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Effects of Corn Stalks and Urea on N₂O Production from Corn Field Soil

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Abstract: Returning corn stalks to the field is an important and widely used soil management practice which is conducive to the sustainable development of agriculture. In this study, the effects of corn stalks and urea on N₂O production in corn field soil were investigated through a 21-day incubation experiment. This study showed that increasing amounts of urea added to soil with a history of corn cultivation leads to increasing overall N₂O emissions, by increasing both the intensity and the duration of emissions. Although N₂O production was affected primarily by urea-derived NH₄⁺-N and NO₃⁻-N, its main source was native soil nitrogen, which accounted for 78.5 to 94.5% of N₂O. Returning corn stalk residue to the field reduced the production of N₂O, and the more urea was applied, the stronger the effect of corn residue on reducing N₂O emissions. Combining the application of corn stalks and urea could reduce the concentration of NH₄⁺-N and NO₃⁻-N derived from urea, and then reduce the substrate required for N₂O production in nitrification and denitrification processes. In addition, the combined application of corn stalks and urea could effectively inhibit the abundance of key N₂O-producing genes AOA *amoA*, *nirS* and *nirK*.

Keywords: N₂O; corn stalks; urea; ¹⁵N isotope; ammonium nitrogen; nitrate nitrogen



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1. Introduction

Mitigating negative global climate change caused by greenhouse gas (GHG) emissions is one of the major challenges in sustainable development [1,2]. Nitrous oxide (N₂O) is the third largest greenhouse gas [3], with a greenhouse effect 298 times greater than that of CO₂ on a 100-year scale [4], and a significant contributor to the destruction of the stratospheric ozone [5–7]. Agricultural soil is the main source of N₂O [8] and contributes approximately 60% of global anthropogenic N₂O emissions [9]. Therefore, a comprehensive understanding of N₂O emission from agricultural soils is crucial for the formulation of reasonable emission reduction strategies. However, most studies on N₂O emissions from agricultural soils have been conducted in temperate or humid ecosystems where water and nutrients are not scarce, while there are relatively few studies on N₂O production in arid areas [4,10]. As one of the world's largest agricultural countries, China produces 21% of the world's corn [11]. Liaoning Province is one of China's 13 main grain-producing areas, and the semi-arid area of northwestern Liaoning accounts for more than 2/3 of corn cultivation in this province [12]. This extensive area of cultivation is also an extensive area of N₂O production. Therefore, exploring the processes associated with N₂O production in corn fields in semi-arid northwestern Liaoning has important practical significance for farmland

greenhouse gas emission reduction. N_2O is produced mainly by microbial nitrification and denitrification processes, among which AOA *amoA* and AOB *amoA* are the key genes of N_2O production in the nitrification pathway, and *nirS* and *nirK* are the key genes of N_2O production in the denitrification pathway [4]. The determination of these genes helps us to better understand the pathway of N_2O production.

Application of nitrogen fertilizer is the main reason for the increase in N_2O emissions from farmland [13–15]. However, the application of nitrogen fertilizer is an important measure to ensure food security, so it is not feasible to reduce N_2O emissions from farmland simply by reducing the amount of nitrogen fertilizer [16]. In order to combat an increasing atmospheric N_2O concentration, other N_2O mitigation strategies are needed, one of which is to reduce N_2O emissions in farmland soil by changing soil properties through the return of corn stalk residue [17]. Corn is planted extensively in northwestern Liaoning Province; the yield of corn stalk is high, and it is a high-quality renewable organic resource [18]. Therefore, returning corn stalks to the field is an important means to promote the sustainable development of agriculture. However, northwestern Liaoning Province is also an important animal husbandry area, and corn stalks are one of the important feed sources, and it may be difficult to return the full amount of corn stalks to the field. At present, there are few studies on the impact of different amounts of corn stalk returning on N_2O emissions in semi-arid areas, and the impact of straw returning on N_2O emissions is still inconclusive. Due to the complexity of different soil types and conditions (soil pH, rainfall, temperature, etc.) [16], returning corn stalks to the field may promote the production of N_2O [19,20], but may also inhibit the production of N_2O [21] or have no effect [22,23]. Therefore, further exploring the effects of different amounts of corn stalks and nitrogen fertilizer on the N_2O production of cornfield soil in semi-arid areas will help to formulate more reasonable N_2O emission reduction measures.

2. Materials and Methods

2.1. Field Site

The field site was located at the National Agricultural Experimental Station for Agricultural Environment, Fuxin County, Liaoning province, China ($42^\circ 11' \text{ N}$, $121^\circ 70' \text{ E}$). The annual average temperature is $7\text{--}8^\circ \text{C}$, the annual average rainfall is about 300–500 mm, and the frost-free period is about 135–165 days. The test soil was a cinnamon soil (Hap-Ustic Luvisol in the FAOWRB system) (60.6% sand, 20.5% silt and 18.9% clay) with an organic matter content of 15.36 g kg^{-1} and a total N of 0.90 g kg^{-1} . Soil bulk density (0–20 cm) was 1.35 g cm^{-3} and the pH (H_2O) was 7.3. The farming system is corn planted once a year. The present experiment started after the corn harvest in the autumn of 2015. A split zone design was adopted, in which the main zone consisted of three rates of corn stalk return (3000 kg ha^{-1} (S_1), 6000 kg ha^{-1} (S_2) and 9000 kg ha^{-1} (S_3)), with this occurring in autumn. The subsurface urea (N 46%) application rates were included as well: 105 kg N ha^{-1} (N_1), 210 kg N ha^{-1} (N_2) and 420 kg N ha^{-1} (N_3). A control treatment (CK) consisted of no nitrogen fertilization and no corn stalk addition for a total of 10 treatments, namely CK, N_1S_1 , N_1S_2 , N_1S_3 , N_2S_1 , N_2S_2 , N_2S_3 , N_3S_1 , N_3S_2 and N_3S_3 . The area of each plot was 30 m^2 , with 3 replicates. Phosphate and potassium fertilizers were superphosphate and potassium sulfate, and the application rates were P_2O_5 150 kg ha^{-1} and K_2O 75 kg ha^{-1} , respectively. All fertilizers were applied at the time of planting in May, and no topdressing was carried out later. The corn variety “zhengdan 958” was planted with a planting density of $60,000 \text{ plants ha}^{-1}$. The cultivation mode was micro-area flat cropping, and the field management mode was carried out according to the local routine operation. Corn was harvested in late September every year, and straws were returned to the field immediately after harvest.

2.2. Incubation Experimental Design

In May 2020, five soil cores (20 cm in depth; drilled by soil auger) were randomly collected from each plot before corn planting and fertilization. The samples were composited, sieved (2 mm) and stored at 4 °C until used for incubation.

Before the start of the incubation experiment, the soil was pre-incubated and soil water content adjusted to 40% of the maximum field water holding capacity. Then, the soil was placed in an incubator at 25 °C for pre-incubation for 14 days to activate the soil microbial activity. Since corn stalks had already been returned to the field after the corn harvest in 2019, only urea was added in the incubation at rates equivalent to field rates (converted by 20 cm surface soil weight), these being 3.4 mg urea vial⁻¹ (N₁), 6.8 mg vial⁻¹ (N₂) and 13.6 mg vial⁻¹ (N₃), respectively. Three additional treatments (N₁, N₂ and N₃) were set up using CK soil for a total of 13 treatments, namely CK, N₁, N₁S₁, N₁S₂, N₁S₃, N₂, N₂S₁, N₂S₂, N₂S₃, N₃, N₃S₁, N₃S₂ and N₃S₃. The ¹⁵N content of the added urea was 98 at%. The incubation vials were made of glass, the volume of which was 110 mL, and each contained 40 g of soil (based on dry soil). The soil moisture content was adjusted to 55% of the maximum field water capacity during incubation. All vials were incubated at 25 °C for 21 days [24].

2.3. Gas and Soil Sampling Analysis

Soil NH₄⁺-N, NO₃⁻-N and N₂O were collected at 1, 2, 3, 5, 7, 10, 14 and 21 days after fertilization, respectively. N₂O concentration was analyzed with a gas chromatograph (Agilent 7890B, Gas Chromatograph, Wilmington, DE, USA). The N₂O accumulation was calculated by summing the products of the average of the N₂O accumulation of two adjacent single days by their interval time [10]. The content of ¹⁵N-N₂O was determined by a Gasbench-IRMS system (ThermoFisher, Waltham, MA, USA). The soil NH₄⁺-N and NO₃⁻-N were extracted with 2 mol L⁻¹ KCl solution [10], filtered, and analyzed with a continuous flow analyzer (AA3, Bran + Luebbe, Norderstedt, Germany). The extraction of soil ¹⁵N-NH₄⁺-N and ¹⁵N-NO₃⁻-N was as described in Yu et al. [25]. Soil ¹⁵N-NH₄⁺-N and ¹⁵N-NO₃⁻-N content were determined by a Stable Isotope Ratio Mass Spectrometer (253 MAT, Termo Finnigan, Bremen, Germany). According to the abundance of ¹⁵N in N₂O, NH₄⁺-N and NO₃⁻-N, the contribution of urea to N₂O accumulation, and the contribution of urea to total NH₄⁺-N and NO₃⁻-N were calculated [26,27]. Soil-derived N₂O, NH₄⁺-N and NO₃⁻-N were calculated as total N₂O, NH₄⁺-N and NO₃⁻-N minus urea-derived N₂O, NH₄⁺-N and NO₃⁻-N, respectively. The mean ¹⁵N content of atmospheric N₂O and soil (0.377 at% ¹⁵N) was deducted in the calculations.

2.4. DNA Extraction

After incubation, soil DNA was extracted using the MoBio Powersoil DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA, USA). The abundances of AOA *amoA*, AOB *amoA*, *nirS* and *nirK* genes were determined by quantitative PCR (qPCR) on an ABI 7500 system (Applied Biosystems, Waltham, MA, USA). The primers listed and the qPCR thermal profile are shown in Supplementary Materials Table S1. The reaction mixture contained 0.5 µL primers, 2 µL DNA template, 7 µL deionized water and 10 µL 2 × Taq Plus Master Mix. All qPCR reactions were performed by melting curve analysis and 1% agarose gel electrophoresis to confirm the amplification of specific products. Three parallel qPCR repeats were performed.

2.5. Statistical Analysis

SPSS Statistics 16.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis of data. One-way ANOVA was used for testing the treatment effects with Duncan ($\alpha = 0.05$). Univariate analysis of variance was used to analyze the response of N₂O accumulation, soil inorganic nitrogen and gene abundance to corn stalk and nitrogen fertilizer application. Pearson correlation analysis was used for the correlation between N₂O accumulation, soil

inorganic nitrogen and gene abundance. The data in the figures and tables are the average value $\pm$ standard error.

3. Results

3.1. N_2O Flux

During the incubation period, the N_2O flux of the CK treatment was always at a low level, and the N_2O flux of all other treatments was greater than CK (Figure 1). It also can be seen from Figure 1 that the flux of N_2O increases with the increase in nitrogen application rate. At the N_1 level, the N_2O flux peaked on the second day after fertilization, while at the N_2 and N_3 levels, the N_2O flux peak appeared on the third and fifth days after fertilization, respectively. At the N_1 and N_2 levels, N_2O flux decreased to a lower level one week after fertilization, while at the N_3 level, it decreased to a lower level two weeks after fertilization. The combined application of corn stalks and urea at N_1 , N_2 and N_3 levels all reduced N_2O flux.

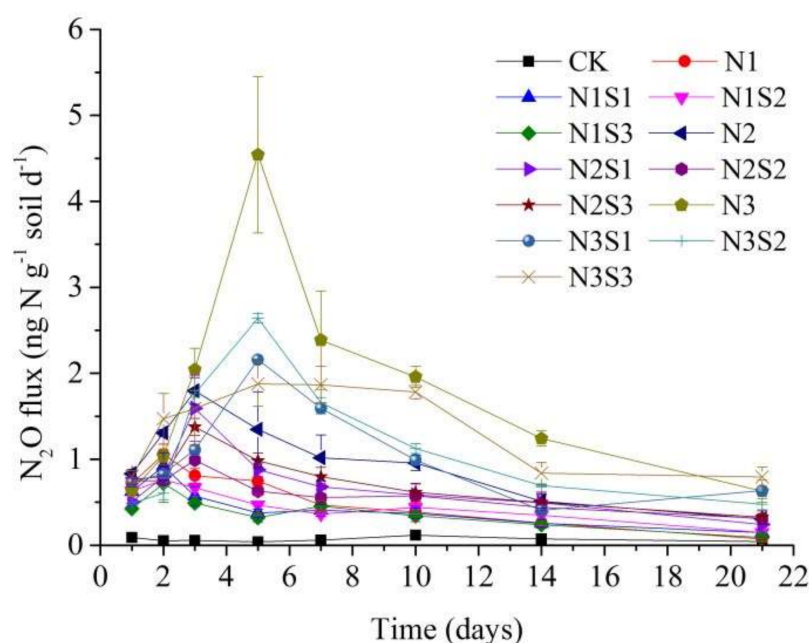


Figure 1. Effects of different treatments on N_2O flux within 21 days of incubation. Bars are $\pm$ one standard errors of means ($n = 3$).

3.2. N_2O Accumulation

As shown in Table 1, the total N_2O , urea-derived N_2O and soil-derived N_2O of all treatments were greater than those in CK. Under urea application, urea-derived N_2O accounted for 5.4–21.4% of the total N_2O , while soil-derived N_2O accounted for 78.6–94.6% of the total N_2O .

Under the N_1 application rate of urea, the addition of corn stalk residue had no significant effect on the accumulation of total N_2O , urea-derived N_2O and soil-derived N_2O . At the N_2 level, the accumulation of total N_2O , urea-derived N_2O and soil-derived N_2O were increased by urea application, but the addition of residue in this case reduced the accumulation of total N_2O , urea-derived N_2O and soil-derived N_2O ; however, this reduction was not affected by the amount of residue added. At the N_3 level, accumulation of total N_2O , urea-derived N_2O and soil-derived N_2O was the highest of the three rates of urea addition; the addition of residue reduced these three measurements, but in the case of N_3 , this reduction was smaller as more residue was added. Under a given amount of residue added (0, S_1 , S_2 or S_3), total N_2O , urea-derived N_2O and soil-derived N_2O increased with an increase in urea application rate.

Table 1. Effects of different treatments on total N₂O accumulation, urea-derived N₂O accumulation and soil-derived N₂O accumulation after 21 days of incubation.

Treatments	Total N ₂ O	Urea-Derived N ₂ O	%	Soil-Derived N ₂ O	%
	ng N g ⁻¹ Soil	ng N g ⁻¹ Soil		ng N g ⁻¹ Soil	
CK	1.43 ± 0.12 h	—	—	1.43 ± 0.12 h	100.00%
N ₁	8.72 ± 0.24 fg	0.77 ± 0.08 e	8.77%	7.96 ± 0.17 fg	91.23%
N ₁ S ₁	7.46 ± 1.06 g	0.48 ± 0.10 e	6.41%	6.98 ± 0.96 g	93.59%
N ₁ S ₂	8.30 ± 0.44 fg	0.59 ± 0.05 e	7.06%	7.71 ± 0.40 g	92.94%
N ₁ S ₃	6.54 ± 0.50 g	0.35 ± 0.05 e	5.42%	6.18 ± 0.45 g	94.58%
N ₂	17.47 ± 0.92 d	2.20 ± 0.20 d	12.57%	15.28 ± 0.73 d	87.43%
N ₂ S ₁	12.90 ± 1.33 e	1.05 ± 0.11 e	8.15%	11.85 ± 1.22 e	91.85%
N ₂ S ₂	11.31 ± 0.23 ef	0.94 ± 0.02 e	8.30%	10.37 ± 0.24 ef	91.70%
N ₂ S ₃	13.79 ± 1.14 e	1.36 ± 0.14 de	9.85%	12.43 ± 1.03 e	90.15%
N ₃	35.99 ± 2.85 a	7.70 ± 1.15 a	21.41%	28.29 ± 1.88 a	78.59%
N ₃ S ₁	19.78 ± 0.15 cd	2.28 ± 0.03 d	11.52%	17.50 ± 0.13 cd	88.48%
N ₃ S ₂	22.81 ± 0.66 c	3.39 ± 0.04 c	14.86%	19.42 ± 0.70 c	85.14%
N ₃ S ₃	27.10 ± 0.88 b	4.48 ± 0.30 b	16.52%	22.62 ± 0.69 b	83.48%

Different lowercase letters within treatments indicate significant differences ($p < 0.05$).

3.3. Soil Inorganic Nitrogen

After the application of urea and corn stalks, the total NH₄⁺-N and urea-derived NH₄⁺-N increased rapidly, then slowly decreased, and fell to a lower level by 21 days (Figure 2A,C), while the soil-derived NH₄⁺-N was always at a lower level (Figure 2E). With the increase in urea application rate, the peak value of total NH₄⁺-N and urea-derived NH₄⁺-N shifted backward, and the content of both NH₄⁺-N increased (Figure 2A,C). The addition of corn stalks significantly delayed the peak value of total NH₄⁺-N and urea-derived NH₄⁺-N, and a small addition of corn stalk had a stronger effect on reducing the content of NH₄⁺-N at the N₃ level (Figure 2A,C). The total NH₄⁺-N was mainly derived from urea (Figure 2A,C,E).

After the application of urea and corn stalks, the total NO₃⁻-N and urea-derived NO₃⁻-N increased rapidly, reaching the maximum on the 21st day (Figure 2B,D), while the soil-derived NO₃⁻-N remained almost unchanged (Figure 2F). With the increase in urea application rate, total NO₃⁻-N and urea-derived NO₃⁻-N increased significantly (Figure 2B,D), while the soil-derived NO₃⁻-N increased only slightly (Figure 2F). After corn stalk addition, the total NO₃⁻-N content increased slightly under all nitrogen addition levels (Figure 2B). The total NO₃⁻-N is mainly derived from urea (Figure 2B,D,F).

3.4. Abundance of Key N₂O-Producing Genes

After the 21-day incubation period, at the N₁ level of urea addition, compared with urea alone, the application of corn stalk residue had almost no effect on the AOA *amoA* gene, but increased the AOB *amoA* gene and decreased the *nirS* and *nirK* genes (Figure 3A–D). At the N₂ level, compared with urea alone, corn stalk application decreased the AOA *amoA* and *nirK* genes, increased the AOB *amoA* and *nirS* genes of N₂S₁ and decreased the *nirS* gene of N₂S₂. At the N₃ level, compared with urea alone, corn stalk application reduced the AOA *amoA* and *nirK* genes, increased the AOB *amoA* genes of N₃S₁ and N₃S₃ and reduced the *nirS* gene of N₃S₁.

After 21 days of incubation, different levels of residue application had different effects on gene abundance under different levels of urea application (Figure 3A–D). When urea was applied without corn stalk, AOA *amoA*, *nirS* and *nirK* genes increased with the increase of urea application amount, while the AOB *amoA* gene increased and then decreased. At the S₁ level, with the increase in urea application, the AOA *amoA* gene first decreased and then increased, and the AOB *amoA*, *nirS* and *nirK* genes first increased and then decreased. At the S₂ level, with the increase in urea application, the AOA *amoA*, *nirS* and *nirK* genes increased, and the AOB *amoA* gene first increased and then decreased. At the S₃ level, with

the increase in urea application, the AOA *amoA* gene remained basically unchanged, the AOB *amoA* gene first decreased and then increased and the *nirS* and *nirK* genes increased.

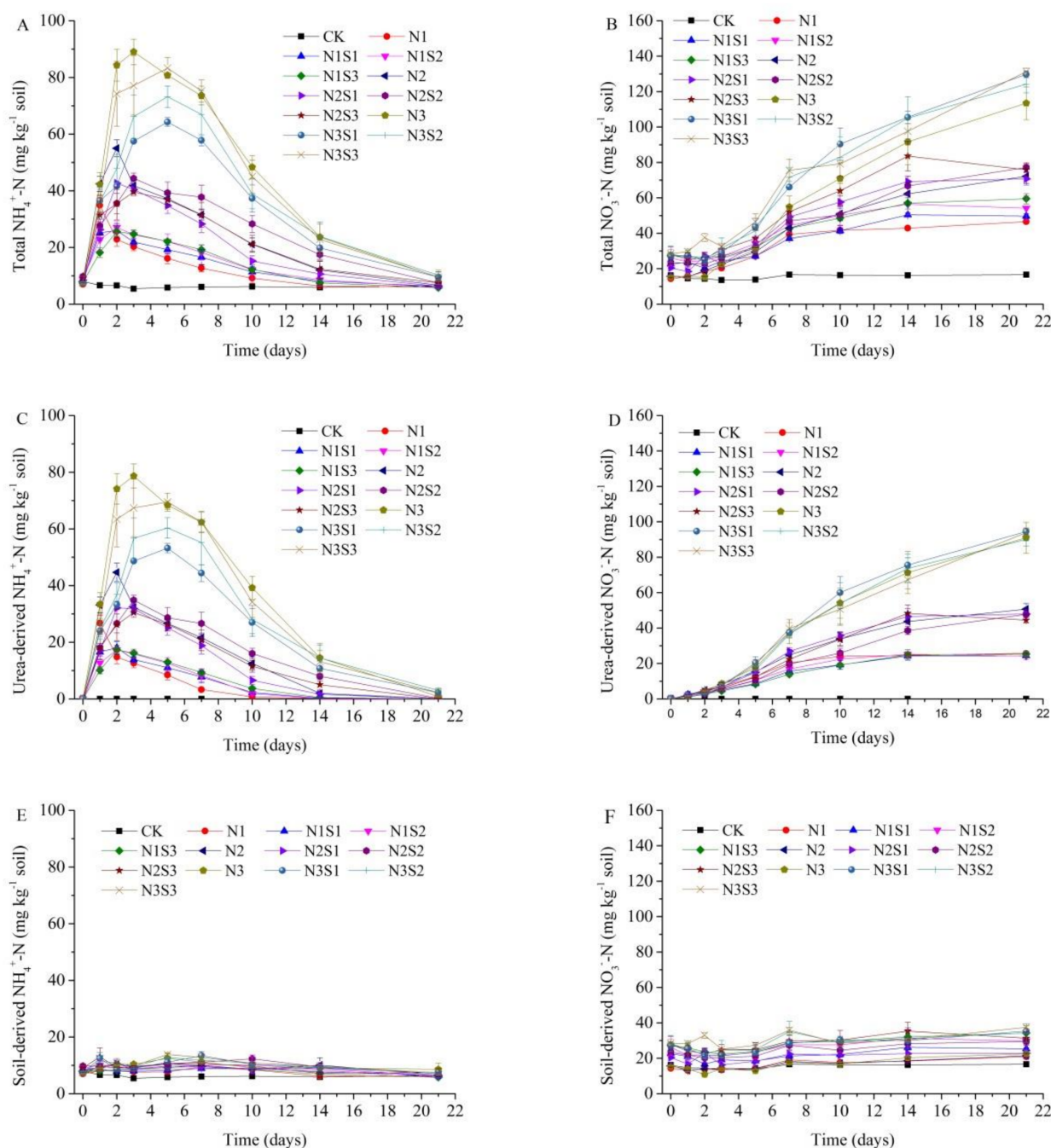


Figure 2. Effects of different treatments on the contents of total $\text{NH}_4^+\text{-N}$ (A) and $\text{NO}_3^-\text{-N}$ (B), urea-derived $\text{NH}_4^+\text{-N}$ (C) and $\text{NO}_3^-\text{-N}$ (D), and soil-derived $\text{NH}_4^+\text{-N}$ (E) and $\text{NO}_3^-\text{-N}$ (F) within 21 days of incubation. Bars are $\pm$ one standard errors of means ($n = 3$).

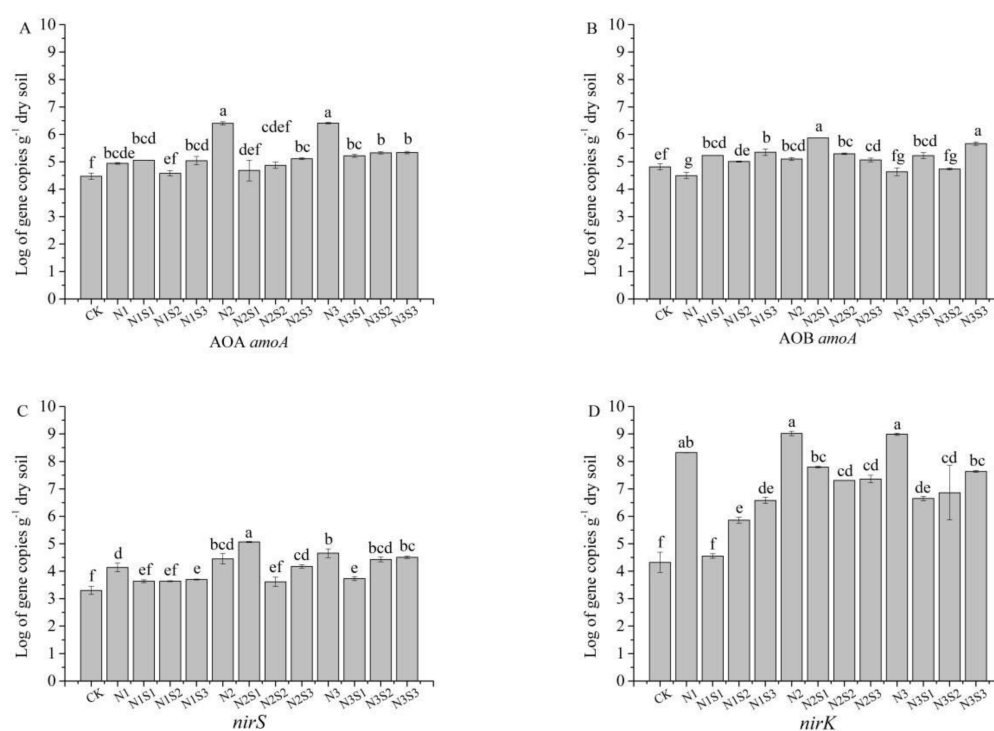


Figure 3. Effects of different treatments on AOA *amoA* (A), AOB *amoA* (B), *nirS* (C) and *nirK* gene, (D) copy number after 21 days of incubation. Bars are $\pm$ one standard errors of means ($n = 3$). Different letters indicate significant difference ($p < 0.05$).

3.5. Pearson Correlation Analysis of N_2O , Soil Properties and Gene Abundance

Total N_2O , urea-derived N_2O and soil-derived N_2O were all significantly positively correlated with other indexes except soil-derived NO_3^- -N and AOB *amoA* gene (Table 2). Total NH_4^+ -N and urea-derived NH_4^+ -N were all significantly positively correlated with other indexes, except soil-derived NO_3^- -N and AOB *amoA* gene. Total NO_3^- -N, urea-derived NO_3^- -N and soil-derived NH_4^+ -N were all significantly positively correlated with other indexes, except the AOB *amoA* gene. Soil-derived NO_3^- -N was significantly positively correlated with total NO_3^- -N, urea-derived NO_3^- -N, soil-derived NH_4^+ -N and the AOB *amoA* gene. AOA *amoA*, *nirS* and *nirK* genes were positively correlated with each other. AOB *amoA* had no significant correlation with other genes.

Table 2. Pearson correlation analysis of N_2O , soil properties and gene abundance after 21 days of incubation.

	A	B	C	D	E	F	G	H	I	J	K	L	M
A	1												
B	0.959 **	1											
C	0.997 **	0.932 **	1										
D	0.915 **	0.836 **	0.924 **	1									
E	0.796 **	0.662 **	0.823 **	0.828 **	1								
F	0.926 **	0.856 **	0.932 **	0.997 **	0.823 **	1							
G	0.891 **	0.778 **	0.91 **	0.865 **	0.953 **	0.867 **	1						
H	0.612 **	0.481 **	0.641 **	0.785 **	0.665 **	0.735 **	0.64 **	1					
I	0.138	0.017	0.171	0.302	0.607 **	0.283	0.337 *	0.387 *	1				
J	0.711 **	0.698 **	0.704 **	0.58 **	0.38 *	0.582 **	0.525 **	0.424 **	−0.196	1			
K	−0.037	−0.142	−0.006	0.037	0.205	0.016	0.122	0.191	0.316 *	−0.221	1		
L	0.611 **	0.546 **	0.62 **	0.498 **	0.47 **	0.497 **	0.567 **	0.385 *	−0.027	0.434 **	0.2	1	
M	0.589 **	0.529 **	0.598 **	0.509 **	0.396 *	0.506 **	0.489 **	0.408 **	−0.051	0.604 **	−0.035	0.686 **	1

The value in the table is the F value, ** indicates that the correlation is significant at $p < 0.01$; * indicates that the correlation is significant at $p < 0.05$. A: Total N_2O ; B: urea-derived N_2O ; C: soil-derived N_2O ; D: total NH_4^+ -N; E: total NO_3^- -N; F: urea-derived NH_4^+ -N; G: urea-derived NO_3^- -N; H: soil-derived NH_4^+ -N; I: soil-derived NO_3^- -N; J: AOA *amoA*; K: AOB *amoA*; L: *nirS*; M: *nirK*.

3.6. Comprehensive Effects of the Combined Application of Residue and Urea on N_2O , Soil Inorganic Nitrogen and Gene Abundance

Corn stalks, urea and their interaction were significantly correlated with total N_2O , urea-derived N_2O , soil-derived N_2O , AOA *amoA*, AOB *amoA*, *nirS* and *nirK* genes (Table 3). The rate of urea application had the greatest impact on total N_2O , urea-derived N_2O , soil-derived N_2O and the *nirS* gene, while the rate of residue application had the greatest impact on AOA *amoA*, AOB *amoA* and *nirK* genes. Corn stalk application was significantly related to total NH_4^+-N , total NO_3^--N , urea-derived NH_4^+-N and soil-derived NO_3^--N , and corn stalk application had the greatest impact on soil-derived NO_3^--N . Urea application was significantly related to total NH_4^+-N , total NO_3^--N , urea-derived NH_4^+-N , urea-derived NO_3^--N and soil-derived NH_4^+-N and had the greatest impact on these measurements. The interaction between corn stalks and urea was significantly related to total NH_4^+-N and urea-derived NH_4^+-N .

Table 3. The response of N_2O , soil properties and gene abundance to corn stalk residue and nitrogen urea application after 21 days of incubation.

	A	B	C	D	E	F	G	H	I	J	K	L	M
Residue	26 ***	24 ***	21 ***	4 *	4 *	6 **	0	1	14 ***	37 ***	52 ***	12 ***	40 ***
Urea	290 ***	135 ***	298 ***	266 ***	81 ***	373 ***	110 ***	12 ***	2	26 ***	19 ***	36 ***	30 ***
Residue $\times$ Urea	10 ***	11 ***	8 ***	3 *	1	5 **	0	0	0	10 ***	16 ***	20 ***	4 **

The value in the table is the F value, *** indicates that the correlation is significant at $p < 0.001$; ** indicates that the correlation is significant at $p < 0.01$; * indicates that the correlation is significant at $p < 0.05$. A: Total N_2O ; B: urea-derived N_2O ; C: soil-derived N_2O ; D: total NH_4^+-N ; E: total NO_3^--N ; F: urea-derived NH_4^+-N ; G: urea-derived NO_3^--N ; H: soil-derived NH_4^+-N ; I: soil-derived NO_3^--N ; J: AOA *amoA*; K: AOB *amoA*; L: *nirS*; M: *nirK*.

4. Discussion

The N_2O fluxes of all treatments increased rapidly and were all higher than that of CK after the application of urea, and then decreased slowly, indicating that the application of urea could promote the production of N_2O , similar to previous studies [27–29]. This was mainly due to the rapid increase in soil mineral nitrogen after urea application (Figure 2A,B) [29]. With the increase in urea application, the appearance of the N_2O peak was delayed, its intensity increased, and the N_2O flux lasted longer (Figure 1). The possible reason was that as the amount of urea increased, the content of mineral nitrogen used for nitrification and denitrification in the soil increased [16], but the initially high NH_4^+-N concentration had a toxic effect on soil nitrifying bacteria [30], thereby inhibiting the appearance time of the N_2O peak, but when the amount of NH_4^+-N subsided, this phenomenon was alleviated [31].

Regardless of how much corn stalk residue was added to soil, the higher the amount of urea, the higher the accumulation of N_2O , and the faster the increase of N_2O with the increase in nitrogen (Table 1), similar to the exponential increase of N_2O with the increase in N observed by Hoben et al. [32]. However, Chen et al. [33] believed that when the nitrogen application rate was greater than 900 mg N kg^{-1} , N_2O would not continue to increase due to the limitation of high ammonium concentrations; perhaps the nitrogen application rate in our experiment did not reach such a maximum threshold value. The production of N_2O was significantly positively correlated with the content of NH_4^+-N and NO_3^--N in the soil (Table 2) [19], indicating that ammonia oxidation and denitrification occurred simultaneously in the soil during the incubation period [29]. The significant positive correlation between N_2O production and AOA *amoA*, *nirS* and *nirK* in this experiment also supports this point. AOA *amoA* is the key gene of N_2O production in the nitrification pathway, and *nirS* and *nirK* are the key genes of N_2O production in the denitrification pathway [4]. Among them, ammonia oxidation may be the main pathway of N_2O production. The production of N_2O was most strongly correlated with the content of NH_4^+-N ; moreover, the high sand content in the experimental soil was conducive to the production of N_2O by nitrification [34]. In addition, the presence of corn stalks and collecting N_2O samples after sealing for 24 h may have increased

oxygen consumption [34,35], thus underestimating the N_2O produced by the ammonia oxidation process. This was different from the study of Hink et al. [36], who believed that the N_2O produced by denitrification in 60% water-filled pore space could be ignored. N_2O production in the present study was mainly affected by urea-derived $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$ (Table 2; Figure 2), but mainly came from the soil-derived $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$ (78.6–94.6%; Table 1), which was similar to the results of previous studies [27,37,38]. It may be that $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$ derived from urea are easier to be used by microorganisms compared to native soil N, thus promoting an increase in the number of microorganisms, accelerating the mineralization of soil nitrogen [20,27] and ultimately making soil N the main source of N_2O . The significant positive correlation between N_2O production and AOA *amoA* in this study also supports this view (Table 2), because AOA produces N_2O resulting from mineralized ammonia [4,36]. However, our experiment cannot distinguish between soil-derived N_2O and corn stalk-derived N_2O .

Compared with nitrogen application alone, low nitrogen (105 kg N ha^{-1}) combined with application of corn stalks had little effect on N_2O accumulation, while medium nitrogen (210 kg N ha^{-1}) and high nitrogen (420 kg N ha^{-1}) combined with application of corn stalks reduced overall N_2O accumulation. This may be because the soil used for the incubation experiment was deficient in nitrogen, and the input of a high C:N residue increased the demand for nitrogen by microorganisms, accelerating the immobilization of mineral nitrogen [34], and thereby reducing the production of N_2O . Chen et al. [33] and Shi et al. [39] believed that the production of N_2O in nitrogen-limited soil is mainly affected by AOA rather than AOB. Our research also found that the production of N_2O in soil is significantly positively correlated with the AOA *amoA* gene. Higher soil nitrogen content was not conducive to the growth and breeding of AOA [39], which further proved that corn stalks combined with urea may aggravate soil nitrogen deficiency. The reduction in N_2O emissions was more effective when high nitrogen (420 kg N ha^{-1}) was combined with a low amount (3000 kg ha^{-1}) of residue. This may be because the dissolved organic carbon (DOC) content in the soil increased with an increase in the corn stalk application, which accelerated denitrification [20,29]. This was also indicated by the observation that *nirS* and *nirK* genes (the key functional genes for N_2O production in the denitrification pathway [4]) were least abundant in the N_3S_1 treatment (Figure 3C,D).

This study also has some shortcomings. The field location experiment time is relatively short, and this study was an incubation experiment. The urea nitrogen content gradient is obvious, the temperature and water content are constant, while actual field conditions are dynamic [33]. In the future, it is necessary to explore the comprehensive effects of long-term combined application of different amounts of corn stalks and urea on N_2O emissions in the semi-arid region of northwestern Liaoning based on actual field conditions.

5. Conclusions

This study showed that under the incubation conditions used here, application of urea was the main cause of N_2O production, which increased with an increase in urea dosage. An increase in urea application delays the emergence of the N_2O emission peak and increases the time of N_2O generation. The production of N_2O is mainly affected by urea-derived $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$, but the main source of N_2O is soil nitrogen itself, accounting for 78.6–94.6%. Returning corn stalks to the field will reduce the production of N_2O . The N_2O production reduction effect is strongest when a large amount of urea (420 kg ha^{-1}) is applied, and with this high urea application, a small return of corn stalks (3000 kg ha^{-1}) to the field has the best N_2O emission reduction effect. The combined application of corn stalks and urea mainly affects N_2O production by changing the concentration of urea-derived $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$ and affecting the abundance of AOA *amoA*, *nirS* and *nirK* genes. In the future, exploring the contribution of ammonia oxidation and denitrification pathways to N_2O production and the role of functional genes related to N_2O production when different amounts of corn stalks and nitrogen fertilizer are combined are essential for formulating reasonable sustainable agricultural development strategies.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/agronomy11102009/s1>, Table S1: Primer sequences of some key N cycling genes used for real time PCR.

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