Nitrogen Cycling

https://doi.org/10.48130/nc-0026-0001

Original Research

Open Access

Synergistic effects of *Pseudomonas stutzeri* NRCB010 and organic fertilizers on soil N_2O emission reduction and microbial community structure in greenhouse vegetable fields

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Received: 31 October 2025

Revised: 16 December 2025

Accepted: 31 December 2025

Published online: 4 February 2026

Abstract

Agricultural soils, particularly intensively managed greenhouse vegetable fields, are a significant source of the potent greenhouse gas nitrous oxide (N_2O). While organic fertilization is essential for soil health, it can exacerbate N_2O emissions. This microcosm study evaluated the efficacy of the denitrifying bacterium *Pseudomonas stutzeri* NRCB010, applied with three organic fertilizers (mushroom residue organic manure, high-temperature pretreated compost, and hydrochar organic manure), to mitigate N_2O emissions and alter the microbial community. The results showed that inoculation with strain NRCB010 significantly decreased cumulative soil N_2O emissions ($p < 0.05$). Among them, the mushroom residue organic manure inoculation treatment achieved the most significant emission reduction, with a mitigating rate of 46.5%, followed by the hydrothermal carbon organic manure inoculation treatment (27.8%) and the high-temperature pretreated compost inoculation treatment (26.3%). Soil N_2O emissions showed a significant negative correlation with the abundance of the *nosZI* gene ($p < 0.05$), a key factor in decreasing N_2O emissions, and a significant positive correlation with nitrate nitrogen content, pH, and electrical conductivity (EC). Furthermore, amplicon sequencing showed that inoculation significantly shifted the soil microbiome, increasing *Pseudomonadota* and decreasing *Verrucomicrobiota*, with the bacterial inoculant having a greater impact on microbial community structure than the type of organic fertilizer. It was concluded that the synergistic application of MR and *P. stutzeri* NRCB010 presents a robust, microbiome-mediated strategy for achieving significant N_2O mitigation and ecological sustainability in protected agriculture.

Keywords: Greenhouse vegetable soil, Mushroom residue organic manure, High-temperature pretreated compost, Hydrochar organic manure, *nosZI* gene

Highlights

- Mushroom residue organic manure with the strain NRCB010 achieves the best N_2O mitigation effect (46.5%).
- The *nosZI* gene is key for emission control, showing a significant negative correlation with soil N_2O emissions.
- Mushroom residue organic manure with NRCB010 provides an efficient technical solution for N_2O emission reduction.
- Inoculation with the strain enhances the expression of the *nosZI* gene, drives reductions in N_2O emissions, and regulates the community interaction network.

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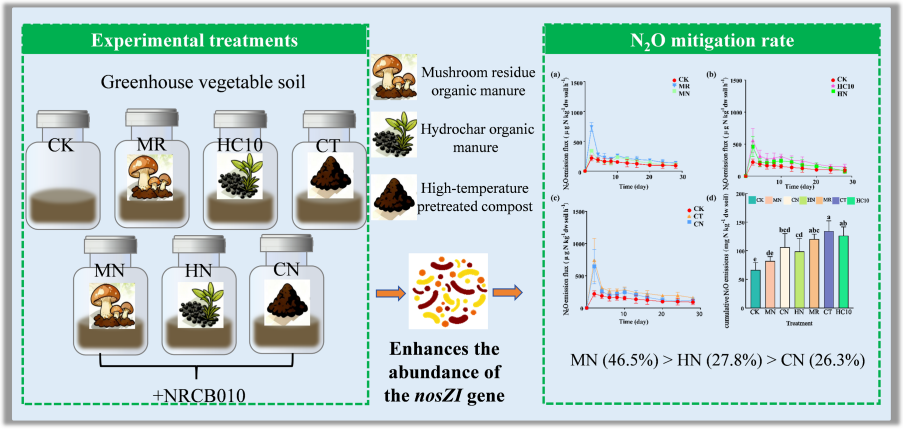
https://doi.org/10.48130/nc-0026-0001

Graphical abstract



Introduction

Compared with grain crop yields, China's vegetable output has exceeded grain output, making vegetables the largest agricultural product since 2011[1]. The vegetable planting area in China has expanded from 6.3 × 10^6 hm^2 in 1989 to 2.2 × 10^7 hm^2 in 2022, accounting for 13% of the total crop sown area that year[2]. In 2022, vegetable output reached 8 × 10^8 t, accounting for more than 50% of the global total[3]. Vegetable fields are highly intensive agricultural land characterized by high water and fertilizer input and intensive rotation. The annual nitrogen fertilizer input exceeds 1,000 kg N·hm^{-2}, far higher than the conventional application rate. A large amount of fertilizer nitrogen is lost from farmland into the surrounding environment, triggering a series of environmental problems, including N_2O emissions[4]. Soil N_2O emissions from protected vegetable fields are a major contributor to agricultural N_2O emissions, with emissions from ordinary plastic greenhouses being approximately 6.9 times that of open-field soil[5]. Among them, protected vegetable fields account for more than 55% of the total vegetable planting area[6].

N_2O is an important greenhouse gas with a global warming potential (GWP) about 273 times that of CO_2 on a 100-year time scale[7]. Agricultural soil is one of the main anthropogenic factors driving increases in atmospheric N_2O concentrations[8]. During crop production, long-term application of traditional chemical fertilizers can affect soil quality, including soil acidification and depletion of organic matter, thereby reducing crop yield and quality over time[9]. Organic fertilizers help maintain soil nutrients and improve soil structure, such as increasing soil total nitrogen (TN), soil organic matter (SOM), available phosphorus (AP), and available potassium (AK) contents, improving soil pH, and increasing soil water content and water-stable aggregate content[10]. Meanwhile, organic fertilizers have a slow-release effect. The combined application of chemical and organic fertilizers can coordinate the nutrient balance during crop growth, thereby optimizing nutrient utilization and improving crop yield. Studies have found that the levels of vitamin C and soluble sugar in tomatoes treated with a combination of Methylobacterium and a carbon-based organic fertilizer increased by 46.53% and 26.65%, respectively[11]. A weighted gene co-expression network analysis suggested that the application of organic fertilizers containing beneficial microorganisms regulates the expression of genes related to sugar and organic acid metabolism, alters sugar-acid metabolism, promotes sucrose accumulation, and citric acid degradation in fruits, and thus improves fruit quality[12]. Compared with the current situation in Denmark, where 70% of nitrogen input into farmland comes from organic fertilizers, China has even greater potential for reducing N_2O emissions by integrating microbial technologies that utilize organic fertilizer resources. Current primary methods to reduce N_2O emissions from farmland soil include adding nitrification inhibitors, applying biochar[13], and inoculating with plant growth-promoting rhizobacteria (PGPR) to mitigate N_2O emissions. Inoculating bacterial agents with N_2O-mitigating effects has emerged as a novel technology for decreasing emissions[14]. However, research on the 'microbe-organic fertilizer' synergistic system still has obvious shortcomings: existing studies mainly focus on the combined effects of a single bacterial strain and a specific organic fertilizer, and there is a lack of systematic investigation on the compatibility between different organic fertilizer substrates (such as livestock manure and decomposed straw) and functional bacterial strains. Gao et al.[15] found that inoculation with strain NRCB010 significantly promoted the growth of tomato seedlings. Yan et al.[16] showed that compared with the application of tobacco-specific compound fertilizer, the use of microbial fertilizer activated mineral elements in soil, promoted tobacco plant growth and development, increased potassium content and quality of tobacco leaves.

A study investigated the effects of co-inoculating *Rhizobium spp.* and *Azospirillum brasilense* on common bean cultivation, and found that the N_2O flux in the inoculation treatment was significantly lower than that in the urea-only treatment, with an emission mitigating efficiency of 51%[17]. Bio-organic fertilizer containing *T. guizhouense NJAU 4742* decreased bacterial *amoA* abundance, increased *nosZ* abundance, and decreased N_2O emissions from soils[18]. The tested strain NRCB010 in this study is a PGPR carrying the *nosZ* gene. As a denitrifying bacterium that encodes the N_2O reductase gene (*nosZ*), it can reduce N_2O to N_2 and is currently the only known biological sink for N_2O[19]. Studies have shown that strain NRCB010 has strong nitrate reduction ability, as well as specific solubilizing capacities for inorganic calcium phosphate and zinc phosphate. Additionally, this strain exhibits resistance to drought and salinity under environmental stress. Therefore, it can promote crop growth while decreasing soil N_2O emissions. Developing bacterial fertilizers with N_2O-mitigating effects is one of the effective technical means for mitigating N_2O emissions from farmland soil[20].

This study investigated the N_2O-mitigating effect of strain NRCB010 inoculated into three different organic manures, aiming to

provide practical guidance and a theoretical basis for mitigating greenhouse gas emissions from protected vegetable fields.

Materials and methods

Candidate strain

The candidate strain was *Pseudomonas stutzeri* NRCB010, isolated from paddy soil in Yixing, Jiangsu Province, China, and registered under the registration number CGMCC 19067. In previous studies, NRCB010 has demonstrated excellent N_2O-mitigating and plant growth-promoting capabilities in both laboratory experiments and field *in situ* experiments, and it carries the *nosZ* gene. The NRCB010 strain was inoculated into nutrient broth medium (NBNS medium: beef extract 3.0 g·L^{-1}, polypeptone 5.0 g·L^{-1}, sodium nitrate 0.3 mmol·L^{-1}, sodium succinate 4.4 mmol·L^{-1}), and cultured with shaking at 28 °C and 180 r·min^{-1} for approximately 24 h. The OD_{600} value was adjusted to 1 for further use.

Sample collection

The soil was collected from a multi-span greenhouse vegetable field in the Full Mechanization Demonstration Base for Vegetable Production in Changshu, Jiangsu Province, China. The soil type is fluvo-aquic soil, and the previous crop was Bok choy, also known as Chinese cabbage (*Brassica rapa* subsp. *chinensis*). The collected soil samples were air-dried, ground, passed through a 2 mm sieve, and stored at 4 °C in a refrigerator before use. Gas samples were collected starting from the 2nd day of incubation, with sampling intervals of every 2 d at the initial active emission stage, and every 3–4 d in the later stage, yielding a total of 10 samplings.

Organic manures

Three types of organic manures were used, provided by Jiangsu Academy of Agricultural Sciences. In this study, the mushroom residue organic manure used mushroom residue as the core raw material; the high-temperature pretreated compost was prepared from livestock and poultry manure via high-temperature composting; the hydrochar carbon organic manure was produced from crop straw through hydrothermal carbonization. Mushroom residue organic manure, has total N, P_2O_5, and K_2O ≥ 5%, organic matter ≥ 45%, pH of 7.37, EC of 5,810 μS·cm^{-1}, total carbon (TC) of 18.59 g·kg^{-1}, total nitrogen (TN) of 1.67 g·kg^{-1}, and carbon-to-nitrogen ratio (C/N) of 11.13. High-temperature pretreated compost has a pH of 9.13, EC of 2,026.67 μS·cm^{-1}, TC of 21.37 g·kg^{-1}, TN of 2.66 g·kg^{-1}, and C/N of 8.03. Hydrothermal carbon organic manure has N ≥ 1.6%, P_2O_5 ≥ 1.2%, K_2O ≥ 2.2%, organic matter ≥ 40%, pH of 9.12, EC of 1,811 μS·cm^{-1}, TC of 24.96 g·kg^{-1}, TN of 2.42 g·kg^{-1}, and C/N of 10.31.

Soil microcosm experiment

A total of seven treatments were set up in the experiment, with 16 replicates per treatment. The three organic fertilizers were added at equal nitrogen application rates, and each treatment also received 16 mL of sterile NBNS liquid medium (diluted 100 times). These seven treatments were no fertilizer (CK), supplemented with 16 mL sterile NBNS medium; mushroom residue organic fertilizer (MR), supplemented with 21 g MR and 16 mL sterile NBNS medium; high-temperature pretreated compost (CT), supplemented with 14 g of CT and 16 mL sterile NBNS medium; hydrothermal carbon organic fertilizer (HC10), supplemented with 15 g of HC10 and 16 mL sterile NBNS medium; the three combined treatments (MN, CN, HN) with each of three organic fertilizers received 16 mL NRCB010 bacterial solution. One hundred g of soil was weighed into a 500 mL culture flask, and organic fertilizer and bacterial solution (if applicable) were then added, then mixed thoroughly. Next, 100 g of soil was added, compacted, and then sterile water added to maintain soil moisture at 80% of the maximum field water-holding capacity. These soils were incubated in a 26 °C biochemical incubator in the dark. The conventional application rate of organic fertilizers in greenhouse vegetable fields in fluvo-aquic soil regions ranges from 300 to 600 t·hm^{-2}. The converted field application rates in this study (364–546 t·hm^{-2}) fall within this range and are consistent with actual production practices.

Quantitative real-time PCR (qPCR)

The gene copy numbers of 16S rDNA, *amoB*, AOA *amoA*, AOB *amoA*, *nirS*, *nirK*, *nosZI*, and *nosZII* in soil samples were determined using a quantitative real-time PCR instrument (Bio-Rad CFX96™ Optics Module, USA). Standard plasmids, with a concentration gradient of 10^2–10^8 copies·μL^{-1}, were used as templates to construct the standard curve for qPCR amplification. The qPCR reaction system had a total volume of 25 μL, consisting of 5 μL of DNA template (diluted with double-distilled water and EASY Dilution at a specified ratio before addition), 12.5 μL of TB Green Premix Ex Taq II, 1 μL each of forward and reverse primers (10 μM), and 5.5 μL of sterile double-distilled water. For the negative control, sterile double-distilled water was used instead of the template DNA. All experimental results showed amplification efficiency greater than 90%, and a single peak in the melting curve. The primer sequences and reaction program for the different genes involved in the reaction are listed in Supplementary Table S1.

Determinations of greenhouse gas and physicochemical properties

N_2O concentrations were analyzed by gas chromatography using an Agilent 7890B gas chromatograph equipped with an electron capture detector. The N_2O emission flux and cumulative emission were calculated according to the formula. The pH of samples was measured using an HI 2211 pH meter (HANNA Instruments, Italy), and EC was measured using a DDS-307A conductivity meter (Inesa Scientific Instrument Co., Ltd, China). The NH_4^+-N content of samples was measured using a colorimetric method at 625 nm, whereas NO_3^--N content was measured using a UV spectrophotometer, and the contents of TN and TC were analyzed using a Vario EL III element analyzer (Elementar, Germany).

DNA extraction and high-throughput sequencing of *nosZI*

Soil DNA was extracted using the HiPure Soil DNA Mini Kit (Magen, China). Based on the aforementioned qPCR analysis results, the *nosZI* region of 28 samples (forward primer sequence *nosZ*-F: CCCGCTGCA CACCRCCTTCGA; reverse primer sequence *nosZ*-R: CGTCGCCSGAGATG TCGATCA) was sequenced on the Illumina Novaseq 6000/Miseq high-throughput sequencing platform. Raw paired-end sequencing data were assigned to each sample via unique barcodes. Quality control and clustering analysis were performed following standard protocols. Amplicon Sequence Variants (ASVs) are defined as exact sequence variants with only single-nucleotide differences, and were obtained using the DADA2 plugin in QIIME2 (https://qiime2.org/). Briefly, after importing the sequencing data into the QIIME2 manifest file, demultiplexing was conducted with the q2-dmux plugin. Subsequently, the demultiplexed sequences were denoised and quality-filtered using the q2-dada2 plugin according to the standard DADA2 protocol, generating an ASV table and representative sequences. Chimeric

https://doi.org/10.48130/nc-0026-0001

sequences and singleton ASVs were removed, and the *nosZI* gene was functionally annotated using the GraftM tool. A total of 3,615,100 sequence reads were obtained from the 28 soil samples. After clustering analysis, 7,712 ASVs were identified.

Data analysis

Data analysis and graphing were performed using Excel 2019 and GraphPad Prism 10.0, while IBM SPSS Statistics 23 was employed to conduct one-way analysis of variance (ANOVA), two-way ANOVA, and least significant difference (LSD) significance test for different treatments, with statistical significance of differences between treatments ($p < 0.05$). RStudio 2023.12.1 was used to generate stacked bar charts to visualize species abundance in bacterial communities and to analyze alpha and beta diversity indices. Beta diversity intra-group variation analysis implemented in R language using the *vegan* package, with PERMANOVA for statistical analysis, and the Bray-Curtis distance algorithm for dissimilarity calculation. The neutral community model was used to quantify the relative contributions of stochastic and deterministic processes to microbial community assembly. The co-occurrence network model was constructed using Gephi 0.9.2, with the calculation of network topological parameters (e.g., number of edges, average degree, connectivity). The structural equation model was analyzed using the *linkET* package in R.

Results and discussion

Soil N_2O emission flux and cumulative emission

The dynamic changes in soil N_2O emission fluxes showed a consistent pattern (Fig. 1a–c), each treatment reached a peak on the second day of incubation. The CK had a peak of 218.23 ± 46.05 μg N·kg^{-1} dw soil·h^{-1}, the MR was 739.22 ± 72.2 μg N·kg^{-1} dw soil·h^{-1}, the MN was 339.39 ± 23.43 μg N·kg^{-1} dw soil·h^{-1}, the HC10 was 558.78 ± 185.57 μg N·kg^{-1} dw soil·h^{-1}, the HN was 460.56 ± 157.62 μg N·kg^{-1} dw soil·h^{-1}, the CT was 744.14 ± 337 μg N·kg^{-1} dw soil·h^{-1}, and the CN was 644.21 ± 266.56 μg N·kg^{-1} dw soil·h^{-1}. The peak N_2O emission of the HC10 treatment was compared with those of the CK and CT treatments using one-way ANOVA followed by the LSD multiple comparison method. The results showed that the peak emission of HC10 was significantly higher than that of the CK, and the peak emission of the MR was significantly higher than that of the CK ($p < 0.05$). These results indicate that applying organic fertilizer alone (especially HC10) significantly increases the peak N_2O emission, and that different organic fertilizers have varying effects on the peak value. The peak N_2O emission of the MN was significantly lower than that of the MR ($p < 0.05$), the CN was significantly lower than the CT ($p < 0.05$), and the HN was significantly lower than the HC10 ($p < 0.05$), demonstrating that inoculation with the NRCB010 strain could significantly decrease the peak N_2O emission of each organic fertilizer treatment. Additionally, the peak N_2O emission of the MN was significantly lower than that of the CN and HN ($p < 0.05$). The cumulative N_2O emissions of the organic fertilizer treatments alone (MR, CT, HC10) were significantly higher than those of the non-inoculated control (CK) ($p < 0.05$). Among them, the HC10 treatment had the highest cumulative emission, followed by the MR treatment. The cumulative N_2O emissions were significantly decreased ($p < 0.05$) after inoculation with the NRCB010 strain (Fig. 1d), with the mitigating rates in the order of MN (46.5%) > HN (27.8%) > CN (26.3%).

Among the inoculation treatments, the MN treatment exhibited the optimal N_2O mitigating effect—achieving a remarkable mitigating rate of 46.5% compared to the non-inoculated control, which

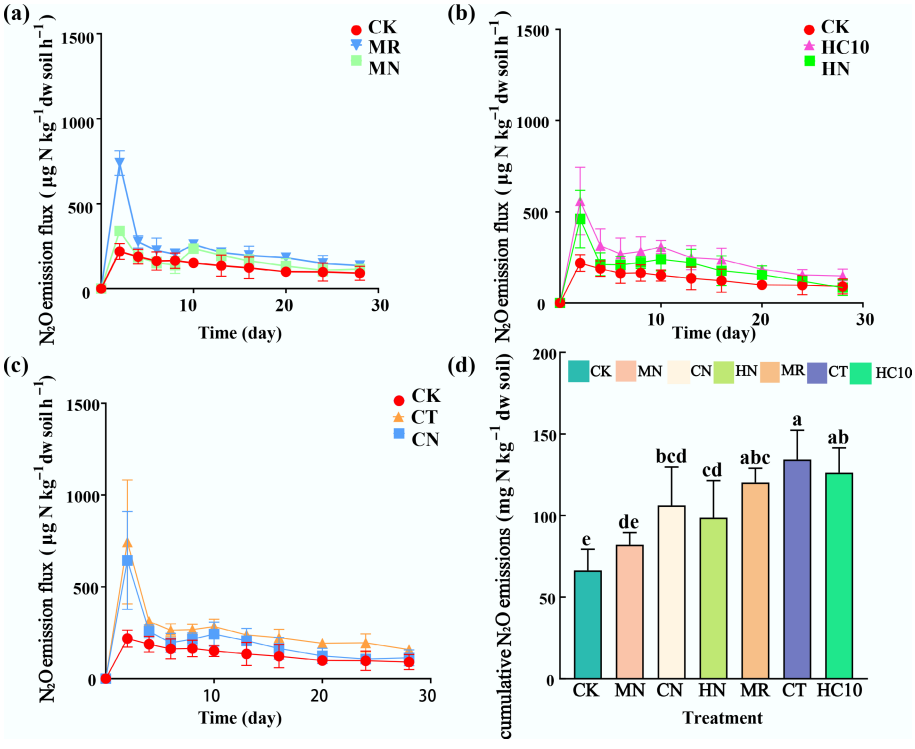


Fig. 1 (**a**)–(**c**) N_2O emission flux, and (**d**) cumulative emission from soil. Different small letters above the bar indicated significant differences among treatments at the 5% level. CK: no fertilizer, MR: mushroom residue organic manure, CT: high-temperature pretreated compost, HC10: hydrothermal carbonized organic manure, MN: mushroom residue organic manure with NRCB010, CN: high-temperature pretreated compost with NRCB010, HN: hydrothermal carbonized organic manure with NRCB010.

is statistically significant. This outstanding performance not only demonstrates that the MN treatment is a highly promising combination for the practical control of N_2O emissions from agricultural soils but also lays a critical foundation for the subsequent in-depth exploration of its underlying mechanisms. The enhanced N_2O mitigation observed in the MN treatment is likely atrributable to the application of the mushroom residue organic manure, as it provides abundant readily available carbon substrates, promotes an optimal C/N ratio, and fosters an improved soil structure. These not only promote the proliferation of *Pseudomonas stutzeri* NRCB010—altering the abundance of the *nosZI* gene and modifying soil physicochemical properties (e.g., pH and EC)—but also contribute to efficient N_2O mitigation through the enhanced *nosZI* gene abundance and synergistic interactions among functional microbial communities. The significant advantage of the MN treatment in N_2O mitigation indicates a more efficient synergistic interaction between the mushroom residue organic manure and the NRCB010 strain. Gao et al.[15] reported that the combination of *Pseudomonas stutzeri* strains and organic fertilizers can reduce N_2O emissions from vegetable soils by increasing the abundance of the *nosZ* gene. Future studies will systematically dissect the key biological processes driving its notable mitigating effect, thereby providing scientific support for the rational design and application of high-efficiency N_2O mitigating technologies.

Soil physicochemical properties

Soil pH values were lower in the organic fertilizer treatments without bacterial inoculation (Table 1). The MR treatment (6.97) was significantly lower than the CK (7.11) ($p < 0.05$). After inoculation with the strain, the pH value of the MN treatment (7.02) was slightly higher than that of MR. The pH values of the CN (7.15) and HN (7.18) treatments were significantly higher than those of CT and HC10 ($p < 0.05$), with the HN treatment having the highest pH value. Regarding EC, the MR treatment (6.18 mS·cm^{-1}) had a significantly higher EC than the other treatments ($p < 0.05$). After inoculation with the strain, the EC value of the MN treatment decreased to 4.45 mS·cm^{-1}, a 28.0% reduction compared with MR ($p < 0.05$). It may degrade some soluble salts in the soil, thereby reducing the soil EC value, and improving the soil physicochemical environment[21]. This improvement reflects the synergistic effect between the strain and the mushroom residue organic manure. MR alleviates soil salinization stress through its high organic matter content, while the strain directly degrades soil-soluble salts via nitrate reduction metabolism under secondary salinization induced by nitrate. Both of them synergistically optimize the soil physicochemical environment, laying a foundation for N_2O mitigation.

Among the non-inoculated treatments, HC10 had the highest NO_3^--N content, while MR had the highest NH_4^+-N content. After inoculation with the strain, the NO_3^--N content in the MN treatment was significantly decreased by 25.2% compared with MR ($p < 0.05$), and the ammonium nitrogen content was slightly lower than that of MR.

The abundance of nitrogen cycling functional genes in soils

On the second day of the experiment, inoculation with strain NRCB010 significantly increased the abundance of the *nosZI* gene in the soil ($p < 0.05$) (Fig. 2g). Among the treatments inoculated with strain NRCB010 (MN, CN, and HN), the abundance of the *nosZI* gene was significantly

Table 1 Physicochemical properties of soil

Time	Treatment	pH (H_2O)	EC (mS·cm^{-1})	NO_3^--N (mg·kg^{-1})	NH_4^+-N (mg·kg^{-1})
2 d	CK	7.11 ± 0.02abc	2.84 ± 0.34e	28.41 ± 2.97e	11.44 ± 0.33b
	MR	6.97 ± 0.07d	6.18 ± 0.23a	68.15 ± 8.22ab	49.39 ± 9.93a
	CT	7.10 ± 0.06abc	3.70 ± 0.34d	48.77 ± 5.17cd	43.71 ± 13.14a
	HC10	7.07 ± 0.07bc	5.25 ± 0.28b	82.96 ± 14.95a	13.74 ± 0.95b
	MN	7.02 ± 0.06cd	4.45 ± 0.25c	50.95 ± 3.36bcd	42.28 ± 18.05a
	CN	7.15 ± 0.07ab	3.34 ± 0.46d	42.65 ± 7.54de	43.76 ± 21.72a
	HN	7.18 ± 0.08a	3.77 ± 0.37d	64.31 ± 24.38bc	13.28 ± 2.82b
10 d	CK	6.81 ± 0.10d	3.45 ± 0.62d	31.78 ± 7.40e	8.62 ± 0.61c
	MR	6.82 ± 0.10d	5.96 ± 0.44a	85.35 ± 6.43a	51.28 ± 15.77a
	CT	7.12 ± 0.06b	3.49 ± 0.15d	46.26 ± 3.69cde	54.40 ± 17.12a
	HC10	6.93 ± 0.02c	4.92 ± 0.41b	39.08 ± 4.16de	26.78 ± 17.36bc
	MN	7.12 ± 0.04b	4.14 ± 0.28c	55.68 ± 20.75b	41.97 ± 25.72ab
	CN	7.24 ± 0.06a	3.25 ± 0.21d	50.64 ± 8.50bcd	51.48 ± 7.24a
	HN	7.15 ± 0.04ab	3.09 ± 0.34d	61.88 ± 8.70b	32.22 ± 12.32ab
20 d	CK	6.67 ± 0.1d	4.14 ± 0.5c	28.91 ± 4.9b	11.39 ± 9.9b
	MR	6.79 ± 0.1c	6.08 ± 0.3a	53.392 ± 10.6a	28.37 ± 13.1a
	CT	6.94 ± 0.02b	3.56 ± 0.3d	49.23 ± 11.6a	30.46 ± 7.7a
	HC10	6.88 ± 0.03bc	4.99 ± 0.6b	61.70 ± 9.5a	22.89 ± 6.4ab
	MN	7.05 ± 0.1a	4.44 ± 0.3c	50.64 ± 8.2a	34.76 ± 17.1a
	CN	7.12 ± 0.1a	3.22 ± 0.3d	48.33 ± 11.5a	26.58 ± 10.6ab
	HN	7.07 ± 0.1a	3.55 ± 0.2d	50.18 ± 9.4a	29.18 ± 2.6a
28 d	CK	7.42 ± 0.1a	2.86 ± 0.5c	34.98 ± 2.5c	8.45 ± 1.6c
	MR	6.97 ± 0.1bc	5.77 ± 1.0a	60.02 ± 12.7a	12.13 ± 1.1c
	CT	7.06 ± 0.1b	3.01 ± 0.1c	51.38 ± 6.1a	43.23 ± 10.4a
	HC10	6.84 ± 0.04c	4.63 ± 0.2b	57.71 ± 4.4a	14.28 ± 1.8c
	MN	6.85 ± 0.1c	4.08 ± 0.4b	51.59 ± 3.8ab	32.18 ± 11.6b
	CN	6.86 ± 0.2c	2.95 ± 0.4c	44.21 ± 8.78bc	35.45 ± 9.3ab
	HN	6.91 ± 0.1bc	2.62 ± 0.2c	45.61 ± 9.4bc	16.63 ± 3.7ac

CK: no fertilizer, MR: mushroom residue organic manure, CT: high-temperature pretreated compost, HC10: hydrothermal carbonized organic manure, MN: mushroom residue organic manure with NRCB010, CN: high-temperature pretreated compost with NRCB010, HN: hydrothermal carbonized organic manure with NRCB010. Different lowercase letters indicate significant differences among treatments at the 5% level.

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higher than that in the non-inoculated treatments (MR, CT, and HC10) ($p < 0.05$). Specifically, the MN treatment exhibited the highest *nosZI* gene abundance (1.8×10^7 copies·g^{-1} dry soil), which was a 125% increase compared with the MR treatment (8×10^6 copies·g^{-1} dry soil). With extended incubation time, the abundance of the *nosZI* gene across all treatments showed a downward fluctuation. On the 28th day

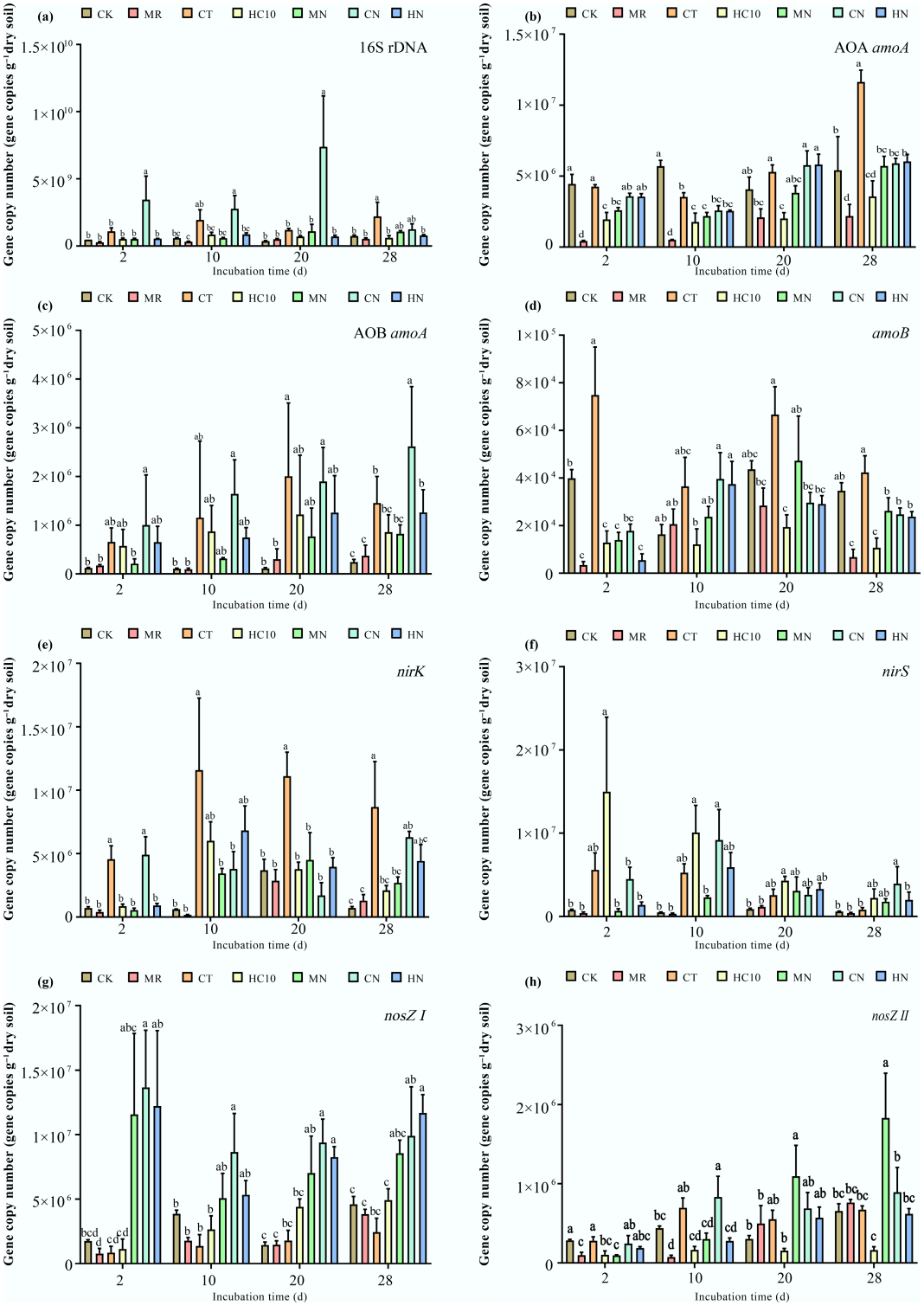

Fig. 2 Abundance of nitrogen cycling functional genes in soils. (**a**) The abundance of 16S rDNA in soils. (**b**) The abundance of AOA *amoA* genes in soils. (**c**) The abundance of AOB *amoA* genes in soils. (**d**) The abundance of *amoB* genes in soils. (**e**) The abundance of *nirS* genes in soils. (**f**) The abundance of f *nirK* genes in soils. (**g**) The abundance of *nosZI* genes in soils. (**h**) The abundance of *nosZII* genes in soils. Different lowercase letters above the bar indicated significant differences among treatments at the 5% level. CK: no fertilizer, MR: mushroom residue organic manure, CT: high-temperature pretreated compost, HC10: hydrothermal carbonized organic manure, MN: mushroom residue organic manure with NRCB010, CN: high-temperature pretreated compost with NRCB010, HN: hydrothermal carbonized organic manure with NRCB010.

of incubation, the abundance of the *nosZI* gene in the MN treatment remained significantly higher than that in other treatments ($p < 0.05$).

Since the N_2O reductase encoded by the *nosZI* gene is the key enzyme for converting N_2O to N_2[22], its high abundance can directly promote the reduction of N_2O to N_2. Therefore, the high expression level of the *nosZI* gene in the MN treatment may be the core mechanism underlying its optimal N_2O-mitigating effect. The decrease in gene abundance over time may be related to the gradual consumption of soil nitrogen and to insufficient microbial metabolic substrates. The results also indicated that inoculation with NRCB010 significantly increased the abundance of the *nosZI* gene in the soil, thereby decreasing soil N_2O emissions. A previous study showed that inoculation with NRCB010 decreases N_2O emissions and promotes tomato growth simultaneously[15]. Additionally, n-hexadecanoic acid, a plant rhizosphere exudate, can not only decrease N_2O emissions from farmland soils by itself, but also synergize with NRCB010 to promote plant growth and the colonization of PGPR in the plant rhizosphere[23].

Community composition and diversity of *nosZI*-carrying microorganisms in soils

To characterize the taxonomic composition of *nosZI*-carrying microorganisms at the phylum, class, and order levels, Fig. 3 illustrates their species composition. The dominant phyla may include *Verrucomicrobiota*, *Pseudomonadota*, *Gemmatimonadota*, *Planctomycetes*, etc. (Fig. 3a). Among them, *Pseudomonadota* and *Verrucomicrobiota* showed the most significant changes. In the treatments inoculated with strain NRCB010 (MN, CN, HN), the relative abundance of *Pseudomonadota* were significantly higher than those in the non-inoculated treatments (MR, CT, HC10) ($p < 0.05$). Specifically, the MN treatment had the highest proportion of *Pseudomonadota* (35%), which was 94.4% higher than that in the MR treatment (18%). Since strain NRCB010 belongs to *Pseudomonas stutzeri* and is classified in *Pseudomonadota*, its colonization and reproduction after inoculation are the main reasons for the increased abundance of this phylum in soils. The relative abundance of *Verrucomicrobiota* in the non-inoculated treatments were significantly higher than those in the inoculated treatments ($p < 0.05$). It is speculated that *Verrucomicrobiota* may prefer nitrogen and SOM-rich environments, and the introduction of the strain altered the soil microenvironment and inhibited bacterial growth in this phylum. At the class level, the relative abundance of *Gammaproteobacteria* in inoculated treatments increased significantly. In contrast, the relative abundance of *Opitutae* in *Verrucomicrobiota* decreased significantly, consistent with the changing trend at the phylum level (Fig. 3b). The dominant orders may include *Pseudomonadales, Enterobacteriales, and Xanthomonadales*. *Pseudomonadales* accounted for a prominent proportion in the MN and MR treatments, while *Enterobacteriales* were more dominant in the CK (Fig. 3c).

The relative abundance of *Verrucomicrobiota* decreased significantly, dropping from 22%–25% in non-inoculated treatments to 10%–15% in inoculated treatments. Given that *Verrucomicrobiota* was enriched in non-inoculated environments with both high nitrogen and organic matter content, it is expected that certain members of this phylum may possess metabolic pathways for N_2O production. The introduction of strain NRCB010 may have promoted the consumption of NO_3^- and SOM, thereby inhibiting the growth or metabolic activity of N_2O-producing microorganisms.

Pseudomonadota is one of the main host phyla for the *nosZI*-carrying microorganisms in soils. In this study, the abundance of the *nosZI* gene in the MN treatment was significantly higher than that in the MR treatment. The enrichment of *Pseudomonadota* directly increased the abundance of the *nosZI* gene and enhanced the N_2O-reducing capacity. Some taxa within *the Verrucomicrobiota* may harbor the *amoA* gene, whose expression is involved in ammonia oxidation and N_2O production. The abundances of AOA *amoA* and AOB *amoA* genes in non-inoculated treatments (MR, HC10) were significantly higher than those in inoculated treatments. Specifically, the abundance of the AOB *amoA* gene in the MR treatment was 18.7% higher than that in the MN treatment. It is hypothesized that taxa carrying the *amoA* gene within *Verrucomicrobiota* proliferate extensively in non-inoculated environments, thereby promoting ammonia oxidation and thus increasing N_2O emissions. In contrast, after inoculation with NRCB010, the soil pH tended to be neutral, and EC decreased, which altered the habitat for *Verrucomicrobiota*, inhibited its growth and the expression of the *amoA* gene, thereby decreasing N_2O production.

Different treatments have significant effects on the community diversity of *nosZI*-carrying microorganisms as reflected in both α and β diversity. In terms of α diversity (Fig. 3d), the HC10 treatment significantly increased the Chao1 and Observed indices as well as Shannon and Simpson indices, whereas the MN and HN treatments significantly decreased community diversity. The HC10 treatment showed the most significant fluctuation in the Shannon index and an increase in the Simpson index, indicating that community stability was poor in the high-temperature pretreated compost environment and that competition among dominant species was intense[24]. The Chao1 indices for the CT treatment were relatively high, suggesting that high-temperature-pretreated compost may be more likely to increase species richness[25]. In terms of β diversity, the bacterial community compositions of the HC10, MN, and HN treatments were clearly separated from those of other treatments, indicating that these treatments significantly altered community composition. Among them, the HC10, MN, and HN groups showed strong internal aggregation, while the CK, MR, and CT treatments overlapped to some extent. The cumulative variance explained by PCoa1 and PCoa2 was 51.4% (Fig. 4e), which could well reflect the differences in community composition. Treatments of the same type were close in distance, while the distance between uninoculated treatments with different organic fertilizers was relatively far, indicating that the NRCB010 strain had a much greater impact on the community composition than the organic fertilizers. The introduction of the strain might be the core factor driving changes in the community structure[26]. The significance levels of β diversity-based community structure differences among treatment groups, determined by PERMANOVA, are shown in Supplementary Table S2.

Community assembly processes and key network modules of *nosZI*-carrying microorganisms

Neutral community modeling[27] analysis revealed that the CT and HC10 treatments exhibited the highest R^2 values, followed by the MR and MN treatments (Fig. 4a). The community structures under these four treatments showed a better fit with the neutral model, and stochastic processes had greater explanatory power for community assembly. The low R^2 values of the CK, CN, and HN treatments indicate that deterministic processes play a more important role. In the CK treatment, the absence of organic fertilizer leads to reduced soil nutrient availability and intense environmental filtering. In the CN and HN treatments, NRCB010 colonization alters soil pH, EC, and carbon-nitrogen supply, thereby reshaping the community structure through interspecific competition and synergistic interactions. The high R^2 values of the CT and HC10 treatments suggest that stochastic processes dominate community assembly, which may be related to the weak selective pressure of these two organic fertilizers on

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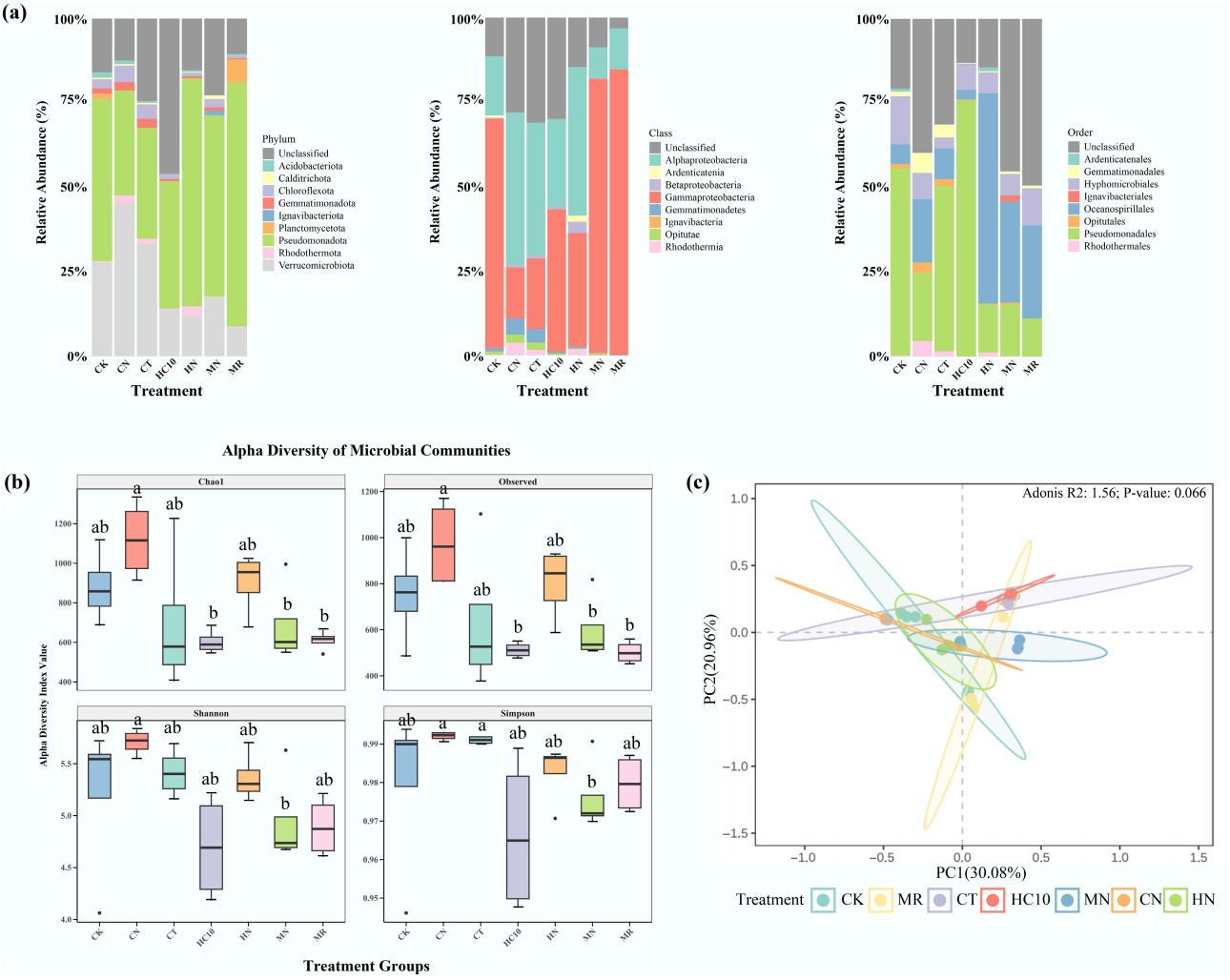

Fig. 3 Community composition of *nosZI* carrying microorganisms at (**a**) phylum, (**b**) class, and (**c**) order levels, and their (**d**) α diversity, and (**e**) β diversity in soils. CK: no fertilizer, MR: mushroom residue organic manure, CT: high-temperature pretreated compost, HC10: hydrothermal carbonized organic manure, MN: mushroom residue organic manure with NRCB010, CN: high-temperature pretreated compost with NRCB010, HN: hydrothermal carbonized organic manure with NRCB010.

microorganisms due to their physicochemical properties—such as the low C/N ratio of CT and the high carbon content of HC10. The MR and MN treatments had the highest Nm values, suggesting that inter-community species migration and diffusion capacities were strongest under these treatments, with higher species exchange frequency and a more uniform species distribution. A synergistic analysis showed that stochastic processes dominated the community assembly in the CT and HC10 treatments, with moderate species diffusion capacity; in the MR and MN treatments, stochastic processes mainly shaped the communities. Although species diffusion capacity was relatively strong in the CK, CN, and HN treatments, deterministic processes remained a key regulator of the community structure.

Through *nosZI* co-occurrence network analysis, the CK (160 nodes, 1,594 edges) showed significantly higher average degree (19.925) than the other treatments, indicating more frequent and intensive bacterial interactions (Fig. 4b). The CN (modularity = 0.822) had significantly higher modularity than the other treatments, suggesting clearer functional module division of the microbial community and potentially more distinct functional subgroups formation. In contrast, the HN (modularity = 0.723) had relatively low modularity with weak differentiation of community functional modules. The MN had the fewest nodes and edges, with both average degree and modularity at low levels, reflecting relatively sparse interactions among bacterial communities and weak modular organization in this treatment. These results indicate significant differences in the interaction patterns and the organizational levels of functional modules within bacterial communities. Such differences may be related to the cooperative metabolism, resource competition patterns, or the establishment of symbiotic relationships among microorganisms. It also implies that different treatments have differential shaping effects on the structure and functional network of microbial communities by influencing the interactions among microorganisms. Studies have shown that under different nitrogen input gradients, changes in the network structures and microbial traits of bacterial and fungal communities reveal that alterations in the life-history strategies of soil bacteria regulate the reduction in the complexity of their co-occurrence networks, uncovering the trait-based regulatory mechanism underlying microbial interspecific interactions and co-occurrence networks[28].

Correlation analysis between environmental factors and N_2O emissions

The correlation analysis indicated that N_2O emissions were significantly negatively correlated with the abundance of *nosZI* (Fig. 5a) and

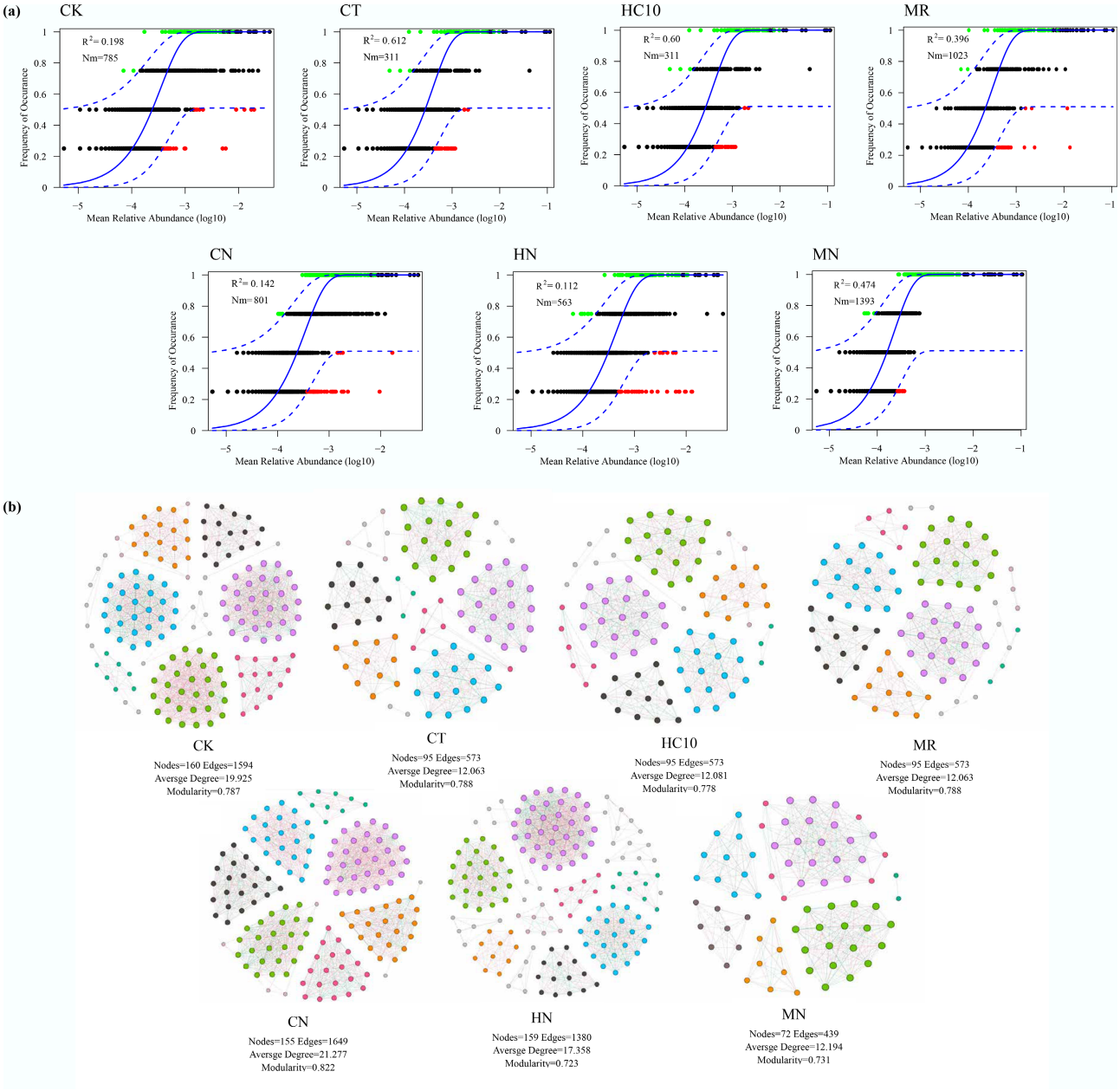

Fig. 4 The community assembly based on (**a**) neutral theory, and (**b**) co-occurrence network of *nosZI* carrying microorganisms. R^2 indicates the model fit, and Nm represents the product of metacommunity size and migration time. CK: no fertilizer, MR: mushroom residue organic manure, CT: high-temperature pretreated compost, HC10: hydrothermal carbonized organic manure, MN: mushroom residue organic manure with NRCB010, CN: high-temperature pretreated compost with NRCB010, HN: hydrothermal carbonized organic manure with NRCB010.

significantly positively correlated with soil NO_3^--N, pH, and EC. EC indirectly modulates the expression level and activity of the *nirS* gene by regulating soil osmotic pressure and the community structure of *nirS*-type denitrifiers, thereby influencing the conversion efficiency of NO to N_2O during denitrification and ultimately regulating N_2O emissions. Greenhouse vegetable soils are highly susceptible to secondary salinization due to multiple factors, including long-term excessive nitrogen fertilization, inappropriate irrigation practices, and weak leaching under the enclosed conditions of greenhouses. The predominant anion in greenhouse vegetable soils is NO_3^-, accounting for more than 60% of the total soluble salts. NRCB010 is an N_2O-reducing bacterium with strong NO_3^- reducing capability. Through its intrinsic NO_3^-- reducing metabolic pathway, NRCB010 can progressively decrease accumulated NO_3^- in soil, which is further converted into gaseous compounds such as N_2. This process not only directly decreases the accumulation of NO_3^- in soil, thereby alleviating the degree of NO_3^--dominated secondary salinization in the greenhouse vegetable soils and improving soil quality, but also decreases the substrate concentration for N_2O production via dissimilatory NO_3^- reduction[15]. Both AOA *amoA* and AOB *amoA* were significantly positively correlated with N_2O emission, suggesting that nitrification contributes to N_2O production in greenhouse vegetable soils.

The importance ranking result of the random forest model reflects the contribution of each variable to the increase in percentage of the model's mean squared error (MSE), thereby indicating the

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variable importance (Fig. 5b). Among them, NO_3^--N has the highest contribution to the increase in MSE and is a core factor affecting soil N_2O emissions; the importance of subsequent variables such as *nosZI* and *amoB* decreases in turn. EC and NO_3^--N are indirect drivers, as they influence N_2O emissions by regulating the abundance of N cycling genes[29]. The structural equation model meets high reliability standards with satisfactory fitting performance, as evidenced by a reasonable degree of freedom, strong explanatory power of the Comparative Fit Index and Goodness-of-Fit Index, and minimal model-data deviation given that the Root Mean Square Error of Approximation and Standardized Root Mean Square Residual are well below the critical thresholds (df = 2.000, CFI = 0.97, GFI = 0.949, RMSEA = 0.012, SRMR = 0.015). The structural equation model further quantified the direct and indirect effects of each factor on N_2O emissions: the *nosZI* gene exhibited a significant negative direct effect on N_2O emissions (−0.44) (Fig. 5c). EC might indirectly promote N_2O emissions by influencing the abundance of the *nirS* gene. Overall, *nosZI* is the key factor governing N_2O emissions from greenhouse vegetable soils.

Synergistic effects of organic fertilizers and inoculation with NRCB010 on N_2O emissions

The results showed significant differences in the N_2O-mitigating effects among the combinations of different organic fertilizers and the strain. Among them, the MR-inoculated treatment (MN) exhibited the greatest N_2O emission reduction, which was significantly higher than those of the CT-inoculated treatment and the HC10-inoculated treatment. This result reveals the key regulatory role of organic fertilizer type in the strain NRCB010 mitigating function. Studies have tested metabolic extracts from soil amendments and a mixture of four plant growth-promoting *Bacillus* strains, combined with different nitrogen fertilizers. The results showed that the microbial inoculants exhibited notable N_2O-mitigating effects, which were dependent on the type of fertilizer[30]. Moreover, these inoculants could increase crop nitrogen uptake and promote crop growth.

From the perspective of the inherent characteristics of organic fertilizers, the physicochemical properties of MR provide a unique and suitable environment for the colonization and functional performance of strain NRCB010. On one hand, the pH of MR is close to neutral, while strain NRCB010 prefers a neutral to slightly alkaline environment. Neutral pH conditions can effectively alleviate acid-base stress on the strain, significantly improving its survival and reproductive efficiency in soil and laying a foundation for the strain to exert its N_2O-reducing function continuously. On the other hand, MR has the highest organic matter content and a moderate C/N ratio. Compared with CT and HC10, MR can not only provide sufficient and easily utilizable carbon sources for the strain to meet its metabolic needs, but also reduce the accumulation of NO_3^-. Since NO_3^- is the core substrate for N_2O production via denitrification, reducing the substrate directly lowers the potential for N_2O generation. Moreover, MR has the unique advantage of improving soil structure. Previous studies have confirmed that microbial organic fertilizers derived from mushroom residue can enhance soil water and fertilizer retention capacity, increase bacterial activity, and even be used to remediate and improve various degraded soils[31].

In this study, the high organic matter content of MR further improved soil aeration, reducing the formation of anaerobic microenvironments. As denitrification occurs under anaerobic conditions, enhanced aeration can directly inhibit denitrifying bacterial activity in soil[32], thereby reducing N_2O production through microenvironmental regulation. The mushroom residue organic manure used in the MN treatment could provide exclusive and highly bioavailable carbon sources for NRCB010. MR is rich in complex organic carbon compounds, including lignocellulosic derivatives, small-molecule organic acids, and amino acids, which are well matched to the metabolic preferences of *Pseudomonas stutzeri*. Compared with simple sugars, these carbon sources can more efficiently stimulate the metabolic activity of the strain[33]. They not only meet the energy requirements for its growth and reproduction, but also upregulate the abundance of *nosZI*, thereby enhancing their N_2O-mitigating efficiency. In contrast, the labile organic carbon in the high-temperature pretreated compost has undergone thermal decomposition, and the carbon structure of the hydrothermal carbon organic fertilizer is recalcitrant to degradation; neither can provide such a specific carbon source. The high organic matter content of mushroom residue organic manure can promote the formation of soil water-stable macroaggregates. The aerobic outer layer of these aggregates can meet the aerobic metabolism and colonization requirements of NRCB010, while the moderately anaerobic inner microzones provide favorable conditions for denitrification[34].

Geng et al. reported that the application of bio-organic fertilizer effectively decreased N_2O emissions from vegetable field soil, with a mitigation efficiency of 34%[18]. In contrast, the maximum N_2O mitigating efficiency of NRCB010 tested in this study reached 46.5%, demonstrating a superior mitigating effect. The synergistic expression of the *nosZ* gene carried by strain NRCB010 and the soil-borne

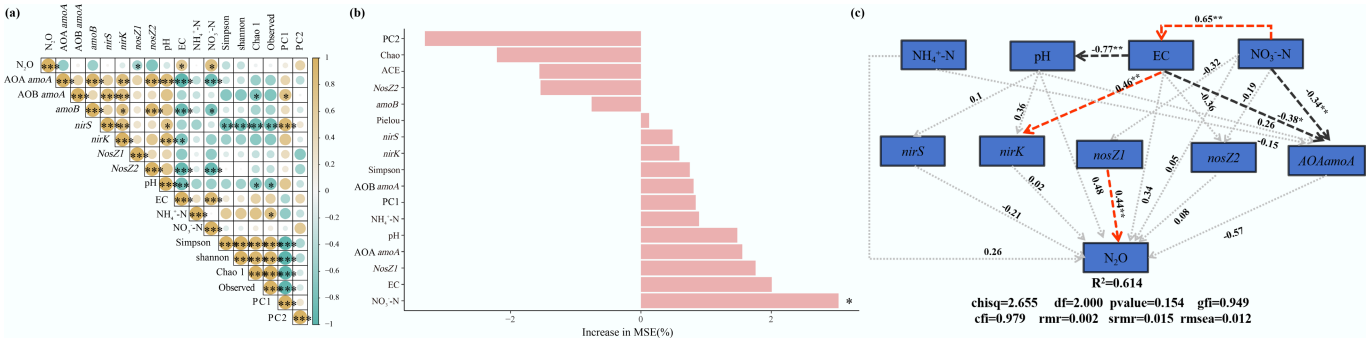

Fig. 5 (**a**) Correlation analysis, (**b**) random forest model, and (**c**) structural equation model analyses of environmental factors and N_2O emissions from greenhouse vegetable soils. The red arrows and black arrows show positive and negative relationships, respectively. The numbers above the lines are standardized path coefficients. The width of the arrows is proportional to the strength of the path coefficients. * Significance at the $p < 0.05$ level, ** significance at the $p < 0.01$ level, and *** significance at the $p < 0.001$ level.

nosZI carrying microorganisms is the key molecular mechanism underlying the efficient N_2O mitigation in the MN treatment. The N_2O reductase encoded by the *nosZI* gene is the key enzyme for converting N_2O into harmless N_2, and its abundance shows a significant negative correlation with soil N_2O emissions[35]. In this study, the abundance of the *nosZI* in the MN treatment was significantly higher than that in other treatments. The appropriate environment provided by MR not only promoted the colonization of NRCB010, but also enhanced the activity of native *nosZI*-carrying microorganisms, thereby enhancing the N_2O reduction capacity and ultimately achieving efficient mitigation[36]. In contrast, the CN and HN treatments showed weaker N_2O mitigating effects. Although the pH of the CN treatment decreased slightly after inoculation, it may still fall outside the suitable growth range of some strains, limiting their activity. Meanwhile, the low C/N ratio of CT readily leads to rapid accumulation of soil inorganic nitrogen, providing sufficient substrates for denitrification and offsetting part of the N_2O mitigating effect. The high NO_3^--N content of HC10 further increases the risk of N_2O production, thereby limiting the N_2O-mitigating impact of the HN treatment.

Overall, the combination of mushroom residue organic manure and NRCB010 not only achieves the optimal N_2O mitigation effect but also offers multiple advantages, including improving soil quality, alleviating soil secondary salinization caused by NO_3^-, and increasing the utilization rate of agricultural wastes. However, limited by the simplified microcosm experimental system, the short experimental term, and the failure to account for field heterogeneity, there are restrictions on the extrapolation of the results. In the future, it is necessary to verify technical stability through long-term field experiments, analyze the mechanisms of strain-soil-crop interactions, optimize application forms, and expand research on adaptability across different environments. This will promote the application of this mitigating technology in actual production and provide more robust technical support for field use.

Underlying mechanism of microbial communities and NRCB010 in regulating N_2O emission

The *nosZI* gene encodes N_2O reductase and is mainly found in dominant bacterial phyla, such as Pseudomonadota[37]. The enzyme encoded by *nosZI* has a higher affinity for N_2O and greater N_2O-reducing efficiency, thereby playing a key role in N_2O consumption. This also provides a theoretical basis for improving the abundance of the *nosZI* gene through genetic engineering methods in the future. The *nirS* gene encodes nitrite reductase, a key rate-limiting enzyme in denitrification. It reduces NO_2^- to NO, and NO is the direct precursor for the subsequent reduction to N_2O[38]. A high abundance of the *nirS* means more active denitrification, increased NO production, and thus higher N_2O generation. Both EC and NO_3^- content were positively correlated with the abundance of the *nirS*[39], suggesting that controlling water and fertilizer input may be an effective measure to inhibit the activity of the *nirS* gene and decrease N_2O emissions[40].

NRCB010 was the primary driver of changes in soil microbial community composition, with its influence far greater than that of organic fertilizers. The colonization and reproduction of the strain directly altered the composition of the dominant bacterial phyla. NRCB010 belongs to the class Gammaproteobacteria of the phylum *Pseudomonadota*; the relative abundance of this phylum increased significantly after the inoculation with NRCB010. Most bacteria in *Pseudomonadota* are aerobic, and their widespread distribution can improve soil aeration[41], and inhibit the growth of anaerobic denitrifying bacteria (e.g., *Verrucomicrobiota*). The proportion of *Verrucomicrobiota* in non-inoculated treatments was about 22%–25%, which decreased to 10%–15% after inoculation. The decline of *Verrucomicrobiota* may further reduce denitrification activity and, in turn, decrease N_2O emissions.

NRCB010 indirectly regulated community structure by altering the soil microenvironment through its metabolic activities. Soil EC decreased from 6.18 to 4.45 mS·cm^{-1}, and pH increased from 6.97 to 7.02 after inoculation; optimizing soil physicochemical properties can promote the growth of beneficial microorganisms. Inoculation with NRCB010 not only achieves short-term N_2O mitigation, but also improves soil ecological functions by optimizing community structure[15], providing support for the long-term sustainable use of greenhouse vegetable fields.

Conclusions

Inoculation with *Pseudomonas stutzeri* NRCB010 significantly decreased the cumulative N_2O emissions from the greenhouse vegetable field. The N_2O emissions were negatively correlated with the abundance of the *nosZI* gene ($p < 0.05$), suggesting that *nosZI*-carrying microorganisms were the most critical factor governing N_2O emissions from the greenhouse vegetable soil. The combination of mushroom residue organic manure with *P. stutzeri* NRCB010 is an effective technical approach for decreasing N_2O emissions from greenhouse vegetable fields, providing practical guidance for greenhouse gas mitigation and soil ecological restoration.

Supplementary information

It accompanies this paper at: https://doi.org/10.48130/nc-0026-0001.

Author contributions

The authors confirm their contributions to the paper as follows: Wenjun Xie: writing – original draft, investigation, data curation; Zijian Qiu: validation, software, methodology, formal analysis; Dandan Li: validation, software, methodology, formal analysis; Zhouzhang Wang: Writing – review and editing; Nan Gao: validation, methodology; Ruonan Xiong: writing – review and editing; Adharsh Rajasekar: writing – review and editing; Xinhua He: writing – review and editing; Weishou Shen: writing – review and editing, validation, supervision, resources, project administration, funding acquisition, conceptualization. All authors provided revision comments on previous versions of the manuscript, reviewed the study results, and approved the final version of the manuscript.

Data availability

The datasets used or analyzed during the current study are available from the corresponding author upon reasonable request.

Acknowledgements

We sincerely thank the editor and reviewers for their insightful comments and suggestions.

Funding

This study was funded by the National Key Research and Development Program of China (Grant No. 2023YFF0805403), and the National Natural Science Foundation of China (Grant Nos 42377311 and 41771291).

Declarations

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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