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Effect of Zinc Oxide Nanoparticles on Nitrous Oxide Emissions in Agricultural Soil

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Abstract: Zinc oxide nanoparticles (ZnO NPs) are widely used and exposed to the soil environment, but their effect on soil nitrous oxide (N_2O) emissions remains unclear. In this study, a microcosm experiment was conducted to explore the effects of different ZnO NPs concentrations (0, 100, 500, and 1000 mg kg^{-1}) on N_2O emissions and associated functional genes related to N_2O amendment with carbon (C) or nitrogen (N) substrates. Partial least squares path modeling (PLS-PM) was used to explore possible pathways controlling N_2O emissions induced by ZnO NPs. In the treatment without C or N substrates, 100 and 500 mg kg^{-1} ZnO NPs did not affect N_2O production, but 1000 mg kg^{-1} ZnO NPs stimulated N_2O production. Interestingly, compared with the soils without ZnO NPs, the total N_2O emissions in the presence of different ZnO NPs concentrations increased by 2.36–4.85-, 1.51–1.62-, and 6.28–8.35-fold following C, N and both C & N substrate amendments, respectively. Moreover, ZnO NPs increased the functional genes of ammonia-oxidizing bacteria (AOB *amoA*) and nitrite reductase (*nirS*) and led to the exhaustion of nitrate but reduced the gene copies of ammonia-oxidizing archaea (AOA *amoA*). In addition, the redundancy analysis results showed that the AOB *amoA* and *nirS* genes were positively correlated with total N_2O emissions, and the PLS-PM results showed that ZnO NPs indirectly affected N_2O emissions by influencing soil nitrate content, nitrifiers and denitrifiers. Overall, our results showed that ZnO NPs increase N_2O emissions by increasing nitrification (AOB *amoA*) and denitrification (*nirS*), and we highlight that the exposure of ZnO NPs in agricultural fields probably results in a high risk of N_2O emissions when coupled with C and N substrate amendments, contributing to global climate warming.

Keywords: nanomaterials; nitrification; denitrification; nitrous oxide; soil

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1. Introduction

In the past few decades, engineered nanoparticles (NPs) have been widely used in industrial, agricultural, and consumer production due to their unique properties (such as antibacterial properties) [1,2]. Meanwhile, most consumed NPs are exposed to the soil environment through the application of wastes and sewage sludge, which causes potential risks for microorganisms due to their toxic effects [3,4]. The presence of NPs in the soil environment affects the activities of nitrifiers and denitrifiers [5–7], which probably influences the release of nitrous oxide (N_2O) from the soil to the atmosphere and contributes to global warming and ozone destruction. However, recent publications have reported that the effect of NPs on soil N_2O emissions depends on their types [8–11]. For example, copper oxide (CuO) [8], silver sulfide (Ag_2S) [9], and high-dose lithium oxide (Li_2O) [10] and iron oxide (Fe_2O_3) [11] NPs could reduce, increase, and not affect soil N_2O emissions, respectively.

Zinc oxide (ZnO) NPs are the third most used type of nanomaterial [4] and can potentially influence N_2O production through at least two distinct mechanisms. First, the toxic action of ZnO NPs or the dissolution of Zn^{2+} can decrease soil microbial activity related to nitrogen (N) cycling [12–15] and N mineralization efficiency [14], which then indirectly reduces N_2O emissions. Second, ZnO NPs inhibited glucose metabolism enzymes and then reduced the electrons and power for denitrifiers to produce N_2O [16–18]. In a mineral medium, Zheng et al. [16] found that ZnO NPs largely inhibited the expression of the *nosZ* gene related to N_2O reductase in the denitrification pathway and then increased N_2O emissions. In a synthetic wastewater system, Ye et al. [18] observed that ZnO NPs reduce N_2O emissions by enhancing N_2O reduction. In the soil environment, few studies have focused on the impact of ZnO NPs on N_2O emissions. Durenkamp et al. [19] observed that combining ZnO and Ag NPs has a minimal impact on N_2O emissions in soil/sewage sludge mixtures. Rashid et al. [14] found that ZnO NPs reduced the nitrogen mineralization of leaf litter in sandy soil and then probably reduced the release of N_2O.

In agricultural fields, the input of additional carbon (C) and N substrates from management practices (such as the application of manure or crop residue) [20–23] has the potential to enlarge N_2O emissions, which probably changes the impact of NPs on N_2O production. However, amendment with a C substrate temporarily creates an anaerobic environment, which further limits the last step of denitrification in soils polluted with ZnO NPs [24,25] and probably results in a high risk of N_2O. In addition, the amendment of C could partly supply electrons and power for denitrifiers and likely offset the negative effect of ZnO NPs on soil nitrifiers and denitrifiers [23]. Meanwhile, the amendment of N fertilizer would increase the *nosZ* gene, which probably mediates the effect of ZnO NPs on N_2O reduction [26]. In this study, microcosm experiments were conducted to explore the effect of ZnO NPs on N_2O emissions and functional genes related to nitrification and denitrification. We hypothesize that: (1) the incorporation of ZnO NPs into soil inhibits the release of N_2O from soil in the absence of C or N substrates and (2) the exposure of ZnO NPs would increase N_2O emissions after C and N substrate amendments.

2. Materials and Methods

2.1. Materials and Experimental Design

Soil samples were collected from farmland without known nanomaterial pollution in the suburbs of Wuhan, China (114°26′ N, 30°19′ E, 25.89 m altitude). Soils in the 0–20 cm layer were sampled at three random sites and then mixed. The sampled soils were passed through a 2-mm sieve and then stored at 4 °C. The soil organic carbon, total nitrogen, pH, and bulk density were 12.4 g kg^{-1}, 1.4 g kg^{-1}, 6.8 and 1.26 g cm^{-3}, respectively.

Four ZnO NPs concentrations and four substrate amendments were conducted in this experiment. Four ZnO NPs levels were 0, 100, 500, and 1000 mg kg^{-1} soil, which was based on the nominal range of ZnO NPs concentrations (100–6400 mg kg^{-1}) in soil [27]. The four amendments included a control (CT), carbon as glucose (C), nitrogen as ammonium (N), and glucose plus ammonium (C AND N). Each treatment included 3 replicates. Deionized water was added to all the soils to achieve 60% water holding capacity, and the soils were then incubated at 25 °C. ZnO NPs (purity > 99% and particle size < 100 nm) were purchased from Aladdin (Shanghai, China). ZnO NPs (0, 1.5, 7.5, and 15 mg) were incorporated into 15 g of air-dried equivalent soil to achieve 0, 100, 500, and 1000 mg kg^{-1} concentrations and then added to 120-mL brown serum bottles. Additionally, 600 mg C glucose per 1 kg air-dried soil was added as the C substrate; the selected quantity equaled 100% of the microbial biomass C of this sampled soil, as suggested by Tian et al. [28]. Nitrogen was added as ammonium sulfate at 56.4 mg N kg^{-1} (equal to 140 kg N ha^{-1}) based on the application amount of nitrogen fertilizer according to local farmers' practices.

2.2. N_2O Emission Measurements

Before N_2O measurement, all bottles were opened and placed in a fuming cupboard for 5 min, which was a sufficient duration for the N_2O in the bottle to adjust to the

background value in the atmosphere (Figure S1); afterwards, the bottles were sealed with silicone plugs and incubated for 1 day to allow N_2O to accumulate in the bottle. Meanwhile, four empty bottles were used to measure the initial N_2O concentrations. On days 1, 2, 3, 5, and 8, 50 µL of sampled gas in the headspace was collected, and the N_2O concentration was analyzed by using a gas chromatography instrument (Agilent 7890B, Agilent, Palo Alto, CA, USA) equipped with an electron capture detector, which attained ±0.1% precision of the measured N_2O. The sampling frequency decreased with incubation days because the peak N_2O emissions occurred in the early period, and 8 days was sufficient for N_2O emissions to decrease to a stable level based on our preliminary experiment. The cumulative N_2O emissions under different treatments were calculated by linear interpolation between daily emissions and the corresponding time [25].

2.3. Soil DOC, NH_4^+ and NO_3^- Measurements

At the end of the experiment, all soils were destructively sampled and divided into two parts. Fresh soil (first portion) was used to determine soil chemical properties. Briefly, 3 g soil was mixed with 2 M KCl (15 mL) and shaken for 60 min. Afterwards, the mixture was filtered with a qualitative filter, and then the ammonium (NH_4^+) and nitrate (NO_3^-) concentrations were measured using a continuous flow analyzer (Skalar SAN^{++} System, Skalar Analytical B.V., Breda, The Netherlands). Another 3 g of fresh soil was added to 15 mL of K_2SO_4, shaken for 1 h, and filtered with a quantitative filter. These filtered solutions were used to detect dissolved organic carbon (DOC) using a total organic carbon analyzer (Multi-N/C 2100S; Analytik Jena, Jena, Germany).

2.4. DNA Extraction and qPCR Analysis

Another part of the soil was freeze-dried at −80 °C for 2 days, and then 0.5 g of soil was used to extract DNA using the FastDNA™ Spin Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) according to the instruction manual. DNA quantification and quality assessment were carried out using a NanoDrop 2000 Spectrophotometer (Thermo Scientific®, Wilmington, DE, USA). The extracted DNA was used to determine the abundances of nitrification (ammonia-oxidizing archaea (AOA *amoA*) and ammonia-oxidizing bacteria (AOB *amoA*) and denitrification functional genes (*nirK*/*nirS*- and *nosZ*-encoded nitrite and nitrous oxide reductases, respectively) by the qPCR method using a LightCycler® 480 II (Roche Diagnostics, Basel, Switzerland) [20]. The 20 µL reaction mixture for qPCR contained 2.0 µL template DNA, 0.5 µL of each primer, 10 µL GoTaq® qPCR Master Mix 2× (Promega, Madison, WI, USA) and 7 µL nuclease-free water (Promega, Madison, WI, USA). The primers and thermal cycling conditions of these genes are listed in Table S1.

2.5. Statistical Analysis

The differences in cumulative N_2O emissions, soil chemical properties (DOC, NH_4^+, and NO_3^-), and functional genes related to N_2O (AOA and AOB *amoA*, *nirK*, *nirS* and *nosZ*) among the four ZnO NPs levels were tested by one-way analysis of variance (ANOVA) using the least significant difference (LSD) test at the level of $p < 0.05$. All statistical analyses were conducted using SPSS 20.0 (SPSS Inc., Chicago, IL, USA). Redundancy analysis (RDA) was applied with CANOCO 4.5 (Microcomputer Power, Ithaca, NY, USA) to analyze the correlations of N_2O emissions and environmental variables (DOC, NH_4^+ and NO_3^-) with functional genes related to nitrification (AOA and AOB *amoA*) and denitrification (*nirK*, *nirS*, and *nosZ*). Partial least squares path modeling (PLS-PM) was used to explore possible pathways controlling N_2O emissions induced by ZnO NPs addition.

3. Results

3.1. Effect of N_2O Emission

In the CT treatment, low daily N_2O emissions (<1.48 µg N kg^{-1} day^{-1}) were detected at ZnO NPs concentrations below 500 mg kg^{-1}, but these values ranged from 1.95 to 5.54 µg N kg^{-1} day^{-1} at the 1000 mg kg^{-1} ZnO NPs level (Figure 1a). The presence of

ZnO NPs accelerated the production of N_2O in the C treatment, and the peaks of N_2O emission were 43.67, 106.24, 221.59 and 199.61 μg N kg^{-1} day^{-1} for 0, 100, 500, and 1000 mg kg^{-1} ZnO NPs concentrations, respectively (Figure 1b). However, incorporating ZnO NPs into soils slightly stimulated the effect of N addition on N_2O emissions, resulting in daily emissions of 1.02–4.14 μg N kg^{-1} day^{-1} in the treatment without ZnO NPs and 2.36–6.35 μg N kg^{-1} day^{-1} in the soils under different ZnO NPs levels (Figure 1c). ZnO NPs amendment considerably stimulated N_2O emissions following both C and N addition; the peaks of N_2O emissions ranged from 459.39 to 680.41 μg N kg^{-1} day^{-1} in the treatment with ZnO NPs, while this variable was 77.07 μg N kg^{-1} day^{-1} without ZnO NPs (Figure 1d).

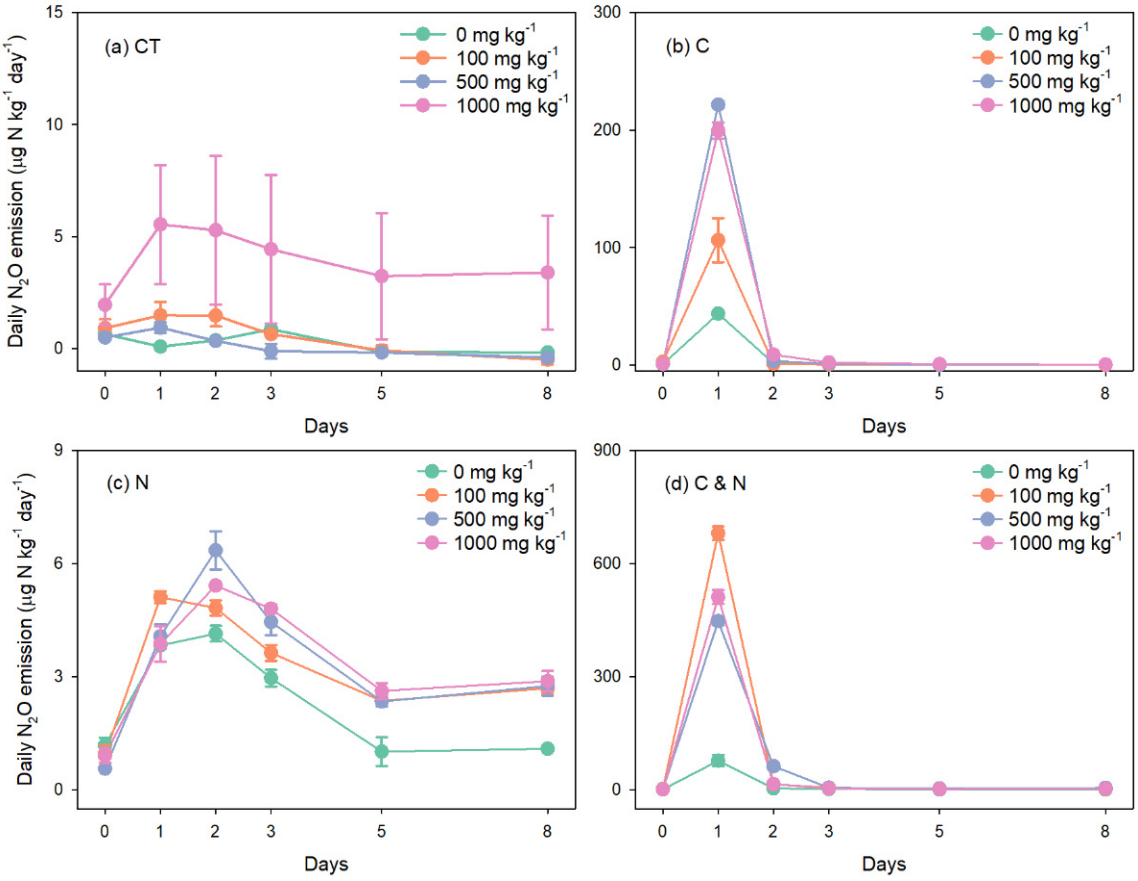

Figure 1. N_2O emissions in the treatments with no addition (**a**), C addition (**b**), N addition (**c**), and C and N addition (**d**) at different ZnO NPs concentrations (0, 100, 500 and 1000 mg kg^{-1}). Error bars represent the standard error of the mean (n = 3).

In the absence of C or N substrates, there were no significant differences in cumulative N_2O emissions among the four ZnO NPs concentrations, although these values were greater at the 1000 mg kg^{-1} level (34.27 μg N kg^{-1}) than at low concentrations (Figure 2). The presence of ZnO NPs significantly increased N_2O emissions after C or N addition (Figure 2), and we found that C, N and their interactions significantly ($p < 0.01$) affected total N_2O emissions (Table 1). Compared with the soils without ZnO NPs, the total N_2O emissions in the presence of different ZnO NPs concentrations increased by 2.36–4.57, 1.51–1.62, and 6.24–8.30 times in the soil amendment with C, N, and C AND N substrates, respectively.

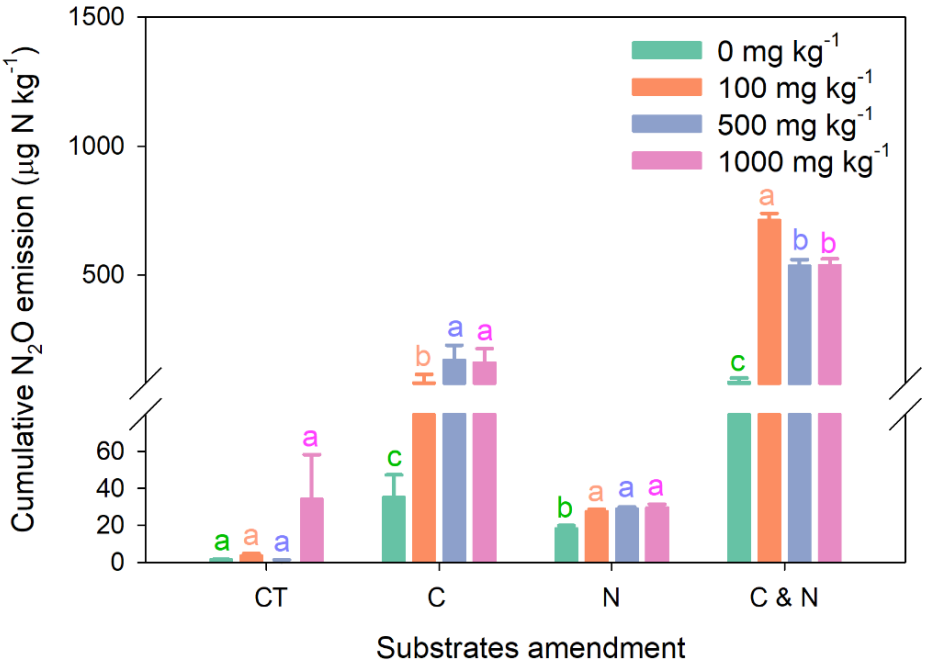

Figure 2. Cumulative N_2O emissions in the treatments with no addition, C addition, N addition and C and N addition at different ZnO NPs concentrations (0, 100, 500 and 1000 mg kg^{-1}). Error bars represent the standard error of the mean (n = 3).

Table 1. Main and interactive effects of C and N substrate amendments on soil N_2O emission, DOC, NH_4^+ and NO_3^- contents and functional genes (AOA and AOB *amoA*, *nirK*, *nirS* and *nosZ*) related to N_2O in the presence of ZnO NPs.

Treatments	N_2O Emission	DOC	NH_4^+	NO_3^-	AOA *amoA*	AOB *amoA*	*nirK*	*nirS*	*nosZ*
C	***	**	*	***	*	*	***	***	**
N	***	ns	**	**	**	***	ns	***	***
C × N	***	**	***	***	*	ns	ns	*	**

* $p < 0.05$, ** $p<0.01$, *** $p < 0.001$.

3.2. Soil DOC, Mineral N Content and Functional Genes Related to N_2O

In the CT, C, and N treatments, ZnO NPs did not significantly ($p < 0.01$) affect the DOC content, there were no significant trends in the NH_4^+ content with increasing ZnO NPs concentration, and the NO_3^- content significantly ($p < 0.01$) decreased with increasing ZnO NPs concentration (Tables 2 and 3). After both C and N addition, ZnO NPs significantly ($p < 0.01$) affected these variables, as DOC and NO_3^- contents decreased but NH_4^+ increased with increasing ZnO NPs concentrations (Tables 2 and 3).

Table 2. Soil DOC, NH_4^+, and NO_3^- contents at different ZnO NPs concentrations (0, 100, 500, and 1000 mg kg^{-1}) in the treatments with no addition, C addition alone, N addition alone, and both C and N addition. Different letters indicate statistically significant differences (LSD, 5%).

Substrates	ZnO NPs Levels	DOC (mg C kg^{-1})	NH_4^+ (mg N kg^{-1})	NO_3^- (mg N kg^{-1})
No addition	0 mg kg^{-1}	34.13 ± 5.54 a	1.43 ± 0.08 ab	61.30 ± 1.56 a
	100 mg kg^{-1}	41.65 ± 1.26 a	1.15 ± 0.12 bc	43.22 ± 0.35 b
	500 mg kg^{-1}	31.33 ± 2.38 a	1.03 ± 0.10 c	44.45 ± 0.64 b
	1000 mg kg^{-1}	34.08 ± 5.97 a	1.55 ± 0.06 a	37.43 ± 0.27 c

Table 2. *Cont.*

Substrates	ZnO NPs Levels	DOC (mg C kg^{-1})	NH_4^+ (mg N kg^{-1})	NO_3^- (mg N kg^{-1})
C addition	0 mg kg^{-1}	45.68 ± 2.49 a	1.57 ± 0.05 b	31.03 ± 1.13 a
	100 mg kg^{-1}	40.72 ± 1.46 a	1.23 ± 0.11 b	15.98 ± 0.05 c
	500 mg kg^{-1}	45.30 ± 10.26 a	1.22 ± 0.16 b	16.95 ± 0.25 c
	1000 mg kg^{-1}	40.88 ± 5.15 a	2.23 ± 0.03 a	19.70 ± 0.26 b
N addition	0 mg kg^{-1}	33.97 ± 1.96 a	24.78 ± 1.58 ab	69.57 ± 0.38 a
	100 mg kg^{-1}	35.85 ± 1.76 a	17.18 ± 1.32 c	65.52 ± 1.03 ab
	500 mg kg^{-1}	26.43 ± 3.80 a	22.73 ± 1.38 bc	58.55 ± 3.29 b
	1000 mg kg^{-1}	36.02 ± 3.64 a	30.03 ± 1.95 a	49.43 ± 0.62 c
C and N addition	0 mg kg^{-1}	41.42 ± 3.51 a	2.80 ± 0.12 bc	64.45 ± 0.09 a
	100 mg kg^{-1}	31.18 ± 3.63 a	1.53 ± 0.08 c	57.08 ± 0.31 b
	500 mg kg^{-1}	17.88 ± 2.83 b	3.97 ± 0.24 b	47.22 ± 0.54 c
	1000 mg kg^{-1}	12.87 ± 1.92 b	15.10 ± 1.11 a	43.62 ± 0.64 d

Table 3. Effect of ZnO NPs on soil DOC, NH_4^+, and NO_3^- contents and functional genes (AOA and AOB *amoA*, *nirK*, *nirS* and *nosZ*) related to N_2O amendment with different substrates.

Treatments	DOC	NH_4^+	NO_3^-	AOA *amoA*	AOB *amoA*	*nirK*	*nirS*	*nosZ*
No addition	Ns	*	***	*	**	ns	*	Ns
C addition	Ns	**	**	**	ns	ns	ns	Ns
N addition	Ns	*	***	ns	ns	ns	*	Ns
C AND N addition	**	***	***	***	***	ns	**	***

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Across all treatments, the functional gene copies related to N_2O followed the order: *nirS* (from 2.53 × 10^6 to 1.51 × 10^7) > AOA *amoA* (from 2.02 × 10^5 to 1.46 × 10^6) > AOB *amoA*, *nirK* and *nosZ* (×10^4) (Figure 3). Increasing ZnO NPs levels tended to decrease the abundance of AOA *amoA* (Figure 3a) but increase AOB *amoA* and *nirS* gene copies (Figure 3b,d). The *nirK* and *nosZ* genes were not significantly affected by ZnO NPs in the CT, C, and N treatments, but *nosZ* significantly increased with increasing ZnO NPs concentrations after amendment with both C and N substrates. In addition, in the presence of ZnO NPs, C, N and their interaction significantly influenced AOA *amoA*, *nirS* and *nosZ* gene copies (Table 1). The RDA results showed that the *nirS* and AOB *amoA* genes were negatively related to DOC on the first redundancy analysis axis, and NH_4^+ and NO_3^- were positively rel'ated to the AOA *amoA* gene but negatively associated with *nosZ* on the second redundancy analysis axis (Figure 4).

3.3. Effect of ZnO NPs on N_2O Emission

The RDA results showed that the *nirS* and AOB *amoA* genes were positively correlated with cumulative N_2O emissions on the first redundancy analysis axis (Figure 4). PLS-PM showed that changes in soil NO_3^- content, nitrifiers (AOB *amoA*) and denitrifiers (*nirS*) induced by ZnO NPs addition significantly affected N_2O emissions (Figure 5a). Both nitrifiers (0.35) and denitrifiers (0.69) had significant positive direct effects on N_2O emissions, while NO_3^- had both positive direct (0.35) and negative indirect (−0.15) effects on N_2O emissions, resulting in a total effect of NO_3^- on N_2O emissions of 0.20 (Figure 5b).

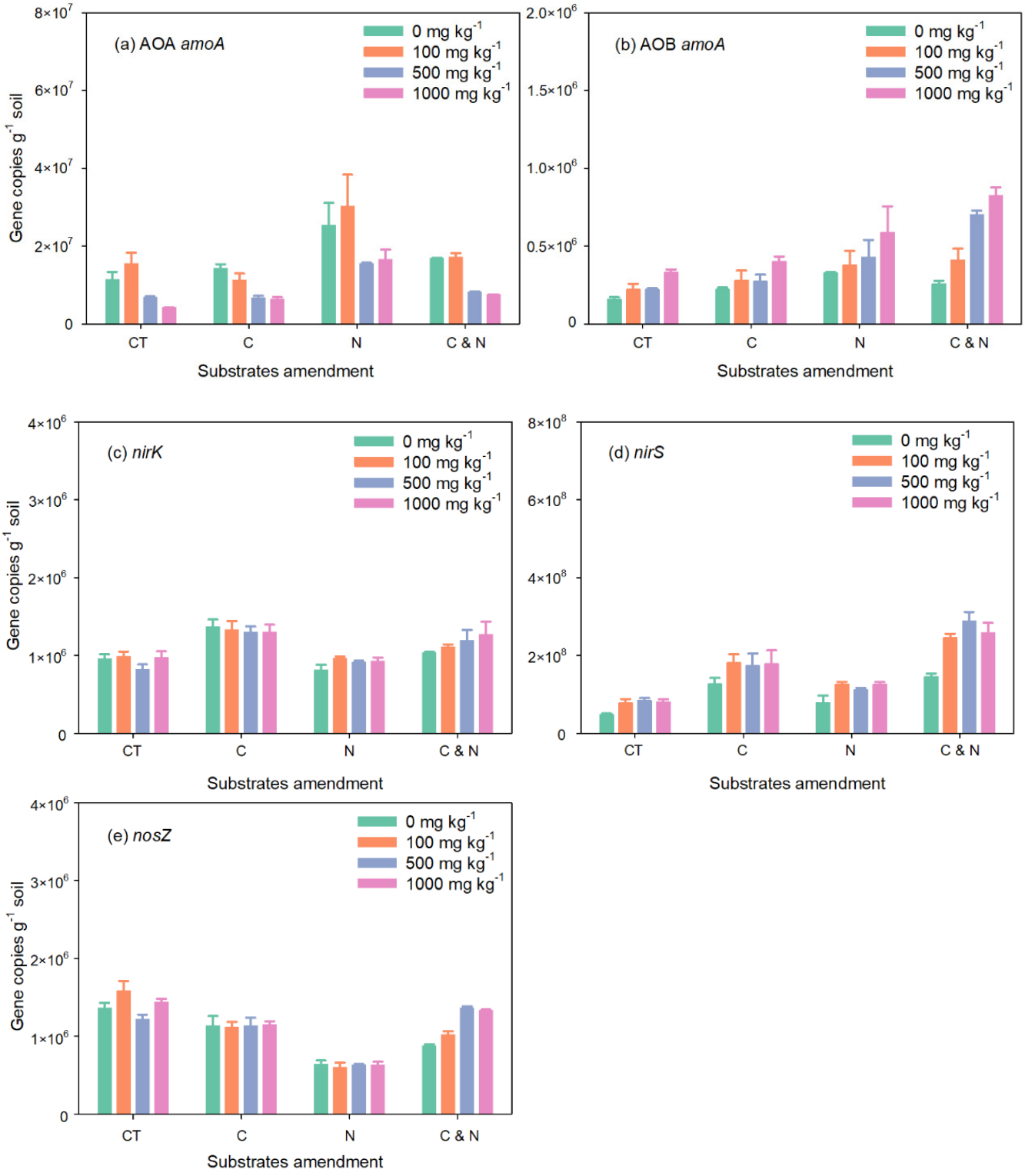

Figure 3. AOA *amoA* (**a**), AOB *amoA* (**b**), *nirK* (**c**), *nirS* (**d**) and *nosZ* (**e**) genes under no addition, C addition, N addition, and C and N addition at different ZnO NPs concentrations (0, 100, 500 and 1000 mg kg^{-1}). Error bars represent the standard error of the mean (n = 3).

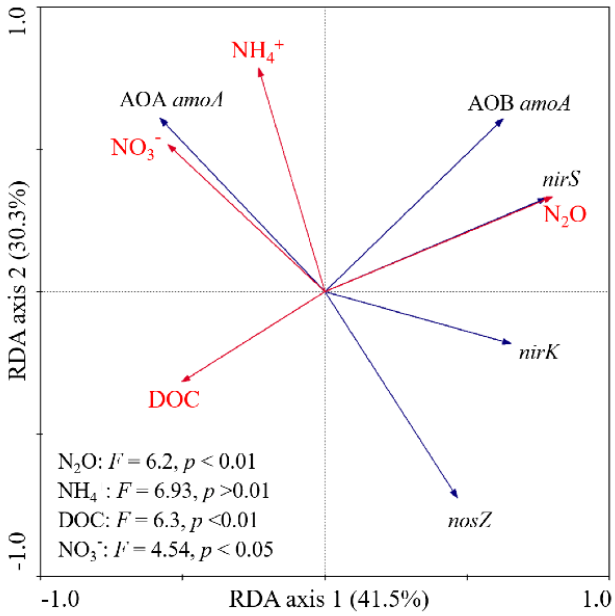

Figure 4. RDA ordination diagram of the relationships between cumulative N_2O emissions and soil chemical variables (DOC, NH_4^+, and NO_3^-) with functional genes related to N_2O (AOA *amoA*, AOB *amoA*, *nirK*, *nirS*, and *nosZ*).

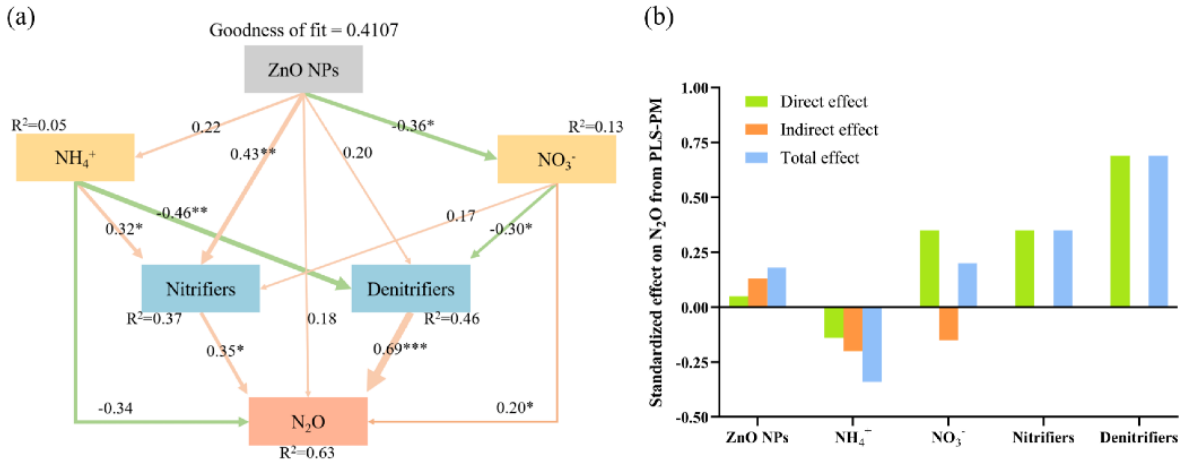

Figure 5. Direct and indirect effects of ZnO NPs on N_2O emissions in PLS-PM. The left figure (**a**) describes the relationships among ZnO NPs, soil NH_4^+, NO_3^-, nitrifiers and denitrifiers with respect to N_2O emissions. Wider arrows indicate higher path coefficients, and orange and green colors indicate positive and negative effects, respectively. *, ** and *** indicate significance at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. The right figure (**b**) describes the standardized direct, indirect and total effects derived from the PLS-PM.

4. Discussion

In the present study, ZnO NPs affected N_2O emissions by controlling nitrification and denitrification pathways. Increasing ZnO NPs levels decreased the *amoA* gene copy number of AOA, which probably reduced the production of N_2O through nitrification. However, this nanomaterial increased the abundances of AOB *amoA* and *nirS*, which induced more N_2O production in the soils. This result disagreed with Phan et al. [29], who found that the presence of ZnO NPs reduced the gene expression levels of AOB *amoA* and *nirK* in a wastewater system. Cumulative N_2O emissions were closely correlated with the *nirS* gene, and the NO_3^- content also decreased with increasing ZnO NPs concentration,

indicating that ZnO NPs affect N_2O emissions probably by enhancing nitrite reduction in the denitrification process. However, Durenkamp et al. [19] observed that ZnO NPs did not significantly affect N_2O emissions from soil/sewage sludge mixtures. In a biological nitrogen removal system, Ye et al. [17] showed that the presence of ZnO NPs inhibited N_2O production by largely reducing the functional genes related to nitrite or nitrate reduction rather than the reduction of N_2O. These different results are probably due to the impact of ZnO NPs on nitrifiers and denitrifiers related to N_2O depending on different environmental conditions. In the soil environment, N_2O is produced through both nitrification and denitrification [23], whereas in other conditions (such as biological nitrogen removal systems), denitrification is the major pathway for N_2O production [17,18].

The impact of NPs on N_2O emissions also depends on their types in the soil environment [8–11,19,30–33]. Zhao et al. [8] observed that CuO NPs (ranging from 10–500 mg kg^{-1}) continuously reduced soil N_2O emissions. Low-dose silver (Ag) NPs unaffected or stimulated N_2O production, but a high dose of this material would inhibit N_2O emission due to toxicity [30–33]. Our results disagree with these studies, and we observed that 100 and 500 mg kg^{-1} ZnO NPs did not affect N_2O production, but 1000 mg kg^{-1} ZnO NPs stimulated N_2O production in the treatment without C and N amendments. This result was consistent with Avila-Arias et al. [10], who found that high-dose Li_2O NPs (1000 mg kg^{-1}) increased soil N_2O emissions, and Wu et al. [9] found that the application of sludge with Ag_2S NPs to soil significantly enhanced the release of N_2O from soils. However, Durenkamp et al. [19] showed that the addition of sewage sludge into soil enriched with both ZnO and Ag NPs did not affect N_2O emissions. Yang et al. [11] also observed no significant effect of Fe_2O_3 NPs (0.5–500 mg kg^{-1}) on N_2O emissions in paddy soils.

The inconsistent effect of these NPs on N_2O emissions is probably due to the different responses of soil nitrification and denitrification processes to nanomaterials. Zhao et al. [8] found that the negative effect of CuO NPs on N_2O emissions was mainly due to this nanoparticle inhibiting the ability of denitrification to accept electrons and then reducing the reductase activities of both nitrate and nitric oxide. Ag NPs decreased soil N_2O production, probably by inhibiting urease activity [34] or functional genes related to nitrification [35–37] and denitrification [38]. However, Ag_2S NPs could improve functional gene-encoded nitrification and denitrification and then increase N_2O emissions in saline soil [9]. In addition, Yang et al. [11] found that Fe_2O_3 NPs did not significantly change the abundances of functional genes related to N_2O production and reduction.

As expected, the amendment of the C substrate significantly stimulated the production of N_2O in the presence of ZnO NPs due to the following: (1) ZnO NPs inhibited the key enzymes of glucose degradation and then reduced the electrons and energy for denitrifiers [16]. However, the addition of glucose probably partly compensates for the negative effect of ZnO NPs on the power available for denitrifiers and then stimulates N_2O production in denitrification. (2) The effect of ZnO NPs on N_2O produced via denitrification pathways relies on O_2 availability, whereas the addition of a C substrate consumes O_2 in the soil through heterotrophic respiration and then temporarily creates an anaerobic environment for denitrification to produce N_2O [23–25,39]. Indeed, in the present study, the amendment of C substrate increased the abundance of *nirK*, which probably stimulated N_2O production through enhanced nitrite reduction.

Our results indicated that in fields polluted by ZnO NPs, some management practices, such as the application of organic manure or crop residue that supply labile C and N substrates probably cause a high risk of N_2O emissions and then contribute to global climate warming. However, two limitations should be noted in this experiment. First, ZnO NPs could dissolve in the soil environment and be consumed by microorganisms [14], which probably resulted in the positive effect of ZnO NPs on N_2O emissions. Indeed, Wu et al. [40] observed a rapid dissolution of ZnO NPs in neutral soil and solubilized them in Zn^{2+}. Further study should be conducted to analyze Zn^{2+} originating from ZnO NPs in the soil and separate the contribution of ZnO NPs and Zn^{2+} to N_2O production and reduction. Second, Zn^{2+} dissolved in the soil could be taken up by plants as a micronutrient,

which could increase the nitrogen use efficiency and then indirectly reduce the substrate for nitrification and denitrification processes [4]. In addition, the use of NPs is a useful method to support plant growth [2], which could indirectly affect nitrification and denitrification processes by regulating the N substrate. Future studies will need to determine the impact of ZnO NPs on N_2O production in the presence of crops.

5. Conclusions

Our findings showed that ZnO NPs accelerated the release of N_2O from soils after C or N substrate amendment. The interaction of C and N caused the total N_2O emissions to substantially increase by 6.28–8.35 times compared with the control treatment without ZnO NPs. Moreover, this stimulatory effect was greater for soils with low ZnO NPs concentrations (100 mg kg^{-1}) than for soils with 500 and 1000 mg kg^{-1} ZnO NPs. Although ZnO NPs reduced the N_2O production rate by decreasing the abundances of AOA *amoA* functional genes, the increase in total N_2O emissions was mainly attributed to ZnO NPs increasing the abundances of functional genes related to AOB and nitrite reductase. This result indicated that ZnO NPs probably induced greater N_2O production than N_2O reduction and thus stimulated the release of N_2O from soils. Overall, our results demonstrate that the amendment of available C and N substrates probably results in a high risk of N_2O emissions from soils in the presence of ZnO NPs, contributing to global warming and ozone depletion.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agriculture11080730/s1, Figure S1: The change in N_2O concentration after all bottles were opened and placed in a fuming cupboard for 5 minutes. The N_2O concentration at 1 min indicated the initial concentration, Table S1: Primers and the thermal cycling conditions of functional genes related to N_2O.

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