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Partial organic substitution for chemical fertilizer reduces N_2O emissions but increases the risk of N loss through nitrification in Tibetan farmland

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The combination of organic fertilizers (OFs) and chemical fertilizers (CF) is a promising agricultural management strategy to improve soil fertility while mitigating N_2O emissions in croplands. However, there is still lacking of in-depth understanding of the effects of different OF and CF blends on N_2O emissions and the underlying drivers. To this end, we conducted a short-term soil incubation to address the influences of partial OF substitutions for CF, i.e., 40% substitution of compost (CP), Yak dung (YD), *Qingke* straw (QS), and sheep dung (SD) on the processes of nitrification and denitrification in sandy loam soils in the Lhasa Valley. We found that CP, QS, and SD reduced cumulative N_2O emissions by 53.43%, 25.96% and 16.64%, respectively compared to pure chemical fertilizer (N), except YD caused a significant higher in total N_2O emissions. Fertilization treatments primarily regulate potential N_2O emissions by affecting denitrification processes. While ammonia-oxidizing archaea (AOA *amoA*) could be the main driver of nitrification, and *nirS* abundance explained most of the cumulative N_2O emissions. In addition, NO_3^-–N tends to accumulate in the farmland soils, indicating an increase in the risk of leaching and nutrient loss. Overall, soil N_2O emission reduction was favored by applying partial organic fertilizer substitution especially after through compost. Co-composting of animal manure and crop residue has more impressive potential for mitigating farmland N_2O emissions.

Keywords Partial organic substitution, Soil incubation, Microbial gene abundance, Nitrification, Denitrification, N_2O emissions

N_2O is a potent greenhouse gas[1,2], with a global warming potential nearly 298 times of carbon dioxide (CO_2) over a 100-year period[3]. Due to extensive use of chemical fertilizers, agriculture as one of the major sources of N_2O, contributes to 25% of the total global N_2O emissions[1,4,5]. The intensity of N_2O emissions determines, to a certain extent, the overall greenhouse gas (GHG) balance of agricultural production systems[6,7]. Therefore, this makes it imperative to find effective and alternative fertilization solutions to mitigating N_2O emissions through reducing use of chemical fertilizers.

It has been documented that combined application of organic fertilizers (OFs) and chemical fertilizers (CFs) may be an effective strategy to reduce N_2O emissions[8,9]. The intensity of soil N_2O emissions depends on the production and reduction processes of N_2O, in which microbial nitrification and denitrification drive N_2O and NO release[7,10]. A number of studies has demonstrated that the combination of OFs and CF can reduced soil inorganic nitrogen surplus and significantly reduced N_2O emissions[8,11]. For example, Wu et al. has proven 45% organic substitution can meet the needs of both sustaining soil fertility and mitigating N_2O emissions. However, some have reported the increasing effect of organic fertilizers on N_2O emissions from low altitude croplands[13,14]. Cui et al. found that OFs supplied carbon source to enhance denitrification, thereby promoting N_2O emissions.

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Similarly, Lin et al. reported that fertilizer application led to increased N_2O emissions, primarily due to the rised abundance of bacterial *amoA* genes. These conflicting results suggest that the factors and processes controlling N_2O emissions are complex and context dependent in the fields. Current research indicates that various factors, including the C/N ratio of OFs[17], soil type[18], and others environmental conditions further influence these processes[19]. As a matter of facts, OFs can stimulate microbial immobilization of fertilized nitrogen and, at the same time, act as a source of N_2O[7,20,21]. To better understand nitrogen transformation and N_2O emissions after partial organic substitution, an in-depth mechanistic understanding is needed to disentangle the contribution of nitrification, denitrification to N_2O emissions.

The identification of nitrification and denitrification genes plays an important role in predicting N_2O emissions[5,18,22]. Ammonia-oxidizing archaea (AOA *amoA*) and ammonia-oxidizing bacteria (AOB *amoA*) convert ammonia (NH_4^+) to nitrite (NO_2^-) through oxidation, which is the main step controlling the rate of nitrification[23,24]. Additionally, Comammox (e.g. *Nitrospira* branch) can oxidize NH_4^+ to NO_3^-[25–27]. *nirS*, *nirK*, and *nosZ* are the functional genes involved in the transformation of denitrifying microorganisms[28,29]. And the composition and activity of these microbial communities may alter the balance between N_2O production and reduction through nitrogen mineralization and denitrification rates[30,31]. Xu et al. found that a strong correlation between AOA *amoA* and *nirS* genes rendered a significant increase in N_2O emission from OFs. While N_2O emissions were reported to be suppressed by organic fertilizers replacing N fertilizers, which was attributed to the reduction of soil mineral nitrogen content and stimulation of denitrifying gene abundance[9]. In contrast, Other studies reported that co-addition of cow manure and CF decreased the abundance of *nirS* and *nirK*, but increased N_2O emissions[33]. Related studies have focused on the link between N_2O emissions and denitrifying bacterial community abundance, and these still present conflicting information[19,34–36]. However, it remains poorly understood what types of metabolic functional genes are involved in the nitrogen transformation process, especially the ones for the increase or decrease in soil N_2O emissions, by changing soil physicochemical properties, microbial composition after partial organic substation for CFs. Knowledge gaps remain regarding the main drivers of N_2O emissions, including nitrification and denitrification genes and environmental factors. Therefore, more efforts are needed to explore multiple microbial functional genes related to the N_2O production process after organic substitution, which can help understand the underlying mechanisms.

On the Tibetan Plateau, crop residue and livestock manure are applied as OFs for agricultural production[37]. Mostly Tibetan sheep dung and partly air-dried Yak dung are the traditional source of nutrients for crops[38,39]. The straw of Tibetan hulless barley (*Hordeum vulgare* L. var. *nudum*), also known as *Qingke*[40,41], is composted with animal manure through microbial action to make organic fertilizers[42]. These OFs application can affect soil nitrogen effectiveness directly by providing external N sources and indirectly by affecting soil nitrogen mineralization[6,10,43]. The organic carbon input from OFs can influence microbial activity, potentially affecting N_2O emissions by modulating nitrification and denitrification processes[44]. Several studies have investigated N_2O emissions from natural grasslands[45–47] and permafrost[48,49] on the Tibetan Plateau. However, the knowledge of N_2O emissions after crop straw and animal manure substitution of chemical fertilizers is still lacking and the underlying drivers of nitrogen transformation and N_2O emissions remain unclear.

In this study, we conducted a short-term incubation experiment to investigate the effect of partial OFs substitution for CF on soil N_2O emissions under equal nitrogen conditions. We analyzed the abundance of functional genes involved in the formation and reduction of N_2O, such as AOA *amoA* and AOB *amoA*, *nirK*, *nirS*, and *nosZ* as well as the community structure and composition of denitrification genes. Attempts were made to address the following questions: (1) What types of organic substitution can effectively reduce soil N_2O emissions? and (2) how do nitrogen transformation processes and associated nitrification and denitrification genes mediate N_2O emissions? We hypothesized that partial organic substitution for chemical fertilizer could mitigate N_2O emissions compared to CF through modulating soil properties and reducing denitrification gene abundance.

Materials and methods

Preparation of soil and fertilizer

The soils for incubation were collected from the top layer (0–20 cm) in August 2023 from the Lhasa Plateau Biological Research Base (29°45′N, 90°96′E, altitude 3650 m), in Caina Township, Lhasa City, Tibetan Autonomous Region. According to the soil system classification, the test soil was alluvial soil with a sandy loam soil texture. All soil samples were sieved through a 2 mm sieve to remove residual roots, litter, and stones. A portion of the samples were stored in a 4 °C refrigerator for laboratory incubation, while the remaining soils were air dried indoors for measuring soil properties. The basic soil properties were as follows: pH 7.24 (1:2.5, soil/water), total C content, 9.89 g kg^{-1}; total N content, 0.63 g kg^{-1}; bulk density, 1.23 g cm^{-3}, and natural moisture content, 7.22% in the field.

OFs include compost, yak dung, *Qingke* straw and Tibetan sheep manure. The raw components of compost were Yak dung, *Qingke* straw, and sheep dung, which were mixed thoroughly and then composted for 68 days using the aerobic composting technology of "air-flow membrane". All OFs materials were dried at 55 °C, grounded and subsequently sieved through a 2 mm mesh for partial organic fertilizer substitution. The chemical properties are shown in Table 1.

Laboratory incubation experiment

Soil incubation included six treatments: ambient soil without fertilization (CK) and five equal N fertilization regimes with 40% organic substitution, i.e. chemical fertilizer (urea) alone (N), compost plus urea (CP), yak dung plus urea (YD), *Qingke* straw plus urea (QS), and Tibetan sheep dung plus urea (SD). We set 12 replicates per treatment. The total nitrogen application rate for all fertilization was designed to be 0.25 mg g^{-1} of dry soil. According to the optimal application ratio of OFs and CF in local practice and referenced strategy to improve

OFs type	pH	C (%)	*N* (%)	C: *N*	*P* (%)	K (%)
QS	7.6	41.53	0.71	58.49	0.09	0.74
YD	9.9	22.20	1.82	12.20	0.50	2.53
SD	9.6	23.51	1.73	13.59	0.46	2.59
CP	8.7	22.99	1.98	11.61	0.53	1.53

Table 1. The chemical properties of different OFs. CP (compost plus urea), YD (Yak dung plus urea), QS (*Qingke* straw plus urea), and SD (Tibetan sheep dung plus urea).

soil fertility while mitigating N_2O emissions[12], the nitrogen ratio of CF to OFs were set at 6:4 in fertilization treatments, i.e., 40% organic substitution for CF. Before incubation, fresh soils were wetted to 60% water holding capacity and pre-incubated in dark conditions at 25 °C for 7 days. Then, 250 g (weight in dry soil) of each treatment was weighed into 500 mL culture bottles, and OF was added to the soil by thorough mixing. All bottles, covered with a thin film with small holes to allow gas exchange, were incubated at 25 °C in the dark for 85 days. Throughout the entire incubation period, soil moisture was kept constant by weighing the soil every other day and subsequently adding water to supplement the water loss through evaporation.

Gas and soil sampling and analyses

N_2O gas samples were collected from culture bottles at 0, 1, 2, 3, 5, 7, 9, 14, 21, 28, 35, 45, 55, 70, and 85 days. At each gas sampling, the culture bottles were tightly sealed with a butyl rubber stopper and cap, and 50 ml of gas samples were taken with a syringe during 0 and 120 min, respectively, and analyzed using a gas chromatograph (Agilent Technologies, 7890B). N_2O fluxes and cumulative N_2O emissions were calculated by the following equations, respectively:

$$F = \frac{V}{m} \times \frac{M}{22.4} \times \frac{?c}{?t} \times \frac{273}{273 + T} \tag{1}$$

$$W = \sum {}^{n}_{i=1} F_i \times t \tag{2}$$

In which, F is the N_2O flux (mg $kg^{-1}day^{-1}$), V is the gas volume in the culture bottle (L), m is the weight of dry soil (g), M/22.4 is the mass density of standard gas (g L^{-1}), $\Delta c/\Delta t$ is the change in concentration per unit time (mg $kg^{-1}day^{-1}$), and T is the incubation temperature (°C). W is the cumulative N_2O (mg kg^{-1}), n is the nth measurement, and t is the interval between two samples (day).

Soil samples were destructively collected on days 0, 1, 45, and 85, with three replicates per time point. Day 0 samples were used to determine initial soil conditions to ensure equal nitrogen input across treatments but were not included in further analyses. Soil physicochemical properties were measured on days 1, 45, and 85, while the determination of functional genes at day 85 linked nitrification and denitrification to cumulative N_2O emissions, minimizing short-term disturbances. Soil pH was measured by the glass electrode method in deionized water at a soil-to-water ratio of 1:2.5. Microbial biomass nitrogen (MBN) was extracted by fumigation with chloroform ($CHCl_3$) and potassium sulfate (K_2SO_4). Soil NO_3^-–N and NH_4^+–N were extracted with 1 mol L^{-1} KCl solution (soil-water ratio 1:10, shaking at 25 °C for 1 h), filtered, and then measured by continuous flow analyzer (AA3). The total phosphorus (TP) and total potassium (TK) were determined by molybdenum-antimony colorimetric method and flame photometer, respectively. Soil moisture content was determined by the ring knife method, and the elemental compositions of soil and organic fertilizer, such as C and N were determined by the Vario Macro CHNS elemental analyzer.

DNA extraction and quantification of functional genes abundance

DNA was extracted from 0.5 g of incubated soil using the FastDNA® Spin Kit (MP Biochemicals, Santa Ana, CA, USA). DNA concentration and mass picking were assayed with a Nanodrop 2000 (ThermoSecientific, Wilmington, DE, USA). DNA samples were diluted to 10 ng DNA μL^{-1} with sterile water and used for quantitative PCR (qPCR) determination. The abundance of key nitrogen cycle microbial functional marker genes (AOA *amoA*, AOB *amoA*, *nirK*, *nirS*, *nosZ*) was quantified using real-time fluorescent qPCR on the Step One Plus Real-Time PCR System (ABI, USA), and all the primers and sequences used for qPCR experiments are shown in Table S1.

Functional gene sequencing

Diversity and community structure containing functional genes for denitrification (*nirK*, *nirS*, and *nosZ* genes) were analyzed by high-throughput sequencing. The library construction process included DNA fragmentation (enzymatic shearing), end repair, adapter ligation, purification and PCR amplification. The primers used for amplification were the same as those listed in Table S1. Thermocycling conditions consisted of 3 min at 95 °C, PCR amplification was performed under the following thermocycling conditions: an initial denaturation at 95 °C for 3 min, followed by 9 cycles (20 s at 98 °C, 15 s at 60 °C and 30 s at 72 °C), and a final extension of 5 min at 72 °C until halted by user. PCR products were purified with Vazyme DNA Clean magnetic beads and quantified using Qubit 3.0 (Thermo Fisher Scientific, Waltham, MA, USA). Mixtures of amplification products were sequenced using the Illumina NovaSeq 6000 sequencing platform (Illumina Inc., San Diego, CA, USA).

Statistical analyses

Prior to conducting the One-way ANOVA, data normality was assessed using the Shapiro–Wilk test. One-way ANOVA and Tukey's HSD test were then performed using IBM SPSS 26.0 statistical package to address the effect of different fertilization treatments on soil properties, abundance of nitrification, and denitrification functional genes. Alpha diversity and Beta diversity analyses of AOA *amoA*, AOB *amoA*, *nirK*, *nirS*, and *nosZ* genes were performed using QIIME2 2020.6 software. Beta diversity of functional genes in different fertilization treatments was assessed by calculating weighted UniFrac distance matrix and Non MetricMulti Dimensional Scaling (NMDS). Line graphs, bar graphs, and box plots were plotted using Origin (2024b) software. Redundancy analysis (RDA) was performed using CANOCO 5.0 to determine the relationship between soil properties, abundance of microbial functional genes and cumulative N_2O emissions. We used the "rfPermute" package in R (version 4.0.3) for Random Forest (RF) analysis to determine the main predictors of soil and microbial properties on cumulative N_2O emissions. The "plspm" package was used to construct partial least squares structural equation modeling (PLS-SEM) to evaluate nitrogen mineralization processes and direct and indirect pathways affecting N_2O emissions.

Results

N_2O emissions in the process of incubation

N_2O emissions showed similar variations among treatments, increasing rapidly within 10 days, then decreasing sharply and remaining stable after 40 days of incubation (Fig. 1a). CP had the lowest emissions, representing by both at peak and cumulative values, whereas YD was the highest, displaying 347.95% and 289.20% higher than CK and N treatment, respectively. As for the cumulative N_2O emissions during incubation, compared to N treatment, YD significantly increased by 1.15-fold, while CP, QS, and SD reduced by 53.43%, 25.96% and 16.64%, respectively (Fig. 1b).

Changes in soil properties during incubation

Compared with CK, OFs and N significantly increased the levels of NO_3^-–N and NH_4^+–N ($p < 0.05$). The NO_3^-–N levels tended to increase substantially, while NH_4^+–N dropped significantly throughout the incubation (Fig. 2a,b), N treatment released NH_4^+ immediately, reaching the highest NH_4^+–N content in the first day. In addition, the partial organic substitution treatments significantly increased soil pH during the early incubation. Among them, N treatment had the highest pH content due to hydrolysis of urea. As incubation progress, soil pH gradually decreased and reached a relatively constant level in all OF substitution treatments. Among them, soil pH in the N treatment was the lowest (Fig. 2c). In the initial incubation, MBN and SOC were significantly higher in fertilized treatments than in CK, but had no difference between N and OFs treatments (Fig. 2d,e). At the end of incubation, the MBN contents were the highest in YD and N treatment (Fig. 2d).

The abundance of functional nitrification and denitrification genes

Compared to CK, OFs and N treatment had lower AOA *amoA*, but higher AOB *amoA* and denitrification genes (*nirK*, *nirS* and *nosZ*). In contrast to N treatment, the nitrification of OFs could be dominated by AOA *amoA* (Fig. 3). The copy numbers of *nirK* and *nirS* were higher in N treatment than in OFs except YD, especially, CP treatment had the lowest gene copy number (Fig. 3c,d). Conversely, *nosZ* gene abundance showed higher performance in OFs than in N treatment except for YD, among them the CP had the highest (Fig. 3e).

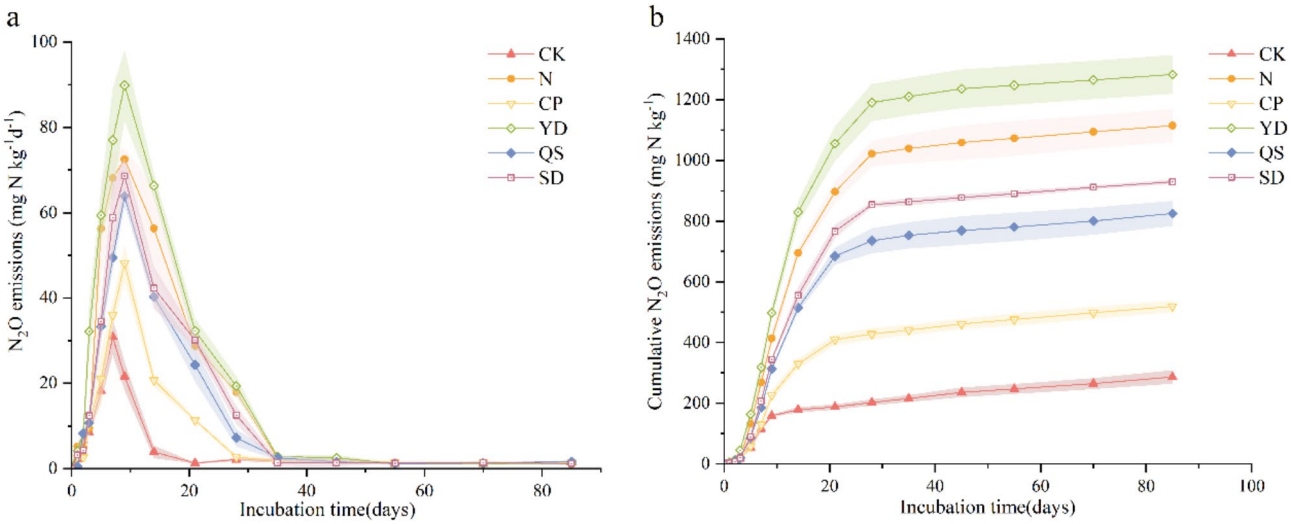

Fig. 1. Changes in N_2O emissions (**a**) and cumulative N_2O emissions (**b**) during incubation as affected by different fertilization treatments. CK (unfertilized control), N (chemical fertilizers alone), CP (compost and chemical fertilizers), YD (Yak dung and chemical fertilizers), QS (*Qingke* straw and chemical fertilizers), and SD (Tibetan sheep dung and chemical fertilizers). The shaded area represents the standard error of the mean.

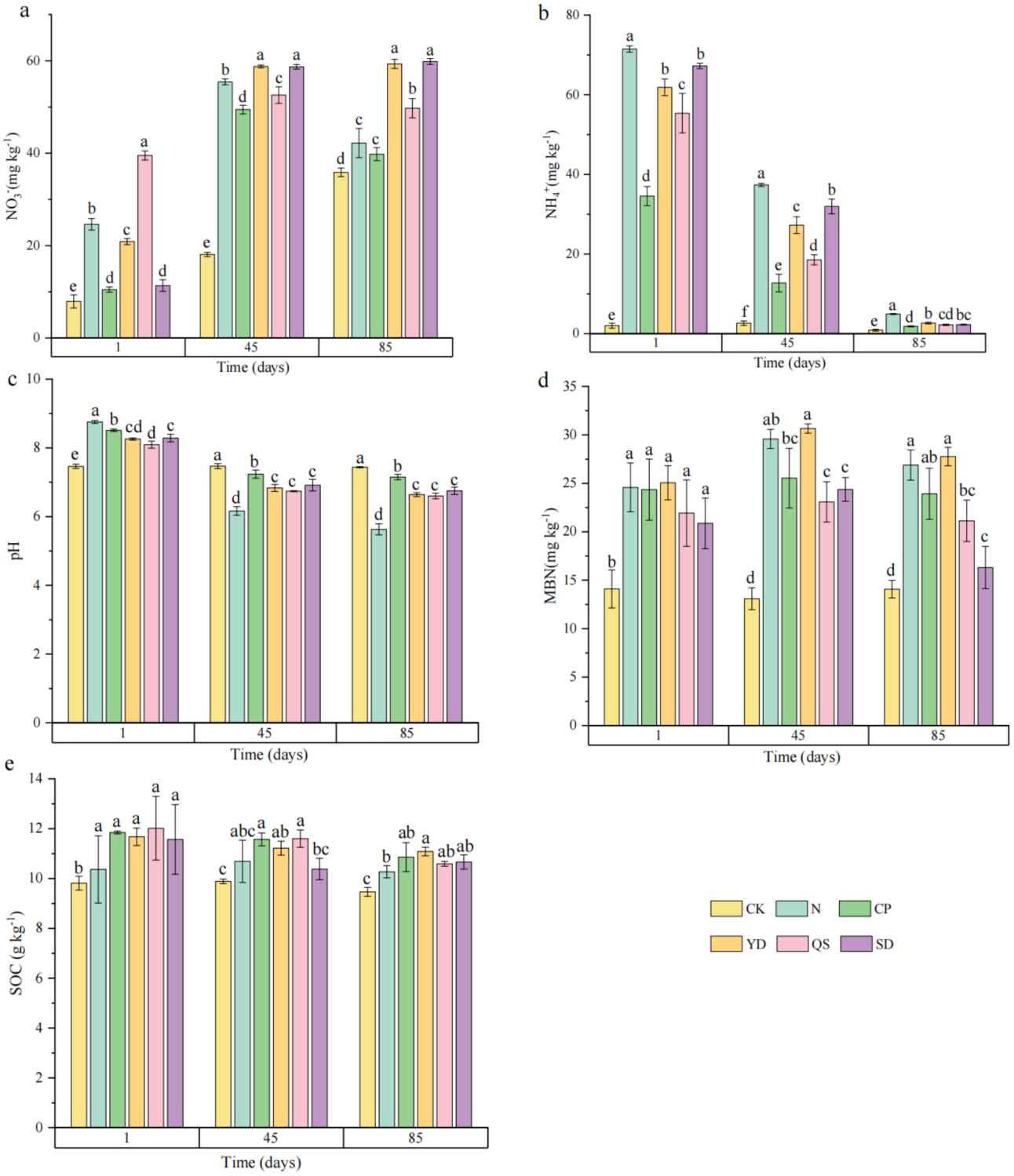

Fig. 2. Changes in soil properties over time under fertilizer treatments. CK (unfertilized control), N (chemical fertilizers alone), CP (compost and chemical fertilizers), YD (Yak dung and chemical fertilizers), QS (*Qingke* straw and chemical fertilizers), and SD (Tibetan sheep dung and chemical fertilizers).

Denitrification gene diversity

Fertilization increased the Chao1 index of denitrification genes (Table 2). Specifically, fertilization treatments increased *nirK*, *nirS*, and *nosZ* genes by 4.26–57.90%, 25.26–77.37%, and 6.02–82.23% compared with CK (Table 2). The denitrification richness (Chao1) was higher in YD than in the other fertilization treatments. However, there was no significant difference in *nirK* abundance between SD and CK treatments. The OFs and CF increased the enrichment of *nirK* by 1.01–18.18% compared to the CK treatment (Shannon). The *nirS* and *nosZ*

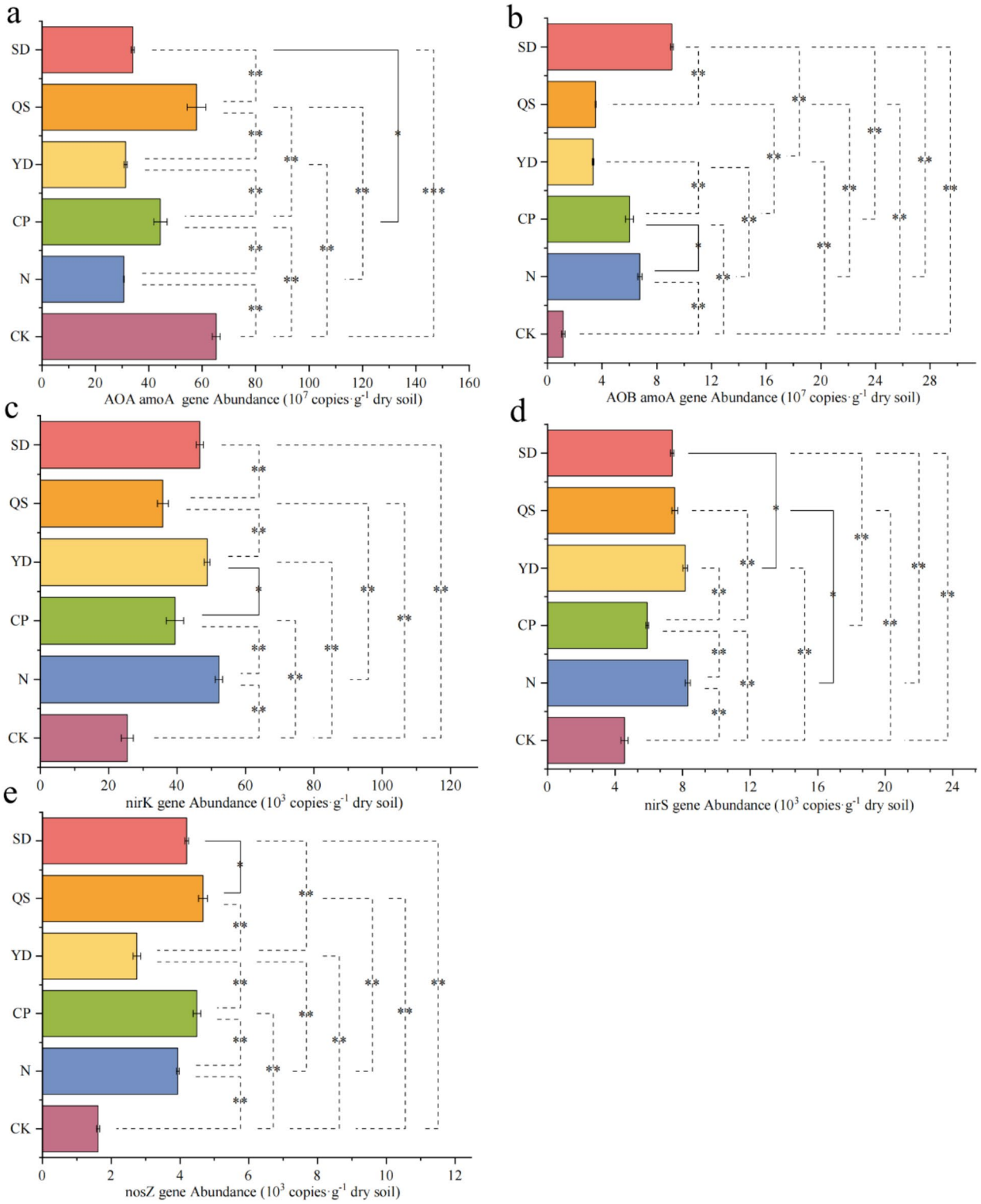

Fig. 3. Changes in nitrification and denitrification functional genes copy numbers under fertilization treatments. $^*p \le 0.05$, $^{**}p \le 0.01$. CK (unfertilized control), N (chemical fertilizers alone), CP (compost and chemical fertilizers), YD (Yak dung and chemical fertilizers), QS (*Qingke* straw and chemical fertilizers), and SD (Tibetan sheep dung and chemical fertilizers).

diversity index of CP and QS treatments were significantly higher than that of the single N treatment ($P < 0.05$). NMDS analysis revealed divergent patterns of denitrification genes in different fertilization treatments, except the close proximity clustering of *nirS* in CP, QS, and SD (Fig. S1). Overall, the combination of OFs and CF significantly increased the diversity of *nirS*.

	Chao1			Shannon		
	nirK	nirS	nosZ	nirK	nirS	nosZ
CK	680.62 ± 16.94e	470.68 ± 9.01e	847.09 ± 41.27d	5.94 ± 0.05e	5.87 ± 0.03d	5.46 ± 0.14e
N	748.66 ± 20.78d	589.58 ± 8.42de	898.11 ± 10.25d	6.37 ± 0.09b	6.14 ± 0.03b	6.85 ± 0.02d
CP	921.55 ± 23.52b	771.89 ± 21.75b	1543.65 ± 33.51a	6.13 ± 0.03cd	6.54 ± 0.04a	8.04 ± 0.07a
YD	1074.72 ± 27.60a	834.86 ± 18.72a	967.06 ± 35.62c	7.02 ± 0.13a	6.02 ± 0.06c	7.36 ± 0.01c
QS	783.25 ± 11.66c	626.16 ± 54.30cd	1072.28 ± 9.12b	6.27 ± 0.09bc	6.49 ± 0.05a	7.79 ± 0.02b
SD	709.59 ± 7.15e	658.18 ± 13.61c	985.00 ± 26.94c	6.00 ± 0.05de	6.15 ± 0.04b	6.78 ± 0.08d

Table 2. The alpha diversity index of *nirK*, *nirS*, and *NosZ* genes under different fertilization treatments. Values are means ± standard error ($n = 3$). Different letters indicate significant difference in a column ($P < 0.05$).

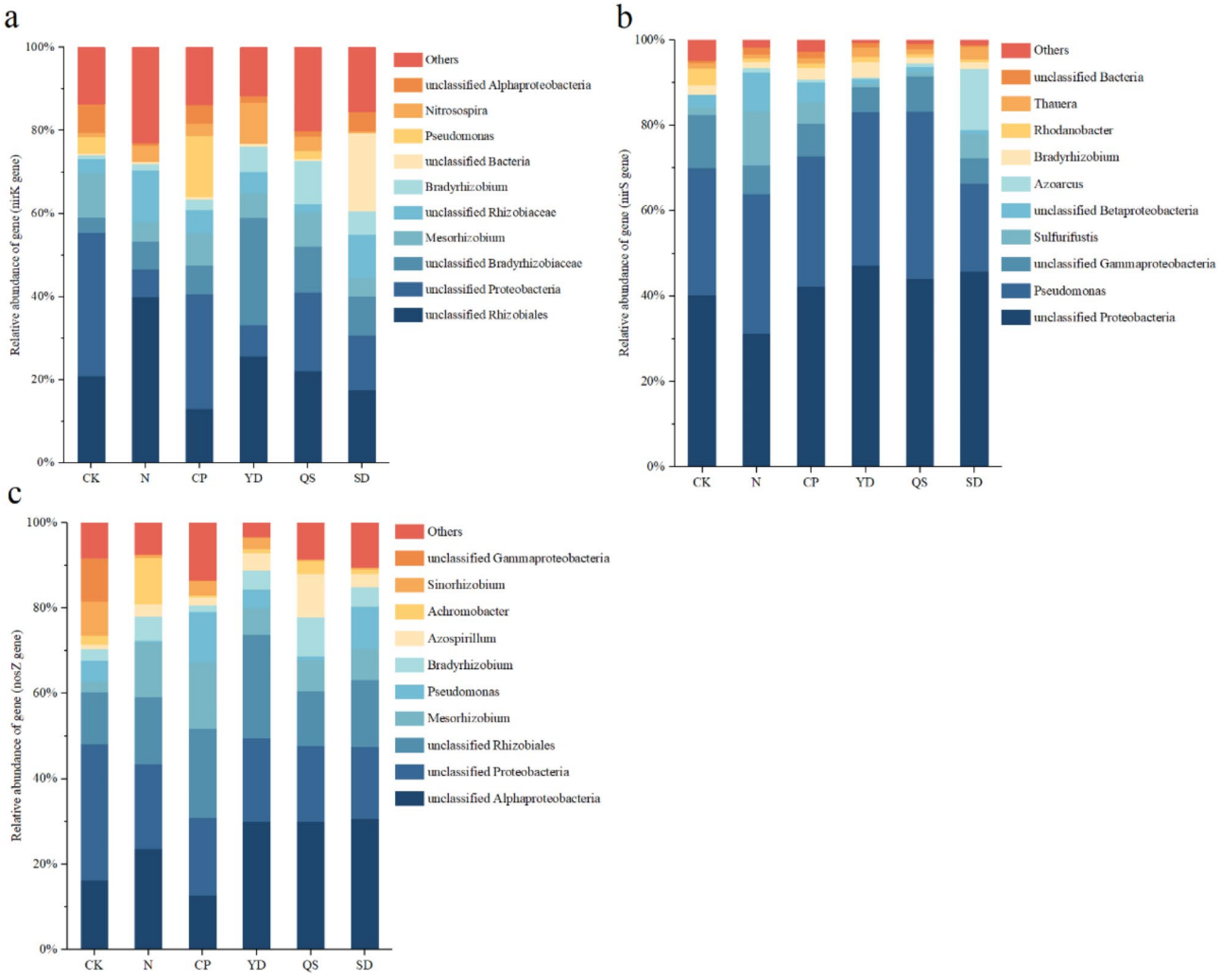

Fig. 4. Community composition of denitrification functional genes at the genus level under fertilization treatment on day 85. CK (unfertilized control), N (chemical fertilizers alone), CP (compost and chemical fertilizers), YD (Yak dung and chemical fertilizers), QS (*Qingke* straw and chemical fertilizers), and SD (Tibetan sheep dung and chemical fertilizers).

Denitrification gene community structure and composition

A total of 40,046–76,700 high-quality *nirK*, *nirS*, and *nosZ* sequences were obtained from all treatments, and clustering of Reads at 97.0% similarity level yielded 2901, 2037, and 3808 OTUs (Fig. S2). The Venn diagram showed that 178, 197, and 225 OTUs in *nirK*, *nirS* and *nosZ*, respectively, were common across treatments (Fig. S2). *Proteobacteria* were the dominant phylum of denitrifier genes (Fig. 4). At the genus level, *nirK* functional genes were present, with unclassified *Rhizobiales* being the most abundant taxa in the soil, having a relative abundance of 13.56-40.95%; followed by unclassified *Proteobacteria* (6.44–34.56%) (Fig. 4a). The relative

abundance of unclassified *Proteobacteria* in *nirS* and *nosZ* genes accounted for 30.26–46.75% and 16.66–33.68%, respectively (Fig. 4b,c). Specifically, partial organic substitution treatments in *nosZ* genes significantly reduced the abundance of unclassified *Proteobacteria* by 0.33–12.85% compared to the N treatment. In addition, the genera *Pseudomonas* and *Bradyrhizobium* coexisted in the *nirK*, *nirS* and *nosZ* genes. Among them, the *Pseudomonas* genus of *nirK* and *nosZ* genes had the highest proportion in CP treatment, accounting for 15.34% and 12.54%, respectively. It is noteworthy that *Mesorhizobium* genus in CP treatment was significantly higher in *nirK* (8.13%) and *nosZ* (16.45%) compared to other treatments. However, *Azospirillum* genus (2.03–9.59%) was found to be widely present in *nosZ* during fertilization treatment.

The relationship between functional gene abundance and soil properties

The result of RDA analysis showed that the explanations of axis 1 and axis 2 contributed 60.01% and 12.82%, respectively to the variation of nitrification and denitrification genes (Fig. 5a). Among them, cumulative N_2O emission was the most influential indicator, followed by pH and NO_3^-–N, accounting for 49.01%, 9.30% and 8.90% of variations, respectively. AOB *amoA* and denitrification genes (*nirS* and *nirK*) were positively correlated with the MBN, SOC, NO_3^-–N, NH_4^+–N, and cumulative N_2O emissions, while negatively associated with soil pH. CP, YD, QS, and SD treatments were largely separated from CK and N treatments along the RDA1 direction (Fig. 5a). RF model showed that *nirS* abundance, NH_4^+–N, and soil pH were the main drivers explaining the cumulative N_2O emissions (Fig. 5b). It is noteworthy that fertilizer treatments had significant positive effects on NO_3^-–N (Fig. 6a). Furthermore, fertilization treatments affected soil pH and NO_3^-–N directly or indirectly through NH_4^+–N, thereby having positive and negative regulatory effects on soil nitrification and denitrification, respectively, and consequently affecting cumulative N_2O emissions (Fig. 6a). In particular, denitrification functional genes, fertilization treatment and NH_4^+–N had the highest total effect on N_2O cumulative emissions, with 0.89, 0.63 and 0.45, respectively (Fig. 6b).

Discussion

This short-term microcosm incubation revealed overall decline in soil N_2O emissions after partial organic substitution for chemical fertilizer, which supported our hypothesis. Compared to CF, partial organic substitution reduced 16.6–53.4% emissions except yak substitution increased 1.15 fold. However, magnifying NO_3^-–N production during the process of incubation implies great risk of N loss. *nir*S, and NH_4^+–N were among the most key factors to regulate N_2O emissions. Thus, based on this study, partial organic substitution is likely to mitigate N_2O emissions but increase the loss of nitrate.

Partial organic substitution affected N_2O emissions and soil properties

The N and OFs treatments increased the cumulative N_2O emissions during incubation compared to the CK treatment. This may be due to the fact that OFs provided more substrate for nitrification and denitrification through mineralization (Fig. 2). However, partial organic substitution treatments except YD reduced soil N_2O

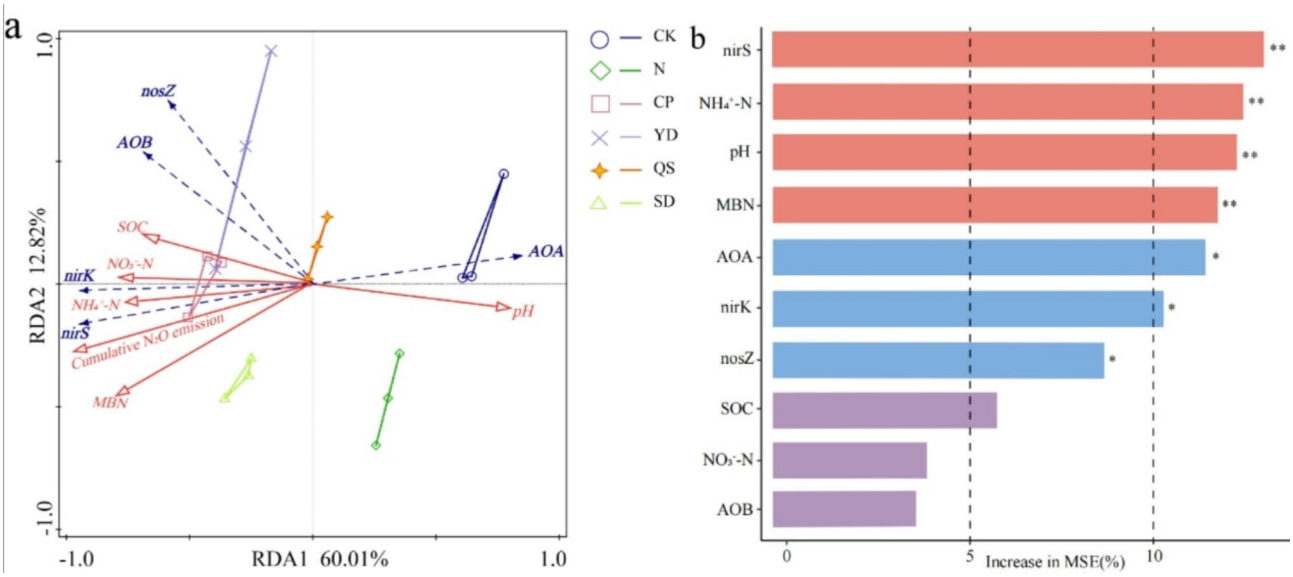

Fig. 5. The relationship between cumulative N_2O emissions, soil properties, and abundance of functional genes. (**a**) Principal component analysis of soil properties, cumulative N_2O emissions, and abundance of nitrification and denitrification genes; (**b**) RF analysis to determine the main explanatory variables for ranking cumulative N_2O emissions. MSE is the mean square error of the random forest model. The larger the MSE value, the greater the importance of the variable. CK (unfertilized control), N (chemical fertilizers alone), CP (compost and chemical fertilizers), YD (Yak dung and chemical fertilizers), QS (*Qingke* straw and chemical fertilizers), and SD (Tibetan sheep dung and chemical fertilizers).

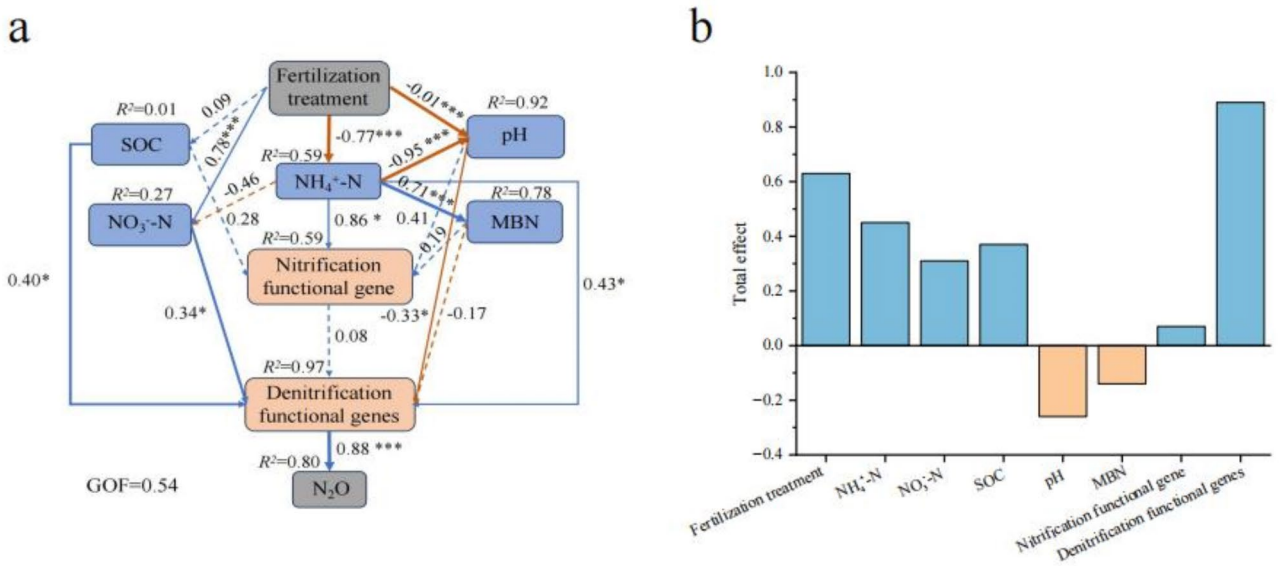

Fig. 6. PLS-SEM was used to show how fertilizer application affects N_2O accumulation through key soil properties, nitrification and denitrification functional gene abundance in the main pathway (**a**) and total effect (**b**). The red and blue lines represent positive and negative effects, respectively, and the dashed lines represent non-significant effects. R^2 values associated with response variables indicate the proportion of variance explained by the relationship with other variables. Significance: *Denotes $p < 0.05$, **denotes $p < 0.01$, ***denotes $p < 0.001$. CK (unfertilized control), N (chemical fertilizers alone), CP (compost and chemical fertilizers), YD (Yak dung and chemical fertilizers), QS (*Qingke* straw and chemical fertilizers), and SD (Tibetan sheep dung and chemical fertilizers).

cumulative emissions compared with N treatment. The cumulative N_2O emissions in CP treatment were the lowest, which reduced by 58.00–147.17% compared to N, YD, QS and SD. The reason for this difference may be because minerlizable N containing substances had already been mineralized and the contents of available nitrogen were lower in CP compared other fertilization treatments during the composting process (Fig. 2a,b) and the compost components are more stable in structure and functionality. This would increase soil nitrogen immobilization and reduce N_2O production by decreasing the substrate for heterotrophic denitrification or ammonia oxidation[15,50]. As a result, available N release slower from composts and caused lower N_2O emissions than other OFs (Fig. 2a,b). This phenomenon is in agreement with the observation of Shah et al., (2016).

The NH_4^+-N content, in consistent with N_2O cumulative emissions[52], was higher in the N treatment than in CP, QS, and SD during the whole incubation period. The possible reason could be that urea produced more NH_4^+–N in the soil by promoting the mineralization of organic N. NH_4^+ stimulated the activities of nitrifiers and providing favorable conditions for nitrification in soils[53–55]. Alternatively, the mineralization of organic nitrogen is greater than the immobilization of mineral nitrogen in the N treatment[56,57]. In other words, urea hydrolysis to NH_4^+–N and nitrified NO_3^-–N in the N treatment[58–60], providing abundant nitrogen substrates for denitrification, which may contribute to N_2O production (Fig. S3). Notably, NO_3^-–N accumulated during the incubation process, with relatively high and stable levels in the later stages of incubation, especially after days 40 when N_2O emissions kept very low (Fig. 2a). This may be attributed to the characteristics of this studied sandy loam soils in favor of nitrate accumulation[61,62]. Also, NH_4^+–N released from the decomposition of organic fertilizers can be converted to NO_3^-–N (Fig. 6a). In addition, although partial organic substitution treatments reduced pH compared to CK, they increased pH compared to N treatment (Fig. 2c). These pH variations may result from the inherent properties of organic fertilizers, urea hydrolysis, and nitrogen mineralization processes that influence NH_4^+–N concentrations. Notably, pH changes can regulate denitrification efficiency, thereby affecting N_2O emissions[56,63–65]. Under favorable conditions, these processes may enhance microbial reduction of N_2O to N_2, contributing to the observed changes in N_2O emissions[15,66]. Therefore, N and OF treatments potentially influence N_2O emissions by altering soil properties and the interaction between nitrification and denitrification. Partial organic substitution, particularly the CP treatment, significantly reduced cumulative N_2O emissions by enhancing nitrogen fixation and stabilizing compost composition.

Partial organic substitution regulated the nitrification and denitrification functional genes

Fertilizer application can alter the potential for soil N_2O emission by affecting the community structure and composition of nitrification and denitrification functional genes[34,67]. AOA *amoA* and AOB *amoA* can convert NH_4^+–N to NH_2OH and are key functional genes in nitrification (Fig. S3). However, these two groups of ammonia-oxidizing bacteria responded differently to the application of OFs. In this study, compared to N treatment, partial organic substitution treatments generally increased the copy number of AOA *amoA* while decreased the copy number of AOB *amoA* (Fig. 3a,b). This may be attributed to the presence of different ammonia oxidizing

bacteria host strains, with AOA *amoA* typically dominating in environment with low ammonium availability or high organic matter content, while AOB *amoA* thriving in conditions with higher ammonium concentrations and greater oxygen availability, reflecting their distinct ecological niches[68–70]. In addition, the copy number of AOA *amoA* was greater than that of AOB *amoA*, consistent with the copy numbers reported by other studies in most soils[18,71–73]. This suggested that AOA *amoA* could be the main driver of nitrification in the alluvial soil with sandy loam texture in this study.

Denitrifying bacteria (*nirK*, *nirS*, and *nosZ*) communities changed significantly under different fertilizer treatments[74,75]. Specifically, both *nirK* and *nirS* genes were significantly lower in the partial organic substitution treatments than in N treatment (Fig. 3c, d), and the *nirS* copy number was more strongly correlated with N_2O emissions than *nirK* (Fig. 5a,b). This may indicate that *nirS* functional genes are more sensitive to N_2O emissions than *nirK*. Similarly, compared with *nirK*, *nirS* functional genes were clearly clustered in OFs (Fig. S1), indicating that *nirS* were more sensitive to fertilizer treatment response than *nirK*. More notably, partial organic substitution treatments except YD effectively increased the *nosZ* copy number, especially in CP treatment (Fig. 3e), reduced N_2O cumulative emissions (Fig. 1b). A possible explanation is that more N_2O was reduced to N_2 during denitrification in CP treatment[76,77]. The alpha diversity indices (Chao1 and Shannon) of nosZ in CP treatment were significantly higher than those in other treatments, supporting this point (Table 2). By contrast, significantly lower *nosZ* in YD may explain its relative higher N_2O emissions. Similarly, species richness (Chao1 index) of *nirK* and *nirS* was higher in YD treatment than other treatments, while *nosZ* was significantly lower (Table 2). It suggests that the Yak dung may have increased nitrite reduction and decreased N_2O reduction capacity. This further explains the high cumulative N_2O emissions from the YD treatment. In addition, previous studies have shown that genera of *Pseudomonas* and *Bradyrhizobium* influence denitrification processes[78,79]. In this study, the higher presence of *Pseudomonas* and *Bradyrhizobium* in the *nirK*, *nirS*, and *nosZ* genes, and significantly higher of *Pseudomonas* in *nirK* and *nosZ* in CP treatment than in other organic substitution treatments, indicated that these genera may be the key bacteria to inhibit large amounts of N_2O emissions from soils.

Linkage among soil properties, functional genes and N_2O emissions

Nitrification and denitrification, as biological processes, are influenced by both soil physicochemical and biological factors[7,67,80]. In this study, the difference in nitrogen mineralization among partial organic substitution treatments with same nitrogen application rate was attributed to chemical composition (Table 1). Addition of OFs to soil causes changes in soil properties, profoundly affecting soil nitrification, denitrification, and N_2O emissions[35,81,82]. Our results also verified that partial organic substitution has differentiated impacts on soil properties and functional gene abundance (Figs. 3 and 4).

The *nirS* and *nirK* denitrification functional genes can reduce NO_2^- to N_2O, while the *nosZ* type denitrifiers mediate the reduction of N_2O to N_2 [7,35,83]. Although the *nirK* gene copy number was greater than *nirS* (Fig. 3), *nirS* was the most important factor to explain accumulative N_2O emissions (Fig. 5b), consistent with previously reported results[15,33,84]. However, there are some studies showing that the increase in *nirK* gene abundance is greater than *nirS* with organic fertilizer application[85]. The reason for the difference between the two results may be due to the difference in the concentration of available substrates in OFs-amended soils[35,86].The contribution of *nirK* to nitrite reduction and *nosZ*-mediated reduction of N_2O to N_2 cannot be ignored. In this study, *Azospirillum*, involved in assimilation and non-differentiation of NO_3^- reduction, was found in *nosZ*[87], while *Mesohizobium* is widely present in *nirK* and *nosZ*, especially in CP treatment (Fig. 4). The nitrogen generated by partial organic substitution can be immobilized by *Mesorhizobium* and *Azospirillum*[10,33,86], thereby reducing N_2O emissions. This is supported by the fact in this study that the CP treatment had the lowest cumulative N_2O missions (Fig. 1b).

Soil NH_4^+–N and NO_2^-–N concentrations are key substrates for nitrification and denitrification[7,10]. Fertilization reduced NH_4^+–N and increased NO_3^-–N contents (Fig. 2a,b), with NH_4^+–N exerting a stronger influence on cumulative N_2O emissions than NO_3^-–N (Fig. 5), consistent with Huang et al. (2020). This may be due to the dual role of NH_4^+–N in N_2O production through nitrification (conversion of NH_4^+ to NO_2^-) and denitrification (conversion of NO_2^- to N_2O). While NO_3^-–N acts only through denitrification[55,86]. Furthermore, NH_4^+–N affects soil pH and MBN content, which in turn regulates denitrifier community composition and N_2O emission potential (Fig. 6).

Soil pH has been recognized as a key soil variable mediating N_2O emissions[64,82,88,89]. Our results of this study showed that the CP treatment had a neutral soil pH and low cumulative N_2O emissions (Figs. 1 and 2). which might be attributed to the fact that the *nosZ* gene was more predominant in soil environments with a neutral pH, facilitating N_2O conversion to N_2[65]. SOC and MBN was also important factors affecting N_2O in our study (Figs. 5 and 6). This could potentially be attributed to the fact that they can impact N_2O emissions via multiple pathways[90,91], including serving as a nitrogen substrate affecting nitrification and denitrification processes[92,93], as well as influencing microbial metabolic activities and community structure associated with N_2O production[5,94].

In our study, denitrification appears to be the primary pathway for N_2O emission. This can be attributed to two aspects. First, nitrification-related functional genes contributed less to N_2O emission (Figs. 5b and 6b). Although AOA *amoA* is likely to be the main driver of nitrification, previous studies have shown that the nitrification-denitrification process is mainly mediated by AOB *amoA*[95,96], which implies that AOA *amoA* may not contribute significantly to N_2O production through nitrification. Instead, in this study, denitrification-related functional genes played a key role in regulating cumulative N_2O emissions (Fig. 6b), especially *nirS*, which was identified as the most important predictor (Fig. 5b). Second, sufficient organic carbon can stimulate denitrification by providing electron donors for NO_3^- reduction, thereby promoting N_2O emission, especially under oxygen-limited conditions[97,98]. In addition, other pathways may contribute to N_2O production. For example, abiotic decomposition of nitrite may also contribute[99]. Therefore, there are many uncertainties in exploring the relationship between N_2O emissions, soil properties, and nitrification-denitrification genes, especially under

different fertilization treatments. Future studies are needed to quantitatively assess the contribution of different pathways to potential N_2O emissions.

Implications for organic fertilizer application

This study is of great significance for understanding and management of N_2O emissions from different organic fertilizer substitutions for chemical fertilizers in agricultural systems, especially in the alluvial soils in the Lhasa River valleys. We found that partial organic substitution for chemical fertilizer, especially the compost substitution had impressive potential to mitigate N_2O emissions. In contrast, direct yak manure substitution would cause increase in N_2O emissions. This result implies that proper composting of organic fertilizers, rather than direct application of untreated yak dung, may be a more environmentally friendly pathway for green agriculture. Partial organic substitution, especially after compost, enables N_2O emission reduction while soil fertility maintenance. This study may corroborate the rationale of traditional manure use habit of Tibetan people, collecting yak dung for domestic fuel and mostly use sheep manure for fertilizer in croplands. In addition, we found that NO_3^-–N accumulated in this incubation experiment even when N_2O emissions were very low, suggesting partial organic substitution also has risk of leading to nitrate leaching, nutrient loss and waterbody contamination in the Lhasa River Valleys. We recommend co-composting animal manure, crop residue for partial organic substitution of chemical fertilizer and using nitrification inhibitors to achieve the goals of both mitigating N_2O emissions and maintaining soil fertility.

Given that denitrification plays a crucial role in determining whether accumulated nitrate is converted to N_2O or further reduced to N_2, our study focused on denitrification functional genes (*nirK*, *nirS*, *nosZ*). However, future studies should include AOA *amoA* and AOB *amoA* communities for a comprehensive understanding of the interactions between nitrification, denitrification, and N_2O emissions under different organic replacement strategies. Moreover, freeze-thaw effects are recognized as major contributors to N_2O emissions in high-altitude ecosystems due to their potential to physically disrupt soil microbial communities and alter denitrification processes[100–103]. Although no significant freeze-thaw events occurred in this farmland study area, it is undeniable that such processes can influence nitrogen cycling in high-elevation ecosystems. Therefore, future research should integrate field experiments to explore additional environmental factors, such as freeze-thaw effects and nitrate leaching, to comprehensively evaluate their impact on nitrogen cycling and greenhouse gas emissions in ecosystems.

Conclusions

This short-term microcosm incubation provided the evidence that application of organic and chemical fertilizer blends generally reduced N_2O fluxes under equal nitrogen addition compared to chemical fertilizer alone. The relatively higher N_2O emissions from yak dung substitution can be evidently reduced through co-composting animal manure and crop residue. Fertilization treatments may modulate potential N_2O emissions primarily by influencing denitrification processes. Denitrification functional genes, especially the *nirS* abundance, NH_4^+–N content and pH were among the predominant factors to determining cumulative N_2O emissions. However, NO_3^-–N was prone to accumulation when applying partial organic substitution, which could render increasing risk of nitrogen leaching and nutrient loss in the alluvial soils. Taken together, soil N_2O emission reduction is favored by applying partial organic fertilizer substitution especially after through co-composting.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request. The raw high-throughput sequencing data have been deposited in the National Center for Biotechnology Information (NCBI) under accession number PRJNA1242584.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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