudomonas sp. SC-H2 showed the most efficient N₂O emissions, and the activity of N₂O emissions was the greatest in Pseudomonas sp. SC-H2, then Enterobacteriaceae bacterium SC-L1, and then Serratia sp. SC-K1 (pH 5) (Table 1, Supplementary Table S2).

Effects of substrate type, temperature and pH on microbial N₂O emissions

According to the N₂O production responses to different nitrogen sources, KNO₃ was the most efficient substrate for N₂O emission, followed by NH₄NO₃, while almost no N₂O emissions were found with NH₄Cl as the substrate. Active N₂O emissions from KNO₃ indicated that the three N₂O emitters were nitrate reducers (Figure 3). N₂O emissions increased as the pH increased from 4.6 to 7.3. Enterobacteriaceae bacterium SC-L1 and Serratia sp. SC-K1 showed a temporary increase at a pH value of 5 but no drastic increase in N₂O emissions, indicating adaptation to acidic environments (Figures 4A,B). At pH values over 6, Pseudomonas sp. SC-H2 emissions increased sharply, making it the most likely N₂O emitter (Figure 4C). For the three strains used, N₂O emissions also increased with increasing temperature from 4 to 30°C (Figures 4D–F).

Disparate responses of N₂O emitters to caffeic acid and sucrose

The three microbial strains exhibited disparate responses to sucrose and E-caffeic acid (Figure 5). In the absence of added sucrose (control treatment), Serratia sp. SC-K1 emitted more N₂O than Enterobacteriaceae bacterium SC-L1 and Pseudomonas sp. SC-H2, while these last two strains emitted N₂O at higher levels with 0.05% sucrose supplementation (Figures 5A,B). Notably, the response of Pseudomonas sp. SC-H2 to 0.05% sucrose was very drastic, resulting in emission ~2x10³ times higher than without sucrose (Figure 5C). This result demonstrated that Serratia sp. SC-K1 is an oligotrophic bacterium, whereas Enterobacteriaceae bacterium SC-L1 and Pseudomonas sp. SC-H2 are eutrophic bacteria.

For the pure strains of Enterobacteriaceae bacterium SC-L1 and SC-K1, a relatively lower concentration of E-caffeic acid (≤0.1 g L⁻¹) increased N₂O emissions of these two strains, and the optimum concentration was 0.1 g L⁻¹ (Figures 5D,E). Among them, Serratia sp. SC-K1 was very sensitive to 0.1 g L⁻¹, and 13-fold higher N₂O production was found than without E-caffeic acid (Figure 5E). For Pseudomonas sp. SC-H2, when the concentration of E-caffeic acid was above 0.01 g L⁻¹, N₂O emissions decreased significantly (p < 0.01) (Figure 5F).
FIGURE 2
Response of N\textsubscript{2}O production of Sphagnum fuscum (SF) and Sphagnum capillifolium (SC) to caffeic acid. Response of N\textsubscript{2}O production of Sphagnum fuscum (SF) to caffeic acid (A). Response of N\textsubscript{2}O production of Sphagnum capillifolium (SC) to caffeic acid (B). Incubation conditions: pH = 5, incubated at 15°C, 4, 8, 15 days, n = 3, without sucrose. Values are means ± s.e. (shown as error bars).

TABLE 1 Identification of the active N\textsubscript{2}O emitters using 16s rRNA gene sequence.

| Isolates | Length (bp) | Accession No. | Most aligned DNA (Accession No.) | Identities |
|----------|-------------|---------------|----------------------------------|------------|
| SC-K1    | 1528        | MW301598      | Serratia sp. HC3-14(JF312984.1)   | 1515/1526(99%) |
|          |             |               | Serratia sp. HC3-9(JF312979.1)    | 1513/1525(99%) |
|          |             |               | Serratia sp. HC4-9(JF312995.1)    | 1512/1525(99%) |
| SC-L1    | 1165        | MW301597      | Serratia liquefaciens strain Noth_10 (MF716557.1) | 1123/1153(97%) |
|          |             |               | Enterobacteriaceae bacterium ENUB8 (JX162036.1) | 1133/1167(97%) |
|          |             |               | Serratia proteamaculans strain 336X(CP045913.1) | 1132/1167(97%) |
| SC-H2    | 1514        | MW301596      | Pseudomonas sp. LH1G9(CP026880.1) | 1513/1518(99%) |
|          |             |               | Pseudomonas sp. 05CF15-5C (LC007966.1) | 1513/1518(99%) |
|          |             |               | Pseudomonas sp. Pi 3-62 (AB365063.1) | 1512/1517(99%) |

Modest responses of N\textsubscript{2}O emitters to acetylene

There was no detectable difference between the 10% C\textsubscript{2}H\textsubscript{2} and control treatment emissions at a pH value of 5.0. However, in Pseudomonas sp. SC-H2 cultured at a pH value of 7.0, N\textsubscript{2}O emissions upon exposure to C\textsubscript{2}H\textsubscript{2} were drastically increased to four-fold higher than that of the control. Without 10% C\textsubscript{2}H\textsubscript{2}, the production level of N\textsubscript{2}O at a pH value of 7.0 was higher than that at a pH value of 5.0 (Figure 6). This result suggested that the peat ecosystem was highly disturbed at a pH value of 7.0, denitrification was greatly accelerated, and the final denitrification step to reduce N\textsubscript{2}O to N\textsubscript{2} was driven by N\textsubscript{2}O reductase.
Functional genes involved in N$_2$O emission

PCR assays detected the narG gene in the three N$_2$O emitter strains, but only Pseudomonas sp. SC-H2 contained nirS and nosZ genes (Table 2; Supplementary Figure S3). In combination with the results of the C$_2$H$_2$ assay, these results suggested that Pseudomonas sp. SC-H2 is a complete denitrifier. The nirK gene was not detected within Enterobacteriaceae bacterium SC-L1 and Serratia sp. SC-K1.

Discussion

Cultured bacterial communities in the leaves distinguishable between two Sphagnum species

Increased atmospheric N deposition can reduce the growth of some Sphagnum species, such as Sphagnum magellanicum (Aerts et al., 2001; Limpens and Berendse, 2003). In contrast, the production of SF increased with elevated N deposition but decreased as N deposition reached 14.0 kg ha$^{-1}$ yr$^{-1}$ as reported by Vitt et al. (2003). SC can also tolerate a high N supply (Bonnett et al., 2010). Our study offered evidence that individual samples of the latter two Sphagnum species had N$_2$O emission potential reasonably associated with their bacterial communities. In particular, the SC sample harbored specific bacterial communities associated with high N$_2$O emission. Surprisingly, the N$_2$O emission of the SC sample was significantly greater than that of the SF sample (Figure 1B) ($p < 0.01$). Such a large difference in N$_2$O emission between the SF and SC species gives precedence to the hypothesis of potential N$_2$O emission differences in different Sphagnum species.

Based on the analysis of bacterial communities using culture-based PCR-DGGE and isolation of N$_2$O emitters, the major Sphagnum-associated bacterial communities of our samples were consistent with boreal mire and tropical peat forest and included Burkholderia, Mucilaginibacter, Rhodanobacter, and Janthinobacterium but their N$_2$O emission activity was different in varied sites due to differences in climate and habitat environments (Hashidoko et al., 2008; Sun et al., 2014). Janthinobacterium spp. did not show high N$_2$O emission potential in subarctic palsa bog unlike in the tropical peatland soil, which suggested that the N$_2$O emission functions of N$_2$O emitters were changing in different climate zones. Previous experimentation has shown that the Sphagnum microbiota supported the host plant and the entire ecosystem under environmental changes (Bragina et al., 2014). Burkholderia spp. were N$_2$O emitters, but their N$_2$O emission functions were significantly lower than the acid-tolerant Janthinobacterium sp. in a deforested tropical peatland soil, which was previously determined by soil pH (Hashidoko et al., 2010). The Burkholderia spp. isolates in SF were similar to another climate zone in Finland, showing the same species of Sphagnum although in a different climate zone (Nie et al., 2015). Within this study, some unique bacterial strains were found in the leaves of SC, including a Pseudomonas sp. and two Enterobacteriaceae family members. In numerous previous studies, Pseudomonas species (P. denitrificans, P. perfectomarinus, P. fluorescens, P. putrefaciens, P. aeruginosa, and P. nautica) were found performing denitrification (Delwiche, 1959; Payne et al., 1971; Balderston et al., 1976; Sørensen et al., 1980; Dooley et al., 1987; Viebrock and Zumft, 1988;
N\textsubscript{2}O emission by three pure N\textsubscript{2}O emitters (SC-L1, SC-K1, SC-H2) upon the gradient pH and temperature. N\textsubscript{2}O emission by SC-L1 (A,D), SC-K1 (B,E), SC-H2 (C,F) upon exposure to different pH from 4.6 to 7.3 (A–C), and different temperatures from 4 to 30 \degree C (D–F) was analyzed. For the impact of pH on N\textsubscript{2}O emission, the N\textsubscript{2}O emitters were incubated at 15 \degree C for 5 days with 0.05% sucrose (n = 3). For the impact of temperature on N\textsubscript{2}O, the N\textsubscript{2}O emitters were incubated for 5 days with 0.05% sucrose (n = 3 and pH = 5).

SooHoo and Hollocher, 1991; Prudêncio et al., 2000). The isolated Pseudomonas sp. was not found in the bands of PCR-DGGE, possibly due to relatively low abundance under acidic conditions (pH 5) (Figure 4C). Anderson and Levine (1986) offered evidence that Enterobacteriaceae and Serratia sp.’s nitrate respiration produces N\textsubscript{2}O, which was also found in our SC sample. Enterobacter sp. was also found as dissimilatory nitrate reduction to ammonium (DNRA) bacteria in agricultural soils (Heo et al., 2020). Pseudomonas sp. SC-H2, Enterobacteriaceae bacterium SC-L1, and Serratia sp. SC-K1 were responsible for N\textsubscript{2}O emissions in our Sphagnum samples (SC). These findings suggest that the variation in the N\textsubscript{2}O emission potential of Sphagnum found in peatlands is associated with species-specific bacterial communities, which are variable under different species and environments.

**Complex environmental factors also impact N\textsubscript{2}O production of active N\textsubscript{2}O emitters**

The top three active N\textsubscript{2}O emitters (Pseudomonas sp. SC-H2, Enterobacteriaceae bacterium SC-L1, and Serratia sp. SC-K1) from SC increased N\textsubscript{2}O production with increasing
temperature up to 30°C (Figures 4D–F), illustrating a potential rise in N₂O emissions following global warming (Pfenning and McMahon, 1997; Voigt et al., 2017a; Chen et al., 2020). For the three active N₂O emitters, N₂O production was relatively high at a pH value of 7.0 (Figures 4A–C), which is much higher than the naturally low pH of Sphagnum microhabitats (Tahvanainen and Tuomaala, 2003). Although N₂O reduction to N₂ by Pseudomonas sp. SC-H2 was obvious, the N₂O production was still high after 5 days of incubation (Figure 6). This result indicated that N₂O emission hotspots are inclined to be in neutral peatlands, as supported by Palmer and Horn (2015). Combining these results with acetylene inhibition assays at pH
value of 5.0 and 7.0 showed that N₂O reduction to N₂ was almost negligible at a pH value of 5 for these three active N₂O emitters. This result is consistent with a previous study of the lack of N₂O reductase (nos) function at low pH (Liu et al., 2014). This result also suggested that N₂O reduction was inhibited in the acidic environment in the peat bogs. Since the Sphagnum microhabitats are very acidic, N₂O reductase activity is repressed, supporting that N₂O reduction is not a pathway decreasing N₂O emissions in the pristine Sphagnum bog system. Under low-pH conditions, N₂O production by Pseudomonas sp. SC-H2 was small, but N₂O could be accumulated. However, the palsa mounds are formed due to the ice core under the Sphagnum peat layer in the subarctic climate, and once they collapse after permafrost thawing, the peat acidity will be neutralized to some extent by mixing with mineral material and minerogenic water flow (Seppälä, 2011; Takatsu et al., 2022).

Sphagnum mosses are important for peat accumulation and form a carbon pool of global significance. Increasing atmospheric N deposition can activate phenol oxidase in peat bogs and destabilize peat carbon (Bragazza et al., 2006). Phenol oxidase requires bimolecular oxygen for its activity (Freeman et al., 2004), and drying increases aerobic conditions in peatlands (Swindles et al., 2019) and can degrade recalcitrant phenolic materials. Tahvanainen and Haraguchi (2013) showed that this phenolic mechanism is affected by pH. Such changes may reduce the generally high C:N ratio, which increases net N mineralization, nitrification, and denitrification rates, while subsequently increasing the potential of N₂O production in peat bogs, while lower C:N ratios (≤25–30) stimulate N₂O emissions (Huang et al., 2004; Klemedtsson et al., 2005; Maljanen et al., 2012). Connected mechanisms and the release of ice-trapped N₂O are further impacted by thawing permafrost (Voigt et al., 2017b). Our findings indicate that N₂O emissions are not exceptionally high under the naturally cold temperatures and low pH of Sphagnum habitats; rather, substantially high pH and temperatures, and perhaps a connected imbalance of microbial communities in such conditions, induced the highest N₂O emissions. The results warrant caution in interpretation and
against unexpected emission potential under rapidly changing conditions. It also calls for a need to monitor the in situ N$_2$O emissions from different permafrost Sphagnum species in the permafrost in future studies.

Responses of N$_2$O emitters to primary metabolites and secondary metabolites of Sphagnum mosses

Without sucrose, the N$_2$O emitters Enterobacteriaceae bacterium SC-L1 and Pseudomonas sp. SC-H2 could not emit N$_2$O because of their low growth. This result indicated that these two strains were heterotrophic microorganisms that needed to gain C sources from Sphagnum moss and form plant-microbial symbionts between plants and microbes. Interestingly, Serratia sp. SC-K1 grew well without sucrose and emitted much more N$_2$O; meanwhile, it could be significantly inhibited by adding a low concentration of sucrose (0.05%). This result indicated that this strain is an autotrophic microorganism adapted to nutrient-poor environments, using carbon dioxide (CO$_2$) as a C source. These autotrophic microorganisms contribute to CO$_2$ uptake and carbon sequestration. Drained peatland ecosystems have an immense potential for C sinks to maintain the C balance, even though droughts are occasionally caused by decreasing photosynthesis (Minkkinen et al., 2018).

Our study showed that N$_2$O emitters (Serratia sp. SC-K1 and Enterobacteriaceae bacterium SC-L1) could resist relatively higher concentrations of caffeic acid ($\leq 0.1 \text{ g L}^{-1}$), while the N$_2$O emitter (Pseudomonas sp. SC-H2) had low resistance to caffeic acid ($\leq 0.005 \text{ g L}^{-1}$) (Figures 5D–F). These results could explain why we could not find the Pseudomonas spp. using DGGE band sequencing. Polyphenol (caffeic acid) from Sphagnum moss inhibits growth and results in a low relative abundance of Pseudomonas spp. The more abundant Serratia sp. SC-K1 and Enterobacteriaceae bacterium SC-L1 were the dominant N$_2$O emitters due to their higher resistance to polyphenolic compounds. The stimulated N$_2$O production in the Sphagnum moss-microbe vial with 0.1 g L$^{-1}$ caffeic acid confirmed Serratia sp. SC-K1 and Enterobacteriaceae bacterium SC-L1 were the dominant N$_2$O emitters. Serratia spp. are gram-negative bacilli and belong to the family Enterobacteriaceae. The interaction of polyphenolic compounds and Enterobacteriaceae bacteria might directly influence N$_2$O emissions in peatland ecosystems. High concentrations of polyphenols are likely to lower N$_2$O emissions. The response of phenol oxidase to N deposition differs by ecosystem type. In peat bogs, elevated N deposition decreased polyphenols’ contents and decreased the polyphenol ratio to N, which may increase N$_2$O production due to an inverse relationship between N$_2$O emissions and the polyphenol to nitrogen ratio (Pimentel et al., 2015).

N$_2$O production of active N$_2$O emitters

The three N$_2$O emitters preferred KNO$_3$ as a substrate over NH$_4$Cl. This result suggested that these three isolates mainly use DNRA or denitrification to produce N$_2$O gas. For the Enterobacteriaceae bacterium SC-L1 and Serratia sp. SC-K1, the nirS, nirK, and nosZ genes were not detected, but the narG gene was, suggesting that they do not have nitrite reductase and are non-denitrifiers consistent with other Enterobacteriaceae bacteria emitting N$_2$O as a final product (Arkenberg et al., 2011). Enterobacter species are often reported as producing N$_2$O by DNRA (Smith and Zimmerman, 1981). This result indicated that they are also important sources for N$_2$O emissions in SC dominant bogs. Pseudomonas sp. SC-H2 harbored nosZ, nirS, and narG. Therefore, Pseudomonas sp. SC-H2 was a typical denitrifier. Microbial heterotrophic denitrification and DNRA compete for shared resources (Jia et al., 2020).

Although the N$_2$O potential was relatively high in the SC sample, the N$_2$O emissions in situ in the peat bogs were generally low in northern Finland, which might be impacted by the complexity of environmental conditions (Dinsmore et al., 2017). The potential N$_2$O emissions in the field (Repo et al., 2009; Voigt et al., 2017b) and laboratory incubations (Elberling et al., 2010) increased with increasing mineral N availability, permafrost thawing, and drainage. A previous study suggested that drainage of bogs alters nutrient cycling and microbial communities to increase N$_2$O emissions (Frolking et al., 2011). Unvegetated (free of vascular plants) peat surfaces resulting from wind erosion and frost action were hot spots for N$_2$O emission in subarctic permafrost peatlands due to the absence of plant nitrogen uptake, a low C:N ratio, and sufficient drainage (Maruschak et al., 2011; Voigt et al., 2017b). Pseudomonas sp. SC-H2 had negligible N$_2$O emissions at low pH (<4.5), while the other two N$_2$O-emitting Enterobacteriaceae bacteria from SC exhibited contrasting patterns in the Sphagnum bogs. Therefore, the contribution of denitrification and DNRA to N$_2$O emissions in boreal peat bogs should be considered in future studies.

Conclusion

In summary, our study identified several N$_2$O emitters in microbial communities of Sphagnum samples from the subarctic permafrost habitat of palsa mires. A composite sample of SC showed high potential to emit N$_2$O, and a composite of SF showed moderate potential to emit N$_2$O. The N$_2$O emission potential was attributed to distinctive bacterial communities inhabiting moss leaves in both cases. Two classes of hyperactive N$_2$O emitters hidden in the SC holobiont were revealed. Pseudomonas sp. SC-H2 was found to harbor narG, nirS, and nosZ genes. N$_2$O reduction to N$_2$ catalyzed by N$_2$O reductase was noteworthy in the neutral pH microenvironment. The other hyperactive N$_2$O emitters, Enterobacteriaceae bacterium SC-L1
and Serratia sp. SC-K1 lacked the *nirS*, *nirK*, and *nosZ* genes but contained the *narG* gene and emitted NO/N<sub>2</sub>O as the final product, possibly via the DNRA pathway. These findings provided some theoretical evidence for the future N<sub>2</sub>O emission study of the *in situ* subarctic palsa under elevated N availability and global warming.

**Data availability statement**

The datasets presented in this study can be found in online repositories. The names of the repository/Repositories and accession number(s) can be found in the article/Supplementary material.

**Author contributions**

YH and YN designed the research, experiments, and acquired the funds. YH, RI, and TT collected the samples in Finland. YN performed experiments and analyzed data. YN, SYL, XT, XL, SL, TT, RI, and QY wrote and edited the paper. All authors read and approved the final manuscript.

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**Conflict of interest**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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**Supplementary material**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.974251/full#supplementary-material

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