Lower soil nitrogen-oxide emissions associated with enhanced denitrification under replacing mineral fertilizer with manure in orchard soils

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Replacing mineral fertilizer with manure suppressed nitrogen-oxide emissions by regulating their production pathway in orchards soil

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ABSTRACT

Emerging evidence suggests that replacing mineral fertilizers with manure was an effective way to suppress soil nitrogen-oxide (N-oxide) emissions. However, the mitigation potential and its microbial driving mechanism of replacing mineral fertilizers with manure on soil N-oxide emissions in orchards soil remains unclear. In the study, annual in-situ observation of N-oxide emissions was conducted respectively in deciduous orchards (pear) and evergreen orchards (citrus) under three treatments: no fertilization (CK), compound fertilizer (CF), and manure plus compound fertilizer (COF). The results showed that although the COF treatment increased the peak fluxes of N_2O, it reduced the cumulative emissions of N_2O and NO by an average of 20% and 17%, respectively, when compared to the CF treatment. Partial replacement of mineral fertilizers with manure enhanced the contribution of AOA to nitrification and reduced the contribution of AOB, resulting in a decrease in the production of N_2O from nitrification. Isotope analysis further suggested that the primary pathway for N_2O emissions in two orchards' soil is bacterial denitrification and nitrifier denitrification (bD/nD), while the COF treatment reducing the ratio of denitrification products. Additionally, the dual isotope mixing model results indicated that partially replacing mineral fertilizers with manure facilitates soil denitrification, resulting in the additional conversion of N_2O into N_2 and, consequently, less N_2O and NO accumulation. The average cumulative N-oxide emissions in pear orchard were 67% higher than in citrus. The difference in soil physicochemical properties and growth habits in pear and citrus orchards may be the key regulators for the observed difference in N-oxide emissions. Taken together, while partial replacement of mineral fertilizers with manure could have mitigation potential, it should be prioritized as an important measure for emission reduction in orchards to achieve the transition towards climate-smart agriculture.

Keywords: Climate change; Greenhouse gas; Soil nitrogen cycle; Mitigation option; Climate-smart agriculture

1 Introduction

As two trace gases of major public concern, nitrous oxide (N_2O) and nitric oxide (NO) directly or indirectly involved in global warming and play a disruptive impact on human and ecosystem health (Ravishankara et al., 2009; Stocker et al., 2013). We are on course to miss the 2 °C target as the acceleration of global N_2O emissions has tracked the path of the worst-possible Intergovernmental Panel on Climate Change (IPCC) scenario (RCP 8.5) since 2010 (Davidson and Kanter, 2014; Tian et al., 2020). On the other hand, NO is involved in the formation of tropospheric ozone and acid rain, both of which have adverse effects on ecosystem functioning (Li et al., 2022). There is growing evidence that biogenic production in cultivated soils is a significant source for N_2O and NO due to large amount of nitrogen (N) fertilizer inputs providing substrates for soil nitrification and denitrification (Davidson, 2009). Statistically, N_2O and NO emissions from N fertilizer utilized in agriculture accounted for approximately 52% (7.3 Tg N yr^{-1}) and 10% (3.7 Tg N yr^{-1}) of total anthropogenic emissions, respectively (Davidson, 2009; Tian et al., 2020). Therefore, it is urgent to take effective measures to mitigate nitrogen oxide (N-oxide) emissions from cultivated soils.

Orchard soil is a hotspot of N-oxide emissions in agricultural sector. Orchards are mainly planted in well-drained neutral, slightly acidic or alkaline soils, with a wide variety and distribution. From 2000 to 2019, the global area of orchards increased by 22.0% (FAOSTAT, 2021). As the third largest agricultural plantation industry, the area of Chinese orchards increased from 5.18 to 12.66 Mha over the last 30 years (China Statistical YearBook, 2019; FAO, 2020). Current average level of N inputs in Chinese orchards is 550 kg N $ha^{-1}yr^{-1}$, which far exceeds the global average annual N fertilizer application rate of 303 kg N $ha^{-1}yr^{-1}$(Xu et al., 2022; Zhang et al., 2013). Furthermore, the amount of N input in orchards accounts for 16.24% of the total input in China (China Statistical Yearbook, 2020). High N input and rapidly expanding the orchard area may lead to potentially obvious N_2O and NO emissions. Previous studies have shown that the direct N_2O emission from Chinese orchards was estimated to be 32–49 Gg N yr^{-1} in the 2000s, accounting for about 14% of the total upland emissions (Xu et al., 2022). Given such significant and unneglectable emissions, developing effective mitigation options for N_2O and NO emissions from rapidly expanding orchards in China is instant.

Replacing mineral fertilizer with manure is a sustainable measure to improve soil fertility and mitigate environmental degradation in agricultural management. Meanwhile, by optimizing the soil microbial-driven internal cycling of nutrients and changing the abundance of functional genes associated with N-cycling, manure substitution for mineral fertilizer may influence the production and consumption of N_2O and NO (Bi et al., 2023; Hu et al., 2022; Xu et al., 2023). For instance, by changing the quantity and quality of inorganic N substrate, replacing mineral fertilizer with manure obviously decreased N_2O emissions in a citrus orchard (Zhou et al., 2022). In contrast, manure substitution for mineral fertilizer stimulated N_2O emissions in an apple orchard because manure application provided sufficient carbon sources for increasing the activity of microorganisms involved in the N cycle, thereby promoting N_2O emissions (Sompouviset et al., 2023). Although several individual studies have investigated the effect of replacing mineral fertilizer with manure on N_2O emission in orchards, a general conclusion has not been made due to the high variation of manuring effects across different experimental sites (Escanhoela et al., 2019; Liu et al., 2017; Shan and Yan, 2013). Furthermore, no study has examined the response of NO emissions to manure substitution for mineral fertilizer and its microbial driving mechanisms in orchards. To make clear the effect of replacing mineral fertilizer with manure on soil N_2O and NO emissions, we need a thorough understanding of the soil properties and microbial activities underpinning N cycle.

In this study, we conducted respective annual in-situ field experiments from pear and citrus orchards under same fertilizer management. We measured N_2O and NO dynamic fluxes and recorded environmental factors, functional gene abundance related to N cycle and the nitrification and denitrification potential based on isotope analysis. The main aims of this study were to: (i) improve understanding of the effect of replacing mineral fertilizers with manure on N_2O and NO emissions in orchards, (ii) evaluate the influence of key microbial processes on N_2O emissions in orchard soils by using the inhibitor and isotope signature methods and (iii) compare the differences in soil N_2O and NO emissions between deciduous (pear) and evergreen (citrus) orchards. We hypothesized that replacing mineral fertilizer with manure mitigates N_2O and NO production by changing the biogenic pathways.

2 Materials and methods

2.1 Field experiment

The field experiment was conducted from November 2020 to November 2021 in pear (deciduous) and citrus (evergreen) orchards, which were located in the Shanxiangyuan family farm in Jurong City, Jiangsu Province, China (108 m a.s.l., 31°97′N, 119°14′E). The climate is a subtropical monsoon, with a long-term mean annual temperature (MAT) of 16.2 ℃ and precipitation (MAP) of 1192 mm (https://data.cma.cn/en). The soil is classified as a Fluvisols (FAO). The initial properties of orchard soil are shown in Table 1.

Trees from the two orchards were planted in 2015. The planting density of trees was 625 tree ha^{-1}, and the row spacing of trees was 4 m. Each orchard was set up with three treatments: no fertilization (CK), compound fertilizer (N: P_2O_5:K_2O = 15:15:15) (CF), and cow manure plus compound fertilizer (COF, 30% substitution for compound fertilizer). The cow manure had a pH of 7.48, a total N of 2.86%, and a C/N ratio of 13. Each treatment was randomly assigned to a block with an area of 16 m^2 per field plot, with three replicates. Following local management, the fertilizer was applied using a circular furrow located 0.7 m away from the tree roots. The total N input of the orchard was 300 kg N ha^{-1} yr^{-1}. Compound fertilizer was applied as a base fertilizer on December 3, 2020, and two top dressing on March 18 and May 18, 2021, with an application ratio of 7:8:10. Manure was divided into three portions, with two portions were applied as basal fertilizer, while the remaining portion was applied as the first topdressing. Phosphorus and potassium deficiencies in the CK and COF treatments were addressed through the supplementation of calcium superphosphate and potassium sulfate. Additionally, all plots were effectively maintained pest and weed-free following local practices (foliar pesticides and manual weed control).

2.2 Gas sampling and flux measurements

Gas samples were collected weekly using the static chamber method throughout the experiment to determine N_2O and NO fluxes. When N fertilizer was applied, gas samples were taken thrice weekly to capture peak N-oxide emissions. A permanent square triangular PVC base (0.60 m side length and 0.20 m height) was installed in each plot. A triangular sampling chamber measuring 0.40 m in height was placed on the base and inserted into a trough at the top end of

the sampling chamber base. It was covered with an insulating membrane to reduce air temperature variation. Four gas samples (1.5 L) were collected per plot and then sent to the laboratory within 24 hours for concentration analysis. N_2O concentrations were analyzed using a modified gas chromatograph (Agilent 7890A). NO concentrations were analyzed using a Model 42i chemiluminescent NO-NO_2-NO_X analyzer (Thermo Environmental Instruments).

The direct emission factors (EF) of N_2O and NO can be calculated as the difference in their total emissions between fertilized and unfertilized treatments, divided by the amount of N applied. The formula is given below:

$$EF = (E_N - E_0) / N_{-fer} \quad (1)$$

where E_N and E_0 represent the cumulative N_2O or NO emissions (kg N ha^{-1}) from the fertilized and unfertilized treatments, and N_{-fer} is the total amount of N applied (kg N ha^{-1}). Annual cumulative emissions of N_2O and NO were approximated by applying the trapezoidal rule to the time interval between measured emission fluxes.

2.3 Soil sampling and physicochemical analysis

Soil samples (0-20 cm) of each plot were collected every two weeks with stainless steel corers to determine physicochemical properties and quantitative microbial analysis. While collecting gas samples, soil temperature and volumetric moisture were recorded at 0-10 cm depth near the sampling site using a handheld thermometer and an MPM 160 moisture content meter. Soil samples were passed through a 2-mm sieve to eliminate gravel and impurities. Subsequently, the homogenized soil samples were stored at 4 °C and -80 °C, respectively, for further analysis. The soil water content was determined by drying in an oven (105 °C, 24 h). The soil core method is used to measure the bulk density (BD) of the surface soil (5 cm). The water-filled pore space (WFPS) was calculated by dividing the volumetric moisture content by the total porosity of the soil, where the calculation formula for the total porosity of the soil is [1-(BD/2.65)]. Soil pH was analyzed separately using a pH electrode (PHS-3C) at a soil-to-water ratio of 1:2.5 (w/v). NH_4^+-N and NO_3^--N concentrations in 2 M KCl extracts were determined using a flow analyzer (Auto-Analyzer 3). Soil total nitrogen and carbon (TN and TC) content was determined using an elemental analyzer (Vario EL Cube, Elementar, Germany).

2.4 Soil DNA extraction and quantitative PCR assay

The abundance of N-cycling key functional genes was measured from soil samples on three fertilization events and June 21st. Microbial DNA was extracted from 0.25 g fresh soil samples using the DNeasy Power soil kit. Genomic DNA integrity was determined via agarose gel electrophoresis, while DNA sample concentrations were measured using a Thermo Fisher Nanodrop 2000 spectrophotometer and appropriately diluted when necessary. Real-time quantitative PCR (qPCR) was used to determine the copy numbers of ammonia oxidizers (AOA and AOB), nitrite reducers (*nir*K and *nir*S) and N_2O-reducers (*nos*Z-I and *nos*Z-II) genes with three replicates. The StepOnePlus™ real-time PCR system from Applied Biosystems (ABI, USA) was employed for qPCR reactions conducted in 96-well plates. Each reaction mixture, containing 20 μl, comprised 2 μl of template DNA, 6.8 μl of sterile water, 0.4 μl of ROX reference dye, 0.4 μl of forward and reverse primers (10 μmol L^{-1}), and 10 μl of SYBR@ Premix Ex Taq. Melting curves were analyzed at the end of the real-time qPCR to confirm the specificity of the PCR products. Amplification efficiencies ranged from 90-95% for all genes, and R^2 values for the standard curve ranged from 0.990-0.998. Table S1 provides details of the gene-specific primers and qPCR cycling thermal conditions.

2.5 Isotope analysis and N_2O source partitioning

Most N_2O emissions from orchards primarily occur in spring and summer, accounting for over 85% of the total annual emissions. Therefore, gas samples from two orchards on 18 May 2021 (second top-dressing fertilizer) were used for N_2O isotopic signature analysis. The analysis of N_2O isotope natural abundance is a frequently utilized tool for determining the relative contribution of specific pathways (Heil et al., 2015). N_2O isotopic signatures of gas samples and air were analyzed using the IRMS isotope ratio mass spectrometer (IRMS, Isoprime100) at the Institute of Environment and Sustainable Development, Chinese Academy of Agricultural Sciences. The measured isotopic signatures of $\delta^{18}O$ and $\delta^{15}N^{SP}$ were determined using Vienna Standard Water (VSMOW) and atmospheric air-N_2 as standards, respectively. Perturbation factors and measurement correction principles were referenced in the previously published literature (Heil et al., 2015). Where N_2O isotopic signatures $\delta^{15}N^{\beta}$ and $\delta^{15}N^{SP}$ values are calculated in the following equations (2) and (3).

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

$$\delta^{15}N^{SP} = \delta^{15}N^{\alpha} - \delta^{15}N^{\beta} \quad (3)$$

Isotopic signature values of N_2O emissions from soil ($\delta^{15}N$, $\delta^{18}O$, or $\delta^{15}N^{SP}$) were calculated from the total isotopic signature values of the gas sample and the two-component ambient air following the (4) equation.

$$\delta_{soil-emitted} = \left(\delta_{sample} \cdot C_{sample} + \delta_{ambient} \cdot C_{ambient}\right) / \left(C_{sample} - C_{ambient}\right) \quad (4)$$

where δ and C represent the isotopic signature and concentration of N_2O in ambient and sample, and the subscripts represent the sample and ambient air, respectively. The N_2O concentration in ambient field air was 271.86 ± 7.41 ppb, and the isotopic signature of N_2O was not calculated when the headspace N_2O concentration at the end of confinement was less than the ambient air concentration.

The four main microbial processes responsible for soil N_2O emissions include nitrification (Ni), fungal denitrification (fD), bacterial denitrification (bD), and nitrifier denitrification (nD) (Hart et al., 1994). Previous studies have proposed quantifying four major microbial processes' contribution to N_2O emissions using the $\delta^{18}O(N_2O/H_2O)$ vs. $\delta^{15}N^{SP}$ dual isotope approach (Lewicka-Szczebak et al., 2017). The contribution of microbial processes and the extent of N_2O reduction were quantified by dividing the four microbial processes into two main parts based on the difference between SP and $\delta^{18}O$ (Buchen et al., 2018). Ni and fD with similar and higher $\delta^{15}N^{SP}$ and $\delta^{18}O$ values were assumed to be Ni/fD in the estimation process, while bD and nD with lower and similar $\delta^{15}N^{SP}$ and $\delta^{18}O$ values were considered as bD/nD. To assess the contribution of N_2O from soil emissions originating from Ni/fD and bD/nD while taking into account the occurrence of N_2O reduction, we relied on the following equation (Buchen et al., 2018) :

$$\delta_{soil-emitted} = X \cdot \delta_{Ni/fD} + (1 - X) \cdot \delta_{bD/nD} + \eta r \cdot ln(1 - Fr) \quad (5)$$

the equation has multiple variables expressed as ratios and isotopic signature values. X represents the contribution of Ni/fD microbial processes to total N_2O production, and ηr represents the net isotopic effect of N_2O reduction. Moreover, Fr is defined as the reduction of

N_2O as $N_2/(N_2O + N_2)$, and $\delta_{Ni/fD}$ and $\delta_{bD/nD}$ represent isotopic signature values of Ni/fD and bD/nD , respectively. Finally, 1-*F*r, which is also called rN_2O, represents the unreduced N_2O fraction expressed as the $N_2O/(N_2O + N_2)$.

2.6 Nitrification and denitrification potential determination

The potential for N_2O production from soil nitrification was evaluated using a modified shaking slurry method during high temperatures and abundant rainfall events (21 June 2021) under the three fertilization treatments (Hart et al., 1994). The following treatments were set up for each soil sample: control (no inhibitor added), acetylene (288 µl, 6 µM) and 1-octyne (2.6 ml, 4 µM). Briefly**,** 3 g fresh-sieved soil (equivalent to dry weight) was placed in 120 ml serum bottles and 20 ml of phosphate buffer (pH = 7.0) containing 1.0 mM NH_4^+-N was added to maximize nitrification. Afterwards, the soil was shaken at 200 rpm, as the constant aerating of the soil slurry at high-speed shaking inhibits denitrification. The culture was aerated at 25 °C for 2 h and then incubated in airtight conditions for 22 h. Acetylene was leveraged as a nitrification inhibitor to differentiate the relative contribution of autotrophic and heterotrophic nitrification processes to N_2O production, and 1-octyne was leveraged as a differential inhibitor to differentiate the contribution of AOA and AOB. Cumulative N_2O emissions were calculated by collecting headspace gas samples from 2 h and 24 h serum bottles.

In addition, we assessed the potential denitrification and denitrification end-product ratio of soil by using the modified acetylene inhibition method (Jones et al., 2022). For each sample, two 25 ml serum vials were weighed with the equivalent of 3 g dry soil weight of fresh-sieved soil (10 mesh sieve). Then, 3 ml of the substrate (prepared from potassium nitrate, succinic acid, glucose, and acetic acid) was added to each serum and vacuumed, adding N_2 or 10% v/v acetylene gas. The serum vials were incubated in a temperature-controlled shaker at 25 °C in the dark for 5 h and then shaken at 200 rpm. Gas samples were collected at the end of the incubation and analyzed using a gas chromatograph (Agilent 7890A). Two serum vials were used to determine potential denitrification and potential N_2O production, respectively. Cumulative N_2O emissions from each serum bottle were determined by calculation, and the denitrification end-product ratio of the soil sample was calculated.

2.7 Statistical Analyses

The results are expressed in terms of mean plus standard error (Mean ± SE, n = 3). The effects of soil physicochemical properties and microbial functional genes on N_2O and NO emissions were investigated by Pearson correlation coefficients. All data were analyzed and plotted using R version 4.1.2 (R Core Team, 2020). The '*Hmisc*' package was used for correlation analysis, the '*multicomp*' package for variance analysis and multiple comparisons, and the '*ggplot*' package for plotting.

3 Results

3.1 Environmental factors

Both orchards exhibited a clear seasonal pattern in soil temperature, with similar fluctuations observed (Fig.1a and f). Seasonal variation of soil WFPS level in both orchards was observed due to rainfall events, ranging from 15.45-93.67% (Fig.1b and g). Soil WFPS of the citrus orchard displayed greater fluctuations in comparison to the pear orchard (Fig.1b and g). Although there were no significant differences in WFPS between the two orchards, the average WFPS of citrus orchards was observed to be higher than that of pear orchards (61.6% vs. 53.8%). The average soil pH of the CF treatment was 6.75 across two orchards, which was lower than the COF treatment (Fig. 1c and h).

The results indicated that soil NH_4^+-N and NO_3^--N concentrations of two orchards varied considerably depending on the N fertilizer utilized, with the CF treatment exhibiting higher levels than the COF treatment (NH_4^+-N: 174.76 vs 164.31 mg N kg^{-1}, NO_3^--N:134.04 vs 117.16 mg N kg^{-1}). After the first fertilizer application in spring, soil NH_4^+-N concentrations in the soils of both orchards increased rapidly and remained high until the second application. In addition, soil NH_4^+-N concentrations were higher in pear orchard soils than in citrus orchard soils (119.77 vs 100.68 mg N kg^{-1}). The average NO_3^--N concentration in fertilized soil was 84.98 mg N kg^{-1} for pear orchards, which was higher than that for citrus orchards, with an average of 62.67 mg N kg^{-1}.

3.2 Soil N-oxide emissions

The seasonal N_2O fluxes of both orchards were highly variable throughout the experimental

period. Similar patterns were observed within the fertilization treatments of each orchard (Fig. 2b and d). During the observation period, the flux peak of the COF treatment was significantly higher than that of the CF treatment. The average N_2O emission of the N-fertilized treatments was relatively similar and high, ranging from 136.41-190.92 g N ha^{-1} d^{-1}. N_2O emissions were primarily concentrated in the spring and summer seasons during fertilizer application (Fig. 2b and d). Additionally, the N input increased annual cumulative N_2O emissions in pear orchards and citrus orchards by 1.99-2.41 and 2.54-3.29 kg N ha^{-1}, respectively (Table 2). The COF treatment led to a decrease in N_2O emissions by 15% and 16% and the EF_{N2O} by 21.3% and 20.4% in pear and citrus orchards, respectively. It is worth noting that the annual cumulative N_2O emission from the citrus orchard was much lower than that from the pear orchard. The correlation analysis conducted revealed that N_2O emissions from the two orchards are positively and significantly correlated with soil temperature, WFPS, NH_4^+-N and NO_3^--N content ($p < 0.05$), while negatively and obviously correlated with the AOA/AOB value ($p < 0.01$) (Fig.4). Furthermore, in the pear orchard, N_2O emissions show a significant negative correlation to both the abundance of the AOA ($p < 0.001$) and *nos*Z-II ($p < 0.05$).

NO fluxes from both orchards demonstrate a consistent pattern of higher levels during the spring and summer seasons (Fig.2a and c). The CF treatment demonstrated a higher flux pulse than the COF treatment. The COF treatment reduced annual cumulative NO emissions by 9.4% and 10.5% in the pear and citrus orchards when compared to the CF treatment (Table 2). Consequently, the COF treatment reduced the mean of EF_{NO} by 21.1% in comparison with the CF treatment. Additionally, the annual cumulative emissions of NO from the pear orchard are higher than those from the citrus orchard. Correlation analysis indicated that NO flux was positively related to soil temperature ($p < 0.001$), pH ($p < 0.05$), NH_4^+-N, and NO_3^--N ($p < 0.01$) while negatively related to the abundance of AOA genes ($p < 0.01$) and the AOA/AOB value ($p < 0.001$) in both orchards (Fig.4). In addition, the abundance of the *nos*Z-II gene in the soil significantly influenced NO emissions in the citrus orchard ($p < 0.05$).

3.3 Abundances of N-cycling functional genes

Throughout the observation period, various fertilization treatments impacted the abundance

fluctuations of functional genes involved in nitrogen cycling (Fig. 3). Nitrogen fertilizer application enhanced the abundance of ammonia-oxidizing bacteria genes in the two orchards (Fig. 3b and h). The COF treatment generally decreased the abundance of ammonia oxidizers, nitrite reductants and N_2O reducers (Fig.3a-d and g-j). When compared to the CF treatment, the COF treatment increased AOA/AOB and (*nir*K+*nir*S)/*nos*Z values. In addition, AOA gene abundances in both orchards showed a downward trend at different time points (Fig. 3a and g). AOA, AOB, *nir*K, and *nir*S abundance in the soil of the pear orchard were higher than those in the citrus orchard (Fig. 3a-d and g-j), while the abundance of *nos*Z-I and *nos*Z-II was lower in pear orchard (Fig. 3e-f and k-l).

3.4 N_2O isotope signatures and production pathways

This study analyzed the N_2O production and reduction processes in the N application treatment using a $\delta^{18}O(N_2O/H_2O)$ versus $\delta^{15}N^{SP}$ model, which distinguishes between Ni/fD and bD/nD. The dual isotope plots reveal that N_2O production in both orchards is a complex process where the isotope δ originates from a mixture of nitrification and denitrification regions (Fig. 5a). The isotopic signature in the pear orchard was close to bD/nD. In contrast, in the citrus orchard, the isotopic signature was closer to the other end. On the other hand, the N_2O isotopic signatures of the CF treatment were located closer to bD/nD, while those of the COF treatment were closer to bD/nD. Therefore, Partial substitution for mineral fertilizer with manure modify the contribution of various microbial processes to N_2O production. The outcomes of the source partitioning using a two-source mixture model showed that N_2O emissions from the orchard soils were primarily caused by bD/nD pathway (Fig. 5b). Furthermore, it was found that the contribution of bD/nD pathway was higher in the COF treatment as compared to the CF treatment. Likewise, partial substitution for mineral fertilizer with manure led to an increase in N_2O reduction (*F*r) and a decrease in residual (rN_2O) proportion in comparison with the CF treatment (Fig.5b).

The results from aerobic sludge indicated that orchard soils reacted differently to two N treatments (Fig. 6). The partial substitution for mineral fertilizer with organic fertilizers increases the contribution of AOA to the autotrophic nitration processes relative to the CF

treatment (Fig.6c, d). Besides, the COF treatment increased the contribution of heterotrophic nitrification to N_2O production from nitrification in the citrus orchard (Fig. 6d). The enhancement of AOA in the pear orchard soil is rapid, while it requires more time in the citrus (Fig. 6c and d). AOB dominates the process of ammonia oxidation in both orchard soils. Autotrophic nitration (AOA + AOB) has a higher contribution compared to heterotrophic nitration (HN). In addition, the denitrification potential was found to be higher in the CF treatment plots than those treated with COF (Fig.6a-b). The product ratios of the COF treatment in both orchards were lower than those of the CF treatment. Furthermore, 87.5% of the N_2O to total products for the CF treatment was greater than 0.73, indicating that most N potential emissions occurred in the form of N_2O rather than N_2 (Fig. 6a-b). Conversely, the COF treatment exhibited a higher N_2O reduction and a final product ratio of denitrification ranging from 0.42 to 0.79, as compared to the CF treatment.

4 Discussion

4.1 Replacing mineral fertilizer with manure suppressed N-oxide emissions

Replacing mineral fertilizer with manure was found to increase N_2O flux peaks in both orchards. However, it effectively reduced the annual cumulative emissions of N_2O and NO by 20% and 17%, respectively. Two potential reasons could account for this phenomenon. Firstly, the NO_3^--N content was higher in the COF treatment than in the CF treatment after fertilization (Fig.1e and j). Consequently, applying manure under suitable soil moisture conditions leads the elevated carbon-nitrogen substrates, thereby triggering N_2O pulse emissions when compared to the CF treatment (Fig. 2). It is consistent with previous research, which shows that manure has a greater impact on triggering N_2O pulse emissions (Escanhoela et al., 2019). However, manure enhances the availability of soil organic carbon and N, stimulates the growth and proliferation of heterotrophic microorganisms, promotes soil microbial fixation of NO_3^--N and reduces N losses from denitrification, consequently leading to a reduction in N_2O cumulative emissions (Bradley, 2001; Burger and Jackson, 2003; Cheng et al., 2017). Secondly, the main factor leading to the decrease in N_2O emissions is the decomposition properties of organic matter. Organic matter with a higher C/N ratio is associated with a lower likelihood of N_2O emissions

(Akiyama and Tsuruta, 2003). In Mediterranean vineyards, composts led to a reduction in N_2O emissions of about 20% when compared to a single mineral fertilizer application. This is attributed to the high C/N value of organic materials (about 20), which results in intense competition for the N matrix during N matrix decomposition, followed by the inhibition of nitrification and denitrification (Fentabil et al., 2016). A meta-analysis also demonstrated that organic fertilizers can stimulate and inhibit soil N_2O emissions, depending on their C/N value, with N_2O emissions can be mitigated when the C/N value exceeds 8.6 (He et al., 2019). Therefore, the C/N ratio of manure was 13 in our study, which has led to a reduction in cumulative N_2O emissions.

The alters of nitrification and denitrification processes under the COF treatment could be the key factor to mitigate N-oxide emissions in orchard soils. On the one hand, AOB dominates the ammonia-oxidizing microbial community in environments in the acidic orchard soil (Fig. 6c). The nitrification process dominated by AOB genes produces more N_2O during nitrification than AOA (Stein, 2019). The result indicated that AOB contributed more to autotrophic nitrification in the CF than the COF treatment and the value of AOB/AOA also supports this finding (Fig. 3, Fig. 6c). Observed variations in the impact of organic fertilizers on the abundance of AOA and AOB genes are due to different ecological niches (Hink et al., 2018). Additionally, Bi et al. (2023) found that an adequate percentage of organic substitutes can reduce N_2O and NO emissions influenced by the AOB community structure. On the other hand, denitrification is more significant than nitrification in the soil after manure application, which coincides with research findings that adding organic fertilizers has a stronger denitrification ability compared to mineral fertilizers (Lin et al., 2019; Yamamoto et al., 2017). The $\delta^{18}O(N_2O/H_2O)$ versus $\delta^{15}N^{SP}$ model showed that bD/nD is the main process responsible for N_2O production in fertilization treatments, with a contribution rate of over 60% and a higher proportion of organic fertilizers (Fig.5b). Previous studies have demonstrated that organic fertilizers increase soil-denitrifying enzyme activity, resulting in more gaseous N_2 produced through soil denitrification catalyzed from N_2O by nitric oxide reductase (NOR) enzymes (Bowles et al., 2014; Chen et al., 2010). Although there is no significant increase in the abundance of *nos*Z genes that encode NOR enzymes, a lower value of (*nir*K+*nir*S)/*nos*Z

denitrifying bacterial communities supported the finding that the higher *F*r and the lower rN_2O in COF treatments than in CF treatments based on N_2O isotope analysis (Figs. 3 and 5b). Hence, more N undergoes a complete reduction to N_2 than is released as N_2O under the COF treatment. Overall, these findings regarding functional genes and N_2O sources demonstrate that partial substitution for mineral fertilizer with manure can effectively decrease N_2O and NO emissions.

4.2 Higher N-oxide emissions in deciduous orchard than evergreen orchard

There were significant differences in the pulse peaks and annual cumulative emissions of N-oxide between two orchards (Fig. 2, Table 2). This difference could be attributed to the soil physicochemical properties of different orchard soils. First, higher WFPS in the citrus orchard stimulated the in-situ reduction of N_2O, resulting in differences in peak pulse time (Fig. 1b and g, Fig. 2). Although the pulse peaks occurred at different times, they appeared after the second topdressing fertilization (Fig. 2). Second, soil C/N value was lower in pear orchards than in citrus orchards (9.2 vs 9.6), indicating higher available N pools in pear orchards (Table 1). If the same exogenous carbon was introduced to both orchards, the difference might have provided more substrate for the N-oxide production of pear orchards. Third, compared to other soil properties, the BD and soil texture were identified as more critical factors affecting N_2O emissions (Gu et al., 2019; Qin et al., 2021). The BD of the citrus orchard (1.36 g cm^{-3}) is higher than the pear orchard (1.33 g cm^{-3}) (Table 2). The higher BD and fine texture of the citrus orchard lead to lower N_2O emissions, which is consistent with the concept that poorly aerated soils have lower N_2O emissions (Gu et al., 2013). Consequently, the variations in WFPS, C/N value, and BD are intimately associated with the differences observed in N-oxides emissions across diverse orchards.

The difference of growth habit can also be contributed to the variation of N-oxide emissions between deciduous (pear) and evergreen (citrus) orchards (Medda et al., 2022). Although there was no significant variance in N_2O emissions between evergreen and deciduous orchards, deciduous orchards (9.53 kg N ha^{-1} yr^{-1}) exhibited higher N_2O emissions than evergreen orchards (6.20 kg N ha^{-1} yr^{-1}) (Zhao et al., 2022). It is consistent with our results, which indicate that the average cumulative emissions of N-oxides in the pear orchard were 67%

higher than in the citrus orchard (Table 2). Simultaneously, EF_{N2O} and EF_{NO} were higher in pear orchards than in citrus orchards, with EF_{N2O} (2.36% vs. 1.85%) showing a similar to previous estimates of 2.29% and 1.74% (Zhao et al., 2022). This difference is mainly caused by the disparity in leaf litter between deciduous and evergreen orchards. Research has shown that the addition of leaf litter stimulates nitrogen mineralization in the soil and increases microbial nitrogen presence (Khalsa et al., 2016). Moreover, the synergistic effect of leaf litter and nitrogen fertilizer enhances microbial activity by providing energy stimulation, thus promoting the emission of N_2O (Pandeya et al., 2020). Hence, the type of orchard plays a crucial role in determining substantial variations in N-oxide emissions.

4.3 Environmental implications and study limitations

This study had significant implications for climate-smart orchard plantings in China. First, partial substitution for mineral fertilizer with manure was a feasible reduction option for decreasing N_2O and NO emissions. Several researches have shown that orchard soil is a potential hotspot of N-oxide emissions in the agricultural sector (Gu et al., 2019; Qin et al., 2021; Xu et al., 2022; Zhao et al., 2022). The irrational field management in Chinese orchards led to substantial greenhouse gas (GHG) emissions, with remarkable regional variation. Therefore, this study is of great significance for designing N-oxide emissions reduction countermeasures. Second, our study for the first time utilized the dual isotopic tracing method to identify microbial sources of N_2O in orchard soil. The result indicated that partial substitution for mineral fertilizer with manure can enhance the bD/nD process in denitrification, thereby leading to more reduction of N_2O (Fig. 5b). It provides theoretical support and a basis for the feasibility of manure application in agricultural practices. Third, our results indicated that the partial substitution for mineral fertilizer with manure can reduce the average of 0.76% EF_{N2O} and 0.04% EF_{NO}, respectively (Table 2). Calculated based on the planting area of 12.81Mha for orchards in China in 2022, it has the potential to reduce emissions by approximately 30.16 Gg N_2O-N and 1.54 Gg NO-N. Therefore, partial substitution for mineral fertilizer with manure can facilitate the target of carbon neutral in 2030. Finally, orchard soil is a hotspot of N-oxide emissions in agricultural sector. Policy and practice in China should prioritize the orchard management, considering the enormous planting area and potential N_2O and NO emissions.

There are three limitations in the study. Firstly, our study solely focused on one substitution rate and did not consider different levels of substitution. Hence, it lays the groundwork for future research. Secondly, our study is limited in scope to data collected from only two orchards in a single year. This may not provide a comprehensive representation of the long-term behavior of the experimental sites. Therefore, future investigations should prioritize conducting extensive, prolonged, and multisite experiments to provide a more precise assessment of GHG emissions within orchard ecosystems. Lastly, the research did not assess the impact of partial substitution for mineral fertilizer with manure on crop yields, as well as conducting a comprehensive evaluation of the environmental benefits.

5 Conclusions

In summary, we investigated the impact of partial substitution for mineral fertilizer with manure on N-oxide emissions in both evergreen and deciduous orchards, concurrently examining the associated microbial mechanisms for their production pathway. Our results showed that the partial substitution for mineral fertilizer with manure decreased cumulative N_2O and NO emissions from orchard soils. This was mainly due to the manure application, which reduced the contribution of AOB to nitrification, stimulating denitrification processes and leading to less N_2O and NO accumulation. In addition, there were significant variations in N-oxide emissions between orchards due to differences in soil physicochemical properties and orchard types. In particular, deciduous orchards exhibited higher background emissions of N-oxide. These findings underlined the necessity of partial substitution for mineral fertilizer with manure in orchards as a critical strategy to mitigate emissions in the agricultural sector.

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Authors' contribution Pinshang Xu: Conceptualization, Formal analysis, Investigation, Data Curation, Writing - Original Draft. Zhutao Li: Investigation, Data Curation. Shumin Guo: Writing - Review & Editing. Jinyang Wang: Project administration, Writing - Review & Editing. Davey L. Jones: Writing - Review & Editing. Zhaoqiang Han: Formal analysis, Writing -

Review & Editing. Jianwen Zou: Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

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Declarations

Code availability The code generated during this study is accessible through the corresponding author upon request.

Data availability The dataset utilized in this study is not publicly accessible; however, it can be obtained from the corresponding author upon request.

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Figure Captions

Fig. 1 Dynamics of soil physicochemical factors under different fertilization treatments from the orchard plantations. Soil temperature (a), WFPS (b), pH (c), NH_4^+ (d), NO_3^- (e) from the pear orchard. Soil temperature (f), WFPS (g), pH (h), NH_4^+ (i), NO_3^- (j) from the citrus orchard. Values are means ± SEM (n = 3). CK, no fertilization; CF, compound fertilizer; COF, cow manure plus compound fertilizer. The arrows indicate the date of fertilization events.

Fig. 2 Seasonal fluxes of N_2O and NO under different fertilization treatments from orchard plantations. NO (a), N_2O (b), and NO (c), N_2O (d) respectively represent the pear orchard and citrus orchard. Values are means ± SEM (n = 3). CK, no fertilization; CF, compound fertilizer; COF, cow manure plus compound fertilizer. The black arrows represent three fertilization events and the grey bars are due to the COVID-19 control not sampled.

Fig. 3 Abundance of functional genes associated with the nitrogen cycle in different fertilizer treatments from the Pear (left panel) and Citrus (right panel) orchard plantations (AOA, AOB, *nir*K, *nir*S, *nos*Z-I, *nos*Z-II). CK, no fertilization; CF, compound fertilizer; COF, cow manure plus compound fertilizer.

Fig. 4 Correlation of N_2O, NO and N_2O+NO fluxes with soil physicochemical properties and functional genes associated with the nitrogen cycle in peach orchards; *, ** and *** indicate statistical significance at $P < 0.05$, 0.01 and 0.001. Soil T, soil temperature; (A/B), (AOA/AOB); (*K*+*S*)/Z, (*nir*K+*nir*S)/(*nos*Z-I+*nos*Z-II).

Fig. 5 Graphical representation of net isotope effect value of 18O (δ18O) vs. $\delta^{15}N^{SP}$ plot method presented to analyze soil-emitted N_2O isotope data (a). The relative contribution of N_2O production from two endmembers nitrification/fungal denitrification and nitrifier denitrification/bacterial denitrification (b). CF, compound fertilizer; COF, cow manure plus compound fertilizer.

Fig. 6 Product ratio of N_2O to (N_2O+N_2) for each treatment at different periods (a, b). Relative contribution of heterotrophic nitrification (HN) versus AOA and AOB dominated autotrophic nitrification in N_2O production by nitrification process for each treatment at different periods (c, d). The left panel is pear orchard, and the right is citrus.

659 **Table 1** The initial properties of the two orchards soil.

Soil	Texture	Clay (%)	Silt (%)	Sand (%)	NH_4^+ (mg N kg^{-1})	NO_3^- (mg N kg^{-1})	TN (g N kg^{-1})	TC (g C kg^{-1})	Bulk density (g cm-3)	pH
Pear	Loam	16	40	44	0.76	1.86	1.01	9.25	1.33	6.18
Citrus	Clay Loam	30	38	32	0.82	1.95	1.27	12.22	1.36	6.14

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661 **Table 2** Annual cumulative emissions and emission factors (EF) for N_2O, NO and (N_2O+NO)
662 under different fertilization treatments in pear and citrus orchards

Orchard	Treatment	N_2O flux (kg N ha^{-1})	NO flux (kg N ha^{-1})	N_2O + NO (kg N ha^{-1})	Direct emission factor (%)		
					EF_{N2O}	EF_{NO}	$EF_{N2O + NO}$
Pear	CK	5.92 ± 0.46 b	0.32 ± 0.07 b	6.24 ± 0.39 b			
	CF	14.25 ± 1.29 a	0.76 ± 0.03 a	15.00 ± 1.32 a	2.78	0.15	2.92
	COF	11.77 ± 2.04 a	0.66 ± 0.08 ab	12.42 ± 2.11 a	1.93	0.11	2.04
Citrus	CK	2.89 ± 1.55 a	0.33 ± 0.12 a	3.22 ± 1.45 a			
	CF	9.51 ± 3.75 a	0.64 ± 0.10 a	10.15 ± 3.83 a	2.21	0.10	2.31
	COF	7.35 ± 2.11 a	0.51 ± 0.05 a	7.86 ± 2.07 a	1.49	0.06	1.55

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