



Article

The Substitution of Sphagnum for Peat as a Culture Substrate Reduces N₂O Emissions from Vegetable Production Systems

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Abstract: Peat-based substrates have been widely used in greenhouse vegetable production (GVP). However, peat is a non-renewable resource, and there is a problem with N₂O emissions when it is used in greenhouse vegetable production due to the application of large quantities of nutrient solutions. Sphagnum (SP) is a precursor substance and a renewable resource for peat formation, and it has good physical and chemical properties. However, there has been no study on the effect of using sphagnum to replace peat in greenhouse vegetable production on N₂O emissions. Therefore, this study used a peat substrate as the control treatment (CK), with sphagnum replacing peat at 25% (25SP), 50% (50SP), 75% (75SP), and 100% (100SP) in six treatment groups. Moreover, lettuce was used as the experimental subject in potting experiments, and the physicochemical properties, N₂O emissions, N₂O isotope δ value, and N₂O-related microbial activity and community structures were determined using different treatments. Compared with the CK treatment, the 25SP treatment significantly reduced N₂O emissions by 55.35%, while the 75SP treatment significantly increased N₂O emissions by 67.76%. The 25SP treatment reduced N₂O to N₂ to the highest extent and demonstrated the lowest contribution of fungal denitrification (FD) and bacterial nitrification (BN) processes, thereby resulting in lower N₂O emissions. In contrast, NH₄⁺ and NO₃[−] were the main substrates for N₂O emissions; the 75SP treatment had higher NH₄⁺ and NO₃[−] contents and a lower relative abundance of the *nosZ* gene, thereby resulting in higher N₂O emissions. In addition, N₂O production and reduction were dominated by bacterial denitrification for all treatments. Thus, this study analyzed the community composition of denitrifying bacterial genera and their association with physicochemical properties. The results indicated that the dominant denitrifying genus in the peat substrate was *Rhodanobacter* and that sphagnum replacement reduced the relative abundance of *Rhodanobacter*. The dominant genus was *Massilia* at 100% sphagnum replacement. More importantly, *Rhodanobacter* was correlated with C/N and electrical conductivity (EC), whereas *Massilia* was affected by NH₄⁺ and the water-filled pore space (WFPS). Therefore, different denitrification-dominant genera were affected by different environmental factors, which indirectly affected N₂O emission. In summary, the 25SP treatment was able to improve nitrogen use efficiency and had no significant effect on lettuce yield. Therefore, 25% sphagnum replacement is the most suitable percentage for peat replacement.



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1. Introduction

Recently, soilless culture systems (SCSs) have gained considerable prominence for their employment in the substitution of soil in greenhouse vegetable production (GVP). Among the various substrate materials employed in SCSs, peat stands out as the most used one [1]. Nevertheless, the cultivation of a peat substrate not only relies on non-renewable resources, but its over-exploitation also results in ecological harm. Hence, it becomes imperative to explore alternative substrate materials that can effectively replace peat in SCSs. Sphagnum, being an essential and renewable resource for peat formation, exhibits numerous advantages such as a high organic matter content, abundant fiber content, a loose and porous structure, as well as excellent aeration and water absorption capabilities [2]. Furthermore, sphagnum can be cultivated artificially, thereby simplifying the cultivation process and proving to be cost-effective [3,4]. Thus, sphagnum emerges as a remarkable substitute for peat [5]. Notably, GVP necessitates the application of nutrient solutions at high levels, which leads to significant emissions of nitrous oxide (N_2O) from peat, soilless substrates [6]. N_2O , being a potent greenhouse gas, exhibits a global warming potential that is 298 times higher than that of carbon dioxide (CO_2), and it ranks as a primary contributor to ozone depletion in the 21st century [7,8]. However, the impact of replacing peat with sphagnum on N_2O emissions remains unknown. Therefore, a comprehensive investigation into the nitrogen transformation mechanism following the substitution of peat with sphagnum becomes invaluable for enhancing nitrogen use efficiency, reducing N_2O emissions, and augmenting crop yield in GVP.

Sphagnum and peat are distinct materials with disparate microbial profiles [9,10], whereas N_2O emissions are closely linked to the microbial processes within the nitrogen cycle. The primary source of N_2O in soilless substrates arises from nitrification and denitrification driven by microorganisms. There are five microbial pathways for N_2O emissions, namely those of bacterial nitrification (BN), archaeal nitrification (AN), fungal denitrification (FD), denitrifier denitrification (DD), and nitrifier denitrification (ND) [6,11]. Nitrification is the process through which $\text{NH}_4^+\text{-N}$ is oxidized to $\text{NO}_2^-\text{-N}$ through the activity of ammonia-oxidizing bacteria (AOB) and archaea (AOA), with N_2O being produced as an intermediate product [12]. Denitrification involves the reduction of $\text{NO}_3^-\text{-N}$ or $\text{NO}_2^-\text{-N}$ to N_2 , with N_2O serving as an intermediate product. This process is controlled by the *nirS* and *nirK* genes, which encode nitrite reductase, as well as the *nosZ* gene, which encodes nitrous oxide reductase [13]. Additionally, fungal denitrification lacks N_2O reductase, thereby resulting in N_2O as the ultimate end product. The *p450nor* gene plays a pivotal role in N_2O production within this process [14]. Recently, the application of N_2O stable isotope technology serves as a valuable tool for quantitatively analyzing the microbial pathways of N_2O production and consumption [15,16]. In this context, the ^{15}N site preference (SP) serves as an indicative measure to elucidate N_2O microbial processes [17]. Furthermore, $\delta^{18}\text{O}\text{-N}_2\text{O}$ assumes a critical role as a key parameter for deciphering N_2O production and consumption. However, N_2O production also relies upon precursors such as $\text{NO}_3^-\text{-N}$, $\text{NO}_2^-\text{-N}$, H_2O , and O_2 . Fortunately, Lewicka-Szczebak et al. [18] postulated that the oxygen (O) in N_2O exclusively originates from H_2O due to the high rate of oxygen exchange between H_2O and oxynitride. Subsequently, Lewicka-Szczebak et al. [19] constructed the $\delta^{18}\text{O}(\text{N}_2\text{O}/\text{H}_2\text{O})$ vs. $\delta^{15}\text{N}^{\text{SP}}$ map to distinguish various microbial processes. Currently, the dual-isotope map of $\delta^{18}\text{O}(\text{N}_2\text{O}/\text{H}_2\text{O})$ vs. $\delta^{15}\text{N}^{\text{SP}}$ has gained widespread application in soil or even soilless substrate environments [20,21]. In the dual-isotope map, the isotope signature values of BN, AN, and FD are classified within the high-value group, while DD and ND are categorized within the low-value group. Currently, certain investigations have examined the microbial processes associated with N_2O emissions by employing multiple methods such as stable isotope analysis, a functional gene abundance assessment, and the characterization of microbial communities [22,23]. These methods provide more information about N_2O production and consumption and present possibilities for investigating the microbial processes occurring in a soilless substrate.

Regardless, the substitution of sphagnum for peat in a soilless substrate inevitably leads to alterations in the physicochemical properties and biological indicators, thereby impacting the process of N₂O emissions and also influencing nitrogen use efficiency and vegetable yield. Hence, this study set five alternative sphagnum substitution schemes to (1) investigate the effects of N₂O emissions and the microbial mechanisms underlying its production and consumption and (2) determine the optimal substitution ratio considering the combined influence of factors such as N₂O emissions, vegetable yield, and nitrogen use efficiency.

2. Materials and Methods

2.1. Experimental Materials and Design

A pot experiment was carried out in a greenhouse light incubator in July 2022 at the Institute of Urban Agriculture, Chinese Academy of Agricultural Sciences, Chengdu, China (104°13' E, 30°42' N). The experimental raw materials included sphagnum, peat, vermiculite, and perlite. The standard soilless substrate consisted of a mixture of peat, vermiculite, and perlite (3:1:1, *v/v*). Table S1 shows the initial physicochemical properties of the raw materials. The vegetable used in this study was Flandria RZ (*Lactuca sativa* var. *ramosa Hort*).

The greenhouse pot experiment comprised five treatments involving substitution of sphagnum for peat, with four replicates per treatment, which were as follows: 0% sphagnum (CK), 25% sphagnum (25SP), 50% sphagnum (50SP), 75% sphagnum (75SP), and 100% sphagnum (100SP). The incubation conditions of the light incubator were as follows: temperature: 22 °C, humidity: 70%, and light intensity: 30,000 Lux. Lettuce seeds were sown on 1 July 2022 into seedling cavity trays raised using pure peat. A total of 30 lettuce seedlings were transplanted into 500mL cultivation pots on 14 July 2022. In addition, to meet lettuce growth needs, the general Hoagland nutrient solution was added into cultivation pots (Table S2). The total nitrogen content for all treatments was 200 mg of N dm⁻³.

2.2. Collection of Substrate and Gas Samples

Substrate and gas samples were collected on 1, 11, 21, and 31 days after the transplantation of the lettuce. The cultivation pots were placed in a rectangular PVC chamber (dimensions = 0.1 × 0.1 × 0.5 m³), and its bottom was sealed with water. The substrates with the lettuce were incubated for one hour, and then the gas samples were pumped into a 250 mL gas bag (LB-201, Dalian Delin Gas Packaging Co., Dalian, China) using a suction pump. Substrate samples were sampled into plastic-sealed bags and 5 mL centrifuge tubes. They were subsequently preserved at −20 °C and −80 °C for the determination of physicochemical properties and microbiological indices, respectively [6,11].

2.3. Analysis of Substrate Samples

The water-filled pore space (WFPS) of the samples was calculated by measuring the water content; the formula for the WFPS is given in Text S1 of the Supplementary Materials. The extract solution of a 1:10 air-dried soilless substrate and distilled water (*w/v*) was used to measure the pH value and EC with a multi-parameter tester (Mettler Toledo Instruments Shanghai Co., Shanghai, China). The extract solution of a 1:5 fresh, soilless substrate and 2 mol L⁻¹ of KCl solution (*w/v*) was used to determine the NH₄⁺-N, NO₃⁻-N, and NO₂⁻-N contents by an enzyme-linked immunosorbent assay instrument (ELISA, ReadMax 1900, Shanghai Flash Spectrum Biotechnology Co., Shanghai, China). The total nitrogen (TN) and total carbon (TC) contents of the lettuce and soilless substrate were determined using an elemental analyzer (EA, iso-prime100, Elementar, Berlin, Germany). The formulae for calculating nitrogen use efficiency are given in Text S1 of the Supplementary Materials.

2.4. Gas Sample Analysis and Microbial Pathways of N₂O Emissions

N₂O content was determined by a gas chromatograph (GC; Agilent 7890A, Santa Clara, CA, USA) equipped with an electron-capture detector (ECD). The N₂O flux (μg m⁻² h⁻¹) was calculated as follows:

$$Flux = \rho * h * \frac{273}{(T + 273)} * \frac{\Delta c}{\Delta t} \quad (1)$$

where ρ denotes the gas density at the standard state (N₂O, 1.978 kg m⁻³), h is the chamber height (0.5 m), T represents the temperature inside the chamber, $\Delta c/\Delta t$ represents the change in gas concentration per unit of time (μL L⁻¹ h⁻¹), an N₂O fluxes (μg m⁻² h⁻¹) are related to the area of each cultivation pot, with 273 units being K. The cumulated N₂O emissions were calculated using the following equation:

$$C_{N_2O} = MN_2O_{flux} * 31 * 24 \quad (2)$$

where MN_2O_{flux} represents the mean N₂O flux, with 31 and 24 units being days and hours, respectively.

The $\delta^{15}N^{bulk}$, $\delta^{15}N^{\alpha}$, and $\delta^{18}O$ of N₂O were determined using an isotope ratio mass spectrometer installed in the pre-concentrator system, chemical traps, and gas chromatograph (GC-IRMS; Delta V Plus, Thermo Fisher Scientific, Bremen, Germany). The isotopic differences of ¹⁵N and ¹⁸O in N₂O are expressed as delta values using the “ δ ” symbol and calibrated using international standards (atmospheric N₂ and Vienna Standard Average Ocean Water):

$$\delta X = \left(\frac{R_{sample}}{R_{standard}} - 1 \right) \left(X = {}^{15}N^{\alpha}, {}^{15}N^{\beta}, {}^{15}N^{bulk}, {}^{18}O \right) \quad (3)$$

$$\delta^{15}N^{SP} = 2\delta^{15}N^{\alpha} - 2\delta^{15}N^{bulk} \quad (4)$$

In the formula, R represents the ¹⁵N/¹⁴N or ¹⁸O/¹⁶O ratio. The measurement accuracy of $\delta^{15}N^{bulk}$, $\delta^{15}N^{\alpha}$, $\delta^{18}O$, and $\delta^{15}N^{SP}$ are 0.1‰, 0.4‰, 0.1‰, and 0.5‰, respectively.

In addition, recent studies have used $\delta^{18}O(N_2O/H_2O)$ vs. $\delta^{15}N^{SP}$ dual-isotope profiles to distinguish the microbial pathway from N₂O reduction [6]. Moreover, the similar range values of $\delta^{18}O$ and $\delta^{15}N^{SP}$ were divided into high-value groups (BN, AN, and FD) and low-value groups (DD and ND), which were denoted as ABF and DND, respectively (Table S4). Furthermore, there then exist two scenarios of the N₂O reduction in SCSs. In scenario i, N₂O is first reduced to N₂ in DND and then mixes with the N₂O produced by NF. In scenario ii, N₂O from the DND and ABF processes is first mixed and then partially reduced to N₂.

$$\text{Scenario i : } \delta_{sub} = x\delta_{ABF} + (1 - x)\delta_{DND} + \eta_R \ln(1 - Fr) \quad (5)$$

$$\text{Scenario ii : } \delta_{sub} = x\delta_{ABF} + (1 - x)[\delta_{DND} + \eta_R \ln(1 - Fr)] \quad (6)$$

Therein, δ is either $\delta^{18}O$ or $\delta^{15}N^{SP}$, and η is either $\eta^{18}O$ or $\eta^{15}N^{SP}$. Subscripts sub and R represent a soilless substrate and N₂O reduction, respectively. x is the contribution of the ABF process, $(1 - x)$ is the contribution of the DND process, Fr represents the extent of N₂O reduction, η_R denotes the net isotope effect values of N₂O reduction, which are $\eta^{15}N^{SP}_R$ or $\eta^{18}O_R$. ABF represents the following: archaeal nitrification, bacterial nitrification, and fungal denitrification. DND represents the following: bacterial denitrification and nitrifier denitrification.

2.5. qPCR Assay and High-Throughput Sequencing

The DNA samples were extracted using the MoBio PowerSoil DNA Isolation Kit (MoBio, Solana Beach, CA, USA) [24]. The amplification conditions for qPCR and primers for the genes are listed in the Supplementary Materials. The copy numbers of the bacterial 16S rRNA gene, fungal ITS gene, AOB *amoA*, AOA *amoA*, *p450nor*, *nirS*, *nirK*, and *nosZ* genes

were determined by a real-time PCR system (ABI 7500, LightCycler480II, 384, Roche, Beijing, China). qPCR reactions were performed using the AceQ[®] qPCR SYBR[®] green master mix (Q112-02, Vazyme, Nanjing, China) at 95 °C for 2 min, with 40 cycles at 95 °C for 15 s and at 60 °C for 30 s. qPCR amplification conditions are described in Text S2 of the Supplementary Materials. The bacterial 16S rRNA and the fungal ITS were sequenced using the Illumina NovaSeq platform (Illumina, San Diego, CA, USA) of Novogene Co., Ltd. (Beijing, China).

2.6. Contribution Analysis of Microbial Pathways for N₂O Emissions

The approximate contribution of AN, BN, and FD in this study was differentiated from the total contribution of the ABF process based on the relative abundance proportion of AOA *amoA*, AOB *amoA*, and *p450nor* genes. The KEGG (Kyoto Encyclopedia of Genes and Genomes) database is a database that systematically analyzes gene functions and links genomic and functional information. All the genera obtained by high-throughput sequencing were compared with the KEGG database to select the denitrification performed by nitrifier bacteria and denitrifier bacteria, which then roughly differentiated the nitrifier denitrification (ND) and denitrifier denitrification (DD) using their relative proportions.

2.7. Statistical Analysis

The experimental data of this study were analyzed and plotted using Excel 2016, origin 2021, and Canoco 5. One-way analysis of variance (ANOVA) was performed to test for differences in physicochemical properties, mineral nitrogen content, N₂O emissions, nitrogen use efficiency, yield, and functional gene abundance among treatments. The least significant difference (LSD) method was also used to test for significant differences. Redundancy analysis (RDA) was used to analyze the effect of the physicochemical properties of soilless substrates on the dominant bacteria genera. Pearson correlation analysis was used to analyze the correlation among different parameters.

3. Results

3.1. Physicochemical Properties, Lettuce Yields, and Nitrogen Use Efficiency of All the Treatments

The physicochemical properties of the soilless substrate in all the treatments are shown in Table 1. The results showed that the WFPS of all the treatments exceeded 60%. Meanwhile, the WFPS of the 50SP (70.6%), 75SP (71.9%), and 100SP (70.3%) treatments were significantly higher than those of the CK and 25SP treatments. Moreover, it is noteworthy to mention that the pH values of all the treatments examined were approximately 6.5 in this study, exhibiting no discernible, significant variations. The EC values in the 75SP (1328.5 $\mu\text{S cm}^{-1}$) and 100SP (1352.4 $\mu\text{S cm}^{-1}$) treatments were significantly higher than those in the CK and 25SP treatments. The C/N ratio in the CK treatment was recorded as 43.2, while a significant reduction in the C/N ratio was observed as the percentage of sphagnum substitution increased, ultimately reaching a value of 25.9 in the 100SP treatment. The CK treatment had the highest bulk density of 0.17 g cm^{-3} , followed by the 25SP, 50SP, 75SP, and 100SP treatments. In this study, there was no significant difference in the lettuce yields among treatments (Figure S1A). Compared with the CK treatment, nitrogen use efficiency was significantly higher in the 25SP, 50SP, and 75SP treatments and highest at 19.0% in the 25SP treatment (Figure S1B).

3.2. Mineral Nitrogen and N₂O Emissions of All the Treatments

In this study, mineral nitrogen of all the treatments was dominated by NO₃[−]-N, followed by NH₄⁺-N and NO₂[−]-N (Figure 1A–C). The contents of NO₃[−]-N, NH₄⁺-N, and NO₂[−]-N varied among the treatments, while the total mineral nitrogen content increased with an increasing substitution rate of sphagnum. Throughout the growth cycle of the lettuce, the contents of NO₃[−]-N and NH₄⁺-N exhibited an initially high level followed by a subsequent decline in the later stages, and the NO₂[−]-N content remained steadfast. More-

over, the contents of NO_3^- -N and NH_4^+ -N were significantly higher in the 50SP, 75SP, and 100SP treatments compared with the CK and 25SP treatments (Table 1).

Table 1. Average of the time series mean of the WFPS, pH, EC and C/N, NO_3^- -N, NH_4^+ -N, and NO_2^- -N contents.

Treatment	WFPS %	pH	EC $\mu\text{S cm}^{-1}$	C/N	NO_3^- mg kg^{-1}	NH_4^+ mg kg^{-1}	NO_2^- mg kg^{-1}	Bulk Density g cm^{-3}
CK	62.5 $\pm$ 1.6 b	6.4 $\pm$ 0.1	841.0 $\pm$ 353.6 b	42.3 $\pm$ 2.3 a	34.3 $\pm$ 10.5 c	2.7 $\pm$ 0.5 c	0.8 $\pm$ 0.3	0.17 $\pm$ 0.04
25SP	62.1 $\pm$ 1.3 b	6.5 $\pm$ 0.1	738.0 $\pm$ 137.4 b	37.0 $\pm$ 2.6 b	41.4 $\pm$ 4.8 c	4.9 $\pm$ 0.7 c	0.9 $\pm$ 0.2	0.15 $\pm$ 0.03
50SP	70.6 $\pm$ 5.2 a	6.5 $\pm$ 0.1	1016.7 $\pm$ 192.2 ab	34.6 $\pm$ 1.5 bc	55.4 $\pm$ 3.2 b	12.6 $\pm$ 2.5 b	1.3 $\pm$ 0.4	0.13 $\pm$ 0.03
75SP	71.9 $\pm$ 6.0 a	6.5 $\pm$ 0.1	1328.5 $\pm$ 377.8 a	30.6 $\pm$ 4.3 cd	61.2 $\pm$ 9.0 b	23.2 $\pm$ 2.0 a	1.3 $\pm$ 0.3	0.11 $\pm$ 0.02
100SP	70.3 $\pm$ 3.5 a	6.4 $\pm$ 0.2	1352.4 $\pm$ 104.6 a	25.9 $\pm$ 2.8 d	74.1 $\pm$ 3.6 a	22.3 $\pm$ 2.6 a	1.1 $\pm$ 0.2	0.08 $\pm$ 0.02

Note: WFPS: water-filled pore space; EC: electrical conductivity; C/N: rate of total carbon to total nitrogen; NO_3^- : nitrate nitrogen; NH_4^+ : ammonium nitrogen; NO_2^- : nitrite nitrogen. Different alphabets represent significant differences in one-way ANOVA among treatment groups, as follows.

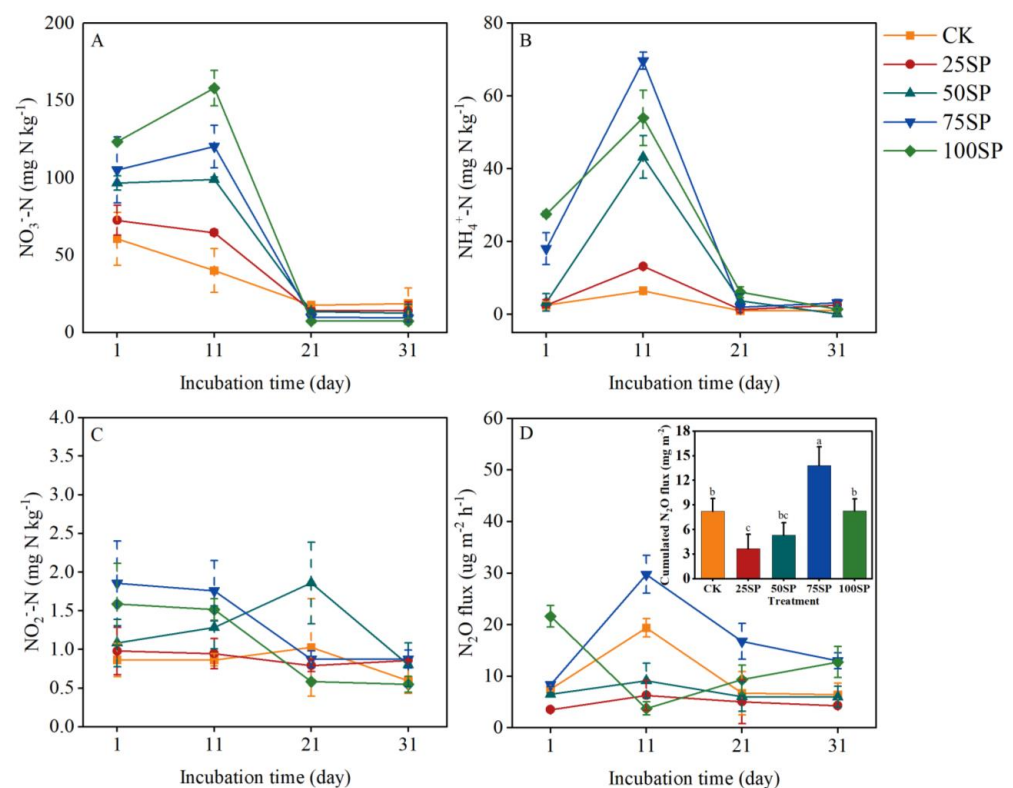


Figure 1. Time series of the NO_3^- -N content (A), NH_4^+ -N content (B), NO_2^- -N content (C), and N_2O flux and cumulated N_2O flux (D) during the whole experimental stage. Different alphabets represent significant differences in one-way ANOVA among treatment groups. Results were shown as means $\pm$ standard deviation ($n = 6$). CK, 25SP, 50SP, 75SP, and 100BC treatments represent 0%, 25%, 50%, 75%, and 100% values of the amount of sphagnum used instead of peat in the soilless substrate, respectively.

The N_2O fluxes of all the treatments showed an increasing and then a decreasing trend, except for 100SP, which showed a decreasing and then an increasing trend (Figure 1D). Compared with the CK treatment (8.2 mg m^{-2}), the accumulated N_2O emissions significantly reduced by 55.4% for the 25SP treatment (3.7 mg m^{-2}) and significantly increased by 67.8% for the 75SP treatment (13.8 mg m^{-2}). N_2O was significantly positively correlated with NH_4^+ -N and EC, while NH_4^+ -N was significantly negatively correlated with C/N and significantly positively correlated with NO_3^- -N, NO_2^- -N, and EC (Table 2, $p < 0.05$).

Table 2. Analysis of the Pearson correlation among different parameters.

	NO ₃ [−]	NH ₄ ⁺	NO ₂ [−]	pH	EC	C/N	WFPS	δ ¹⁵ N ^α	δ ¹⁵ N	δ ¹⁸ O
NO ₃ [−]	1	0.78 **	0.57 **	−0.7 **	0.91 ***	−0.49 **	0.58 **	−0.44 **	−0.15	−0.24
NH ₄ ⁺	0.78 **	1	0.56 **	−0.28	0.76 **	−0.61 **	0.18	0.01	−0.16	0.13
NO ₂ [−]	0.57 **	0.56 **	1	−0.34 *	0.57 **	−0.4 **	0.25	−0.28	−0.24	−0.23
N ₂ O	0.16	0.41 *	0.21	−0.08	0.35 **	−0.28	0.06	−0.11	−0.14	0.05

Note: * correlation is significant at the 0.05 level; ** correlation is significant at the 0.01 level; *** correlation is significant at the 0.001 level.

3.3. Isotope Analysis of All the Treatments

The emitted N₂O isotope values, including those of δ¹⁵N^{bulk}, δ¹⁵N^α, and δ¹⁸O, were extracted from the measured N₂O isotope values according to the Keeping plot method, and δ¹⁵N^{SP} was calculated by δ¹⁵N^{bulk} and δ¹⁵N^α according to Equation (4) (Figure S2 and Table 3). For the CK, 25SP, 50SP, 75SP, and 100SP treatments, the δ¹⁸O values were 35.2, 38.9, 36.7, 35.8, and 37.7 ‰, and the δ¹⁵N^{SP} values were 16.1, 12.8, 13.7, 14.4, and 14.7 ‰, respectively. The dual-isotope map of δ¹⁸O vs. δ¹⁵N^{SP} in conjunction with Equations (5) and (6) was used to obtain the contribution of the ABF and DND processes and the extent of N₂O reduction in the two scenarios (Table 2). In this study, it was observed that the DND process was the main process of the N₂O emissions in all the treatments. Notably, the contribution of the DND process was lowest in the CK treatment (60%) and highest in the 25SP treatment (75.5%). Furthermore, when considering scenarios i and ii, the extent of N₂O reduction was lowest in the CK treatment, with extents of 67.2 and 84.6%, whereas the highest extent of N₂O reduction was observed for the 25SP treatment, with extents of 79.3 and 87.7%, respectively.

Table 3. δ¹⁵N^{bulk}, δ¹⁵N^α, δ¹⁸O, δ¹⁵N^{SP}, contributions of ABF and DND, and the extents of N₂O reduction in scenario i and scenario ii in different treatments.

Treatment	δ ¹⁵ N ^{bulk} ‰	δ ¹⁵ N ^α ‰	δ ¹⁸ O ‰	δ ¹⁵ N ^{SP} ‰	ABF %	DND %	Scenario i %	Scenario ii %
CK	−5.5	−13.6	35.2	16.1	40.0	60.0	67.2	84.6
25SP	−4.7	−11.0	38.8	12.8	24.5	75.5	79.3	87.7
50SP	−3.2	−10.7	36.7	13.7	30.0	70.0	74.2	85.7
75SP	−2.6	−9.8	35.7	14.4	33.59	66.4	71.1	84.7
100SP	−0.1	−7.4	37.7	14.7	32.25	67.8	75.1	87.3

3.4. qPCR Assay of All the Treatments

There were significant differences in the abundance of the 16S rRNA, ITS, and N-related functional genes among the treatments (Figure 2A). The gene copy number of the 16S rRNA gene was significantly higher than those of the ITS genes in all the treatments. Meanwhile, the 16S rRNA gene copy number in the 25SP treatment was significantly higher than those in the CK treatment, while the ITS gene copy number in the 100SP treatment was significantly higher than those in the CK treatment. In addition, the gene copy number of the denitrification genes (*nirK*, *nirS*, *nosZ*, and *p450nor*) were significantly higher than those of the nitrification genes (AOA *amoA* and AOB *amoA*) in all the treatments (Figure 2A). For the different treatments, the relative abundance of functional genes was analyzed (Figure 2B), and the results showed that the 75SP treatment had a significantly higher relative abundance of *nirK* genes than the CK treatment, while its relative abundance of *nosZ* genes was significantly lower than in the CK treatment.

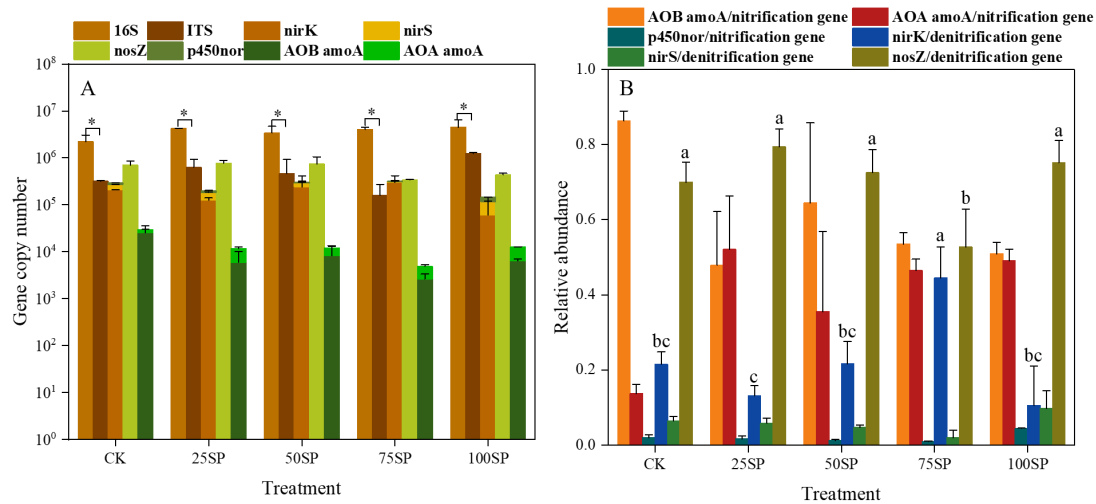


Figure 2. Gene copy number of 16S rRNA and ITS, AOB *amoA*, AOA *amoA*, *nirK*, *nirS*, *nosZ*, and *p450nor* (A) and the relative abundance of AOB *amoA*, AOA *amoA*, *nirK*, *nirS*, *nosZ*, and *p450nor* (B) in different treatments. * represents a significant difference between genes. Different alphabets represent significant differences in one-way ANOVA among treatment groups.

3.5. Microbial Community Composition of Genera

This study analyzed the microbial community composition at the genus level using high-throughput sequencing (Figures 3 and 4C). The results showed that the proportion of denitrifying bacterial genera was 98.1%, 98.0%, 98.0%, 97.7%, and 97.8% for the CK, 25SP, 50SP, 75SP, and 100SP treatments, respectively. The dominant genera in the CK, 25SP, and 50SP treatments were *Rhodanobacter* and *Dokdonella*, and their relative abundance was 22.2% and 10.9%, 20.1% and 9.6%, 15.4% and 8.83%, respectively. Meanwhile, in the 75SP and 100SP treatments, the denitrifying dominant genera were *Rhodanobacter* (15.3%), *Devosia* (8.3%) and *Massilia* (10.0%), and *Rhodanobacter* (6.8%), respectively.

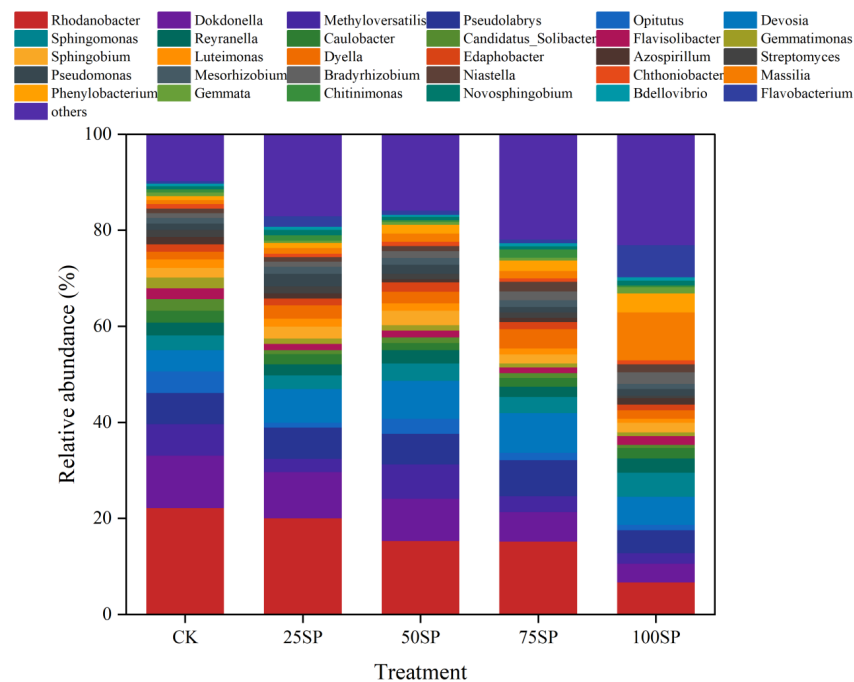


Figure 3. Microbial genera community composition and relative abundance in different treatments.

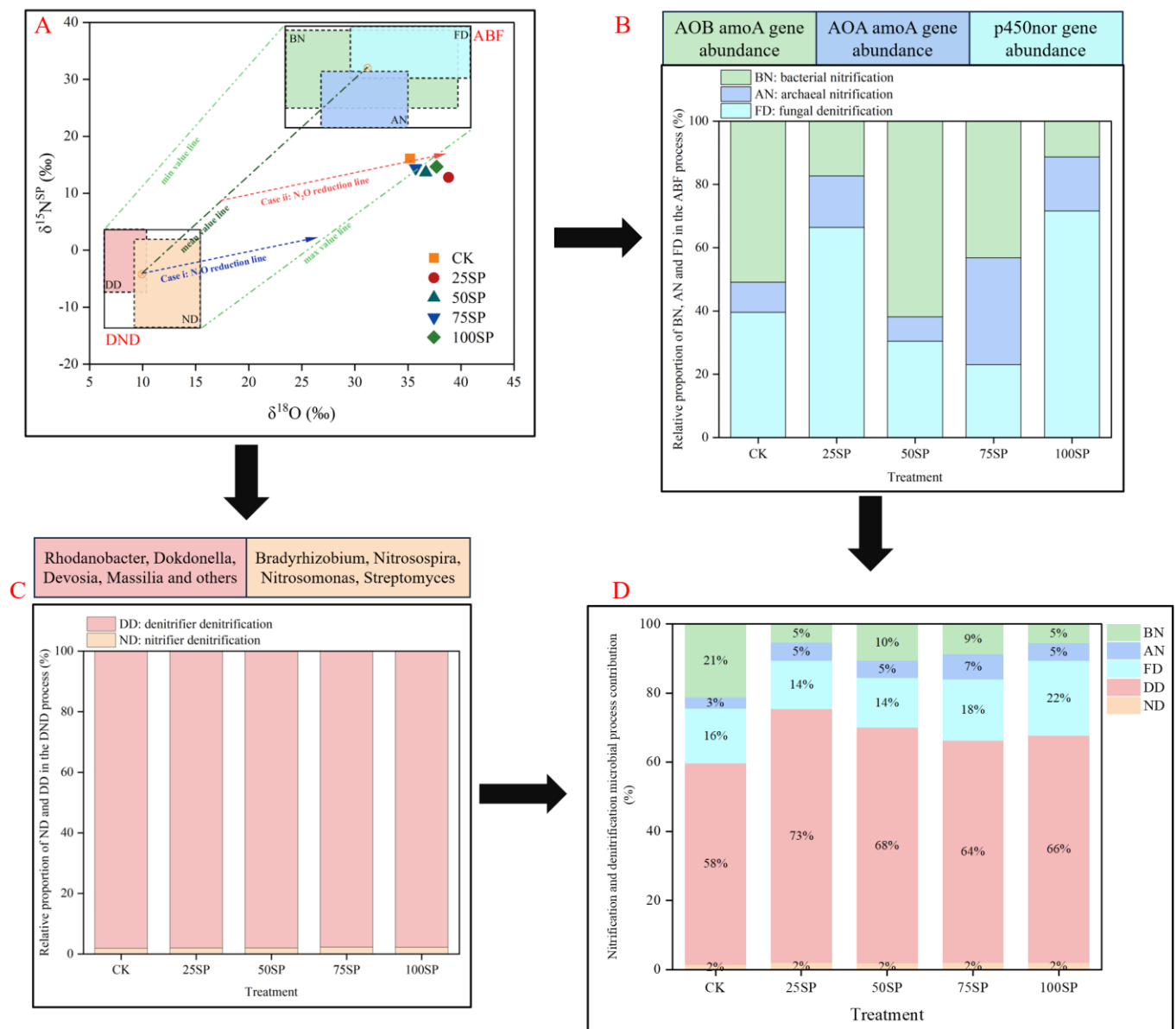


Figure 4. Dual-isotope map of $\delta^{18}\text{O}$ vs. $\delta^{15}\text{N}_{\text{SP}}$ (A); relative proportion of BN, AN, and FD in the ABF process (B); relative proportion of ND and DD in the DND process (C); and relative contribution of BN, AN, FD, ND, and DD (D).

The genera that perform nitrifier denitrification were *Bradyrhizobium*, *Nitrospira*, *Nitrosomonas*, and *Streptomyces*; the relative proportions of these genera in the denitrifying genera of the CK, 25SP, 50SP, 75SP, and 100SP treatments were 2.6%, 2.7%, 2.2%, 3.1%, and 3.0%, respectively (Figure 4C). The contributions of the microbial pathways to N_2O emissions from the different treatments were obtained from stable isotope analysis, functional gene abundance, and microbial community composition (Figure 4D). The main microbial pathway toward N_2O emissions for all the treatments was that of the DD process, which contributed more than 50% of the total amount, while the contribution of the ND process was negligible (making up less than 2.5% of the total amount). Simultaneously, compared with the CK treatment, the 25SP treatment decreased the contribution of the BN, AN, and FD processes, while the 75SP treatment increased the contribution of the AN and FD processes.

3.6. Correlation Analysis between Microbial Communities and Physicochemical Properties

Redundancy analysis (RDA) explained 82.38% of the correlation between the microbial communities and physicochemical properties; C/N, EC, NO_3^- , and the WFPS were the main explanatory factors of the microbial communities. The *Rhodanobacter* was significantly positively correlated with C/N and EC and significantly negatively correlated with the pH, and the *Dokdonella* was significantly negatively correlated with NH_4^+ , the WFPS, and NO_2^- . Correlation heat map analysis further confirmed these results. Meanwhile, the *Massilia* was significant positively correlated with NH_4^+ and the WFPS (Figure 5B, $p < 0.05$). Remarkably, microbial genera were not correlated with isotope δ -values.

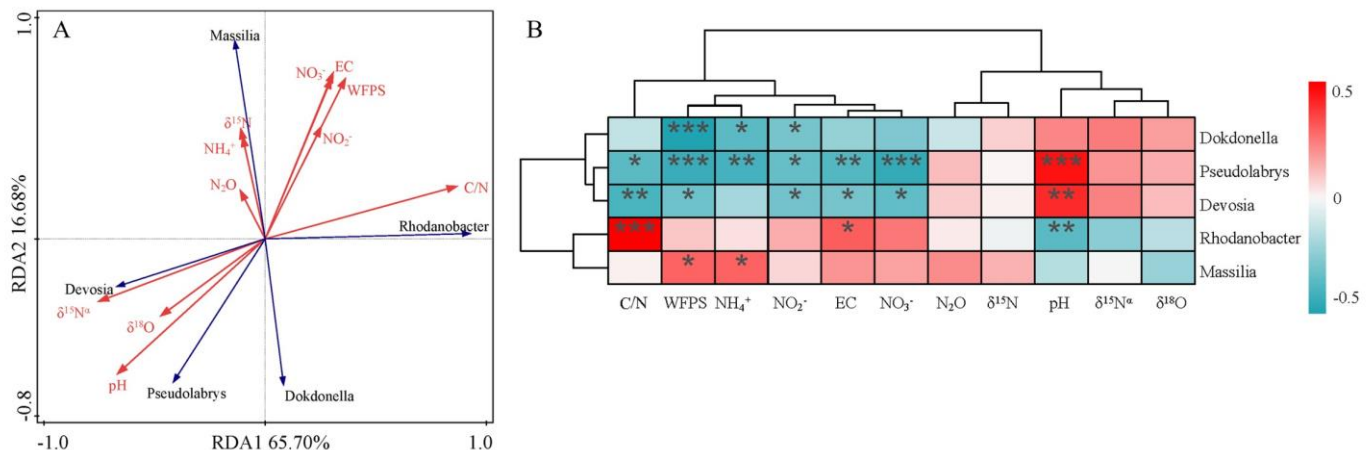


Figure 5. Redundancy analysis (RDA) (A) and correlation heat map analysis (B) of the physicochemical properties of soilless substrates with dominant bacteria genera. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$.

4. Discussion

4.1. Effects of Different Sphagnum Substitution Rates on N_2O Emissions

There were N_2O emissions from the use of a peat substrate for greenhouse vegetable production. N_2O is primarily produced and consumed through nitrification and denitrification by converting mineral nitrogen and also is influenced by the soil's physicochemical properties [25]. In this study, N_2O emissions were positively correlated with NH_4^+ -N content and EC (Table 2). Therefore, the 75SP treatment exhibited significantly higher N_2O emissions compared with those observed for the CK treatment, which were likely attributable to its higher NH_4^+ -N content and EC (Table 1). However, there was no significant difference in N_2O emissions between the CK treatment, 50SP treatment, and 100SP treatment, although the NH_4^+ -N content and EC were significantly higher in the 50SP and 100SP treatments compared with the CK treatment. This result suggested that N_2O emissions may be influenced by other factors [26]. Furthermore, the N_2O emissions from the 25SP treatment were significantly lower than those from the CK treatment. Meanwhile, we have also observed a significant difference solely in the C/N ratio between the 25SP and CK treatments. Most studies considered that the C/N ratio can alter the microbial processes and thus affect N_2O emissions [27,28], so the reason for lower N_2O emissions in the 25SP treatment may be attributed to its comparatively lower C/N ratio. Notably, the WFPS is also one of the factors influencing microbial activity [29]. In the present study, the WFPS observed for all the treatments exceeded 60%, indicating that denitrification should be the dominant pathway for N_2O production [30]. In order to further corroborate the hypothesis, this study investigated the microbial mechanisms of N_2O emissions and the response of the mineral nitrogen and physicochemical properties of the soilless substrate to microorganisms.

4.2. Microbial Mechanisms of N₂O Production and Consumption

The dual-isotope map of $\delta^{18}\text{O}$ vs. $\delta^{15}\text{N}^{\text{SP}}$ has been widely used to distinguish the microbial pathways of N₂O production and consumption [31]. In this study, consequently, the $\delta^{18}\text{O}$ vs. $\delta^{15}\text{N}^{\text{SP}}$ map combined with Equations (5) and (6) was used to obtain the contribution of the ABF and DND processes and the extent of N₂O reduction to N₂ [32]. The results showed that the DND process dominated all the treatments, and the substitution of sphagnum for peat had a facilitating effect on the DND process. This was consistent with the previous findings based on WFPS analysis [6], and the promotion effect of sphagnum substitution for peat on the DND process was closely correlated with its enhancement of the WFPS (Table 1). Furthermore, in this study, different treatments had different extents of N₂O reduction depending on whether scenario i or ii of N₂O reduction occurred. The substitution of sphagnum for peat enhanced the extent of N₂O reduction, with the 25SP treatment exhibiting the strongest extent of N₂O reduction. This could be attributed to the 25SP treatment providing a more favorable C/N environment for the growth of N₂O-reducing microorganisms [33]. It is worth noting that the DND process is primarily driven by bacteria. Moreover, scenario i of N₂O reduction is more likely to occur under the process dominated by bacteria [6,34]. Therefore, we believe that scenario i may be more closely aligned with the actual situation in this study.

The results based on a qPCR assay showed that the abundance of the 16S rRNA gene was significantly higher than that of the ITS gene, and the gene copy number of denitrification-related genes (*nirS*, *nirK*, *nosZ*, and *p450nor*) was significantly higher than that of nitrification-related genes (AOA *amoA* and AOB *amoA*). This signifies that N₂O production and consumption were predominantly governed by bacterial activity, with bacterial denitrification being the primary mechanism. This further confirms the analysis results of the $\delta^{18}\text{O}$ vs. $\delta^{15}\text{N}^{\text{SP}}$ map. In addition, the 75SP treatment had a significantly higher *nirK* gene, which is associated with N₂O production, than the that of the CK treatment, while *nosZ*, which is a gene associated with N₂O reduction, was significantly lower in the 75SP treatment than in the CK treatment, with this perhaps being a reason for the higher N₂O emissions in the 75SP treatment. The reason for this result may be that the 75SP treatment has a stronger inorganic nitrogen content, which in turn provides more substrates for the denitrification process. Meanwhile, we further analyzed the microbial community composition. The results showed that denitrifying bacteria genera accounted for more than 96% of the genera in different treatments. The dominant denitrifying bacterial genus in the CK treatment was *Rhodanobacter* and *Dokdonella*. As the proportion of sphagnum replacing peat increases, *Massilia* gradually supplanted *Dokdonella* as the predominant denitrifying bacterial genus. Previous studies have identified *Rhodanobacter* as a genus with a fully denitrifying capacity that is present in a variety of habitats [35,36], while *Massilia* is the common dominant genus among all the mosses tested based on the previous library of research [37]. Therefore, the substitution of peat with sphagnum can alter the structure and composition of microbial communities in the substrate and enhance the denitrification potential through increasing the dominance of *Massilia*.

In this study, based on the contribution analysis of the microbial pathways, the relative contributions of the BN, AN, FD ND, and DD processes were roughly differentiated (Figure 4D). The five microbial processes have different effects on N₂O emissions, the BN and AN processes mainly produce N₂O as a byproduct of bacterial and fungal nitrification, while the FD process lacks N₂O reductase resulting in N₂O as a final product. Compared with FD, BN produced a higher amount of N₂O. This is due to the fact that BN not only generated N₂O through nitrification but also increased the substrate for denitrification by converting NH₄⁺-N to NO₃⁻-N, thereby resulting in secondary N₂O emissions [11]. In addition, the extent of N₂O reduction is the main factor determining the N₂O emissions, and N₂O reduction only occurs in the DD and ND processes, so the DD and ND processes also affect the N₂O emissions. In this study, different rates of substitution with sphagnum had a different effect on the relative contributions of microbial processes. The 25SP treatment had the lowest contributions of BN and FD, the strongest contribution of DD, and the

highest extent of N_2O reduction. This may be the reason why the 25SP treatment has the lowest N_2O emissions. In contrast, 75SP had the highest N_2O emissions probably because of its stronger contributions of FD and AN compared with those of the CK treatment.

4.3. The Correlation between Microbial Action and Environmental Factors due to Sphagnum Substitution

Redundancy analysis (Figure 5A) showed that environmental factors explained 82.4% of the variation of the denitrifying bacteria genera in this study, with C/N explaining most of the variation, followed by EC and NO_3^- -N. This was similar to previous correlation analyses of soil properties with bacterial communities [38]. It can be concluded that sphagnum substitution significantly alters C/N, EC, and NO_3^- -N, which indirectly affect the variation in denitrifying bacteria genera communities, ultimately resulting in different N_2O emissions [39]. Correlation heat map analysis (Figure 5B) showed that *Rhodanobacter* was positively correlated with C/N and EC and negatively correlated with the pH. This was consistent with previous studies on *Rhodanobacter* [40,41]. This result indicated that the growth of *Rhodanobacter* requires the environment to provide sufficient sources of carbon and a suitable pH. Sphagnum substitution reduces the C/N ratio of the peat substrate, which in turn reduces the supply of the carbon source and decreases the activity of *Rhodanobacter*. In addition, the activity of *Massilia* was mainly influenced by NH_4^+ -N and the WFPS [42,43]. Sphagnum substitution significantly increased NH_4^+ -N and the WFPS, which enhanced the activity of *Massilia*. Remarkably, N_2O emissions in this study were directly affected by NH_4^+ -N and EC, while the dominant microbial genera had no direct correlation with N_2O emissions. Therefore, sphagnum substitution may affect the microbial community structure indirectly by regulating environmental factors to influence N_2O emission.

In this study, the pH values among various treatments were essentially identical, thereby suggesting that sphagnum is a prerequisite substance for peat formation and has a similar pH to that of peat [44]. However, sphagnum significantly changed other physicochemical properties. Firstly, the higher substitution rate of sphagnum significantly elevated the WFPS of the soilless substrate, indicating that sphagnum possesses superior water retention [45]. Secondly, sphagnum increased the EC of the soilless substrate, implying a higher concentration of inorganic nutrients within the moss. Notably, there has been a notable augmentation in the levels of NH_4^+ -N and NO_3^- -N contents, thereby providing plants with an enriched nitrogen supply for their growth. However, these nutrients did not exert a significant impact on the yield of lettuce, thus indicating that sphagnum can serve as a viable substitute for peat. In addition, the nitrogen use efficiency of the 25SP, 50SP, and 75SP treatments was significantly higher than that of the CK treatment, thereby indicating that a proper substitution rate of sphagnum could enhance the nitrogen uptake by lettuce. Considering the N_2O emissions from each treatment, it can be observed that the 25% substitution of peat with sphagnum is the optimal substitution ratio, which is followed by a substitution ratio of 50%.

5. Conclusions

In this study, the substitution of peat with sphagnum was able to improve nitrogen use efficiency without a discernible impact on lettuce yield. However, varying substitution rates had different effects on N_2O emissions, with a 25% substitution rate resulting in the highest reduction, which amounted to a decrease of 55.4%. Compared with the CK treatment, a rate of 25% for sphagnum substitution enhanced the extent of N_2O reduction and reduced the contribution of bacterial nitrification and fungal denitrification processes, resulting in reduced N_2O emissions. Moreover, the substitution of sphagnum has altered the community structure of bacteria, thus leading to a decrease in the dominant genus *Rhodanobacter* within the peat and an increase in the dominant genus *Massilia* within the sphagnum. *Rhodanobacter* was correlated with the EC and C/N of the soilless substrate, while *Massilia* was correlated with NH_4^+ -N and the WFPS of the soilless substrate. In summary, a substitution rate of at least 25% for sphagnum can be used to replace the peat

substrate, and 50% is an equally good substitution, with 25–50% being the optimal ratio for sphagnum to substitute for peat. *Rhodanobacter* and *Massilia* have different responses to environmental factors. More importantly, the combined analysis of the $\delta^{18}\text{O}$ vs. $\delta^{15}\text{N}^{\text{SP}}$ map, functional genes, and microbial community provide a novel perspective and an effective tool to assess the multiple microbial processes of N_2O production and consumption.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agronomy14020369/s1>. Table S1: Initial characteristics of raw materials; Table S2: Composition of nutrient solution; Table S3: Primer sequences of different genes; Table S4: $\delta^{15}\text{N}^{\text{SP}}/\eta^{15}\text{N}^{\text{SP}}_{\text{red}}$ and $\delta^{18}\text{O}/\eta^{18}\text{O}_{\text{red}}$ values for different N_2O production and consumption pathways with the minimum, maximum and mean of end-member values used in the double isotopocule plot approach; Figure S1: The yield (A) and nitrogen use efficiency (B) of different treatments; Figure S2: The values of substrate-driven N_2O isotopocule deltas, $\delta^{15}\text{N}$, $\delta^{15}\text{N}^{\alpha}$, and $\delta^{18}\text{O}$ obtained according to Keeling plot method for all treatments; Text S1: WFPS and Nitrogen use efficiency; Text S2: qPCR reactions efficiency and standards.

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