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

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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$_2$O is produced mainly by microbial nitrification and denitrification processes, among which AOA amoA and AOB amoA are the key genes of N$_2$O production in the nitrification pathway, and nirS and nirK are the key genes of N$_2$O production in the denitrification pathway [4]. The determination of these genes helps us to better understand the pathway of N$_2$O production.

Application of nitrogen fertilizer is the main reason for the increase in N$_2$O 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$_2$O emissions from farmland simply by reducing the amount of nitrogen fertilizer [16]. In order to combat an increasing atmospheric N$_2$O concentration, other N$_2$O mitigation strategies are needed, one of which is to reduce N$_2$O 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$_2$O emissions in semi-arid areas, and the impact of straw returning on N$_2$O 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$_2$O [19,20], but may also inhibit the production of N$_2$O [21] or have no effect [22,23]. Therefore, further exploring the effects of different amounts of corn stalks and nitrogen fertilizer on the N$_2$O production of cornfield soil in semi-arid areas will help to formulate more reasonable N$_2$O 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°11′ N, 121°70′ E). The annual average temperature is 7–8 °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$_2$O) 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$_1$S$_1$, N$_1$S$_2$, N$_1$S$_3$, N$_2$S$_1$, N$_2$S$_2$, N$_2$S$_3$, N$_3$S$_1$, N$_3$S$_2$ and N$_3$S$_3$. 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$_2$O$_5$ 150 kg ha$^{-1}$ and K$_2$O 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 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, 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 (α = 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 ± standard error.

3. Results

3.1. \( \text{N}_2\text{O} \) Flux

During the incubation period, the \( \text{N}_2\text{O} \) flux of the CK treatment was always at a low level, and the \( \text{N}_2\text{O} \) flux of all other treatments was greater than CK (Figure 1). It also can be seen from Figure 1 that the flux of \( \text{N}_2\text{O} \) increases with the increase in nitrogen application rate. At the \( N_1 \) level, the \( \text{N}_2\text{O} \) flux peaked on the second day after fertilization, while at the \( N_2 \) and \( N_3 \) levels, the \( \text{N}_2\text{O} \) flux peak appeared on the third and fifth days after fertilization, respectively. At the \( N_1 \) and \( N_2 \) levels, \( \text{N}_2\text{O} \) 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 \( \text{N}_2\text{O} \) flux.

![Figure 1](image-url)

**Figure 1.** Effects of different treatments on \( \text{N}_2\text{O} \) flux within 21 days of incubation. Bars are ± one standard errors of means (\( n = 3 \)).

3.2. \( \text{N}_2\text{O} \) Accumulation

As shown in Table 1, the total \( \text{N}_2\text{O} \), urea-derived \( \text{N}_2\text{O} \) and soil-derived \( \text{N}_2\text{O} \) of all treatments were greater than those in CK. Under urea application, urea-derived \( \text{N}_2\text{O} \) accounted for 5.4–21.4% of the total \( \text{N}_2\text{O} \), while soil-derived \( \text{N}_2\text{O} \) accounted for 78.6–94.6% of the total \( \text{N}_2\text{O} \).

Under the \( N_1 \) application rate of urea, the addition of corn stalk residue had no significant effect on the accumulation of total \( \text{N}_2\text{O} \), urea-derived \( \text{N}_2\text{O} \) and soil-derived \( \text{N}_2\text{O} \). At the \( N_2 \) level, the accumulation of total \( \text{N}_2\text{O} \), urea-derived \( \text{N}_2\text{O} \) and soil-derived \( \text{N}_2\text{O} \) were increased by urea application, but the addition of residue in this case reduced the accumulation of total \( \text{N}_2\text{O} \), urea-derived \( \text{N}_2\text{O} \) and soil-derived \( \text{N}_2\text{O} \); however, this reduction was not affected by the amount of residue added. At the \( N_3 \) level, accumulation of total \( \text{N}_2\text{O} \), urea-derived \( \text{N}_2\text{O} \) and soil-derived \( \text{N}_2\text{O} \) 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 \( \text{N}_2\text{O} \), urea-derived \( \text{N}_2\text{O} \) and soil-derived \( \text{N}_2\text{O} \) increased with an increase in urea application rate.
Table 1. Effects of different treatments on total N<sub>2</sub>O accumulation, urea-derived N<sub>2</sub>O accumulation and soil-derived N<sub>2</sub>O accumulation after 21 days of incubation.

| Treatments  | Total N<sub>2</sub>O ng N g<sup>-1</sup> Soil | Urea-Derived N<sub>2</sub>O ng N g<sup>-1</sup> Soil | % | Soil-Derived N<sub>2</sub>O ng N g<sup>-1</sup> Soil | % |
|------------|---------------------------------|---------------------------------|---|-------------------------------|---|
| CK         | 1.43 ± 0.12 h                   | —                               | — | 1.43 ± 0.12 h                 | 100.00% |
| N<sub>1</sub> | 8.72 ± 0.24 fg                  | 0.77 ± 0.08 e                   | 8.77% | 7.96 ± 0.17 fg                 | 91.23% |
| N<sub>1</sub>S<sub>1</sub> | 7.46 ± 1.06 g                  | 0.48 ± 0.10 e                   | 6.41% | 6.98 ± 0.96 g                 | 93.59% |
| N<sub>1</sub>S<sub>2</sub> | 8.30 ± 0.44 fg                  | 0.59 ± 0.05 e                   | 7.06% | 7.71 ± 0.40 g                 | 92.94% |
| N<sub>1</sub>S<sub>3</sub> | 6.54 ± 0.50 g                  | 0.35 ± 0.05 e                   | 5.42% | 6.18 ± 0.45 g                 | 94.58% |
| N<sub>2</sub> | 17.47 ± 0.92 d                  | 2.20 ± 0.20 d                  | 12.57% | 15.28 ± 0.73 d                | 87.43% |
| N<sub>2</sub>S<sub>1</sub> | 12.90 ± 1.33 e                  | 1.05 ± 0.11 e                  | 8.15% | 11.85 ± 1.22 e                | 91.85% |
| N<sub>2</sub>S<sub>2</sub> | 11.31 ± 0.23 ef                 | 0.94 ± 0.02 e                  | 8.30% | 10.37 ± 0.24 ef               | 91.70% |
| N<sub>2</sub>S<sub>3</sub> | 13.79 ± 1.14 e                  | 1.36 ± 0.14 de                 | 9.85% | 12.43 ± 1.03 e               | 90.15% |
| N<sub>3</sub> | 35.99 ± 2.85 a                  | 7.70 ± 1.15 a                  | 21.41% | 28.29 ± 1.88 a               | 78.59% |
| N<sub>3</sub>S<sub>1</sub> | 19.78 ± 0.15 cd                 | 2.28 ± 0.03 d                  | 11.52% | 17.50 ± 0.13 cd                | 88.48% |
| N<sub>3</sub>S<sub>2</sub> | 22.81 ± 0.66 c                  | 3.39 ± 0.04 c                  | 14.86% | 19.42 ± 0.70 c               | 85.14% |
| N<sub>3</sub>S<sub>3</sub> | 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 NH<sub>4</sub><sup>+</sup>-N and urea-derived NH<sub>4</sub><sup>+</sup>-N increased rapidly, then slowly decreased, and fell to a lower level by 21 days (Figure 2A,C), while the soil-derived NH<sub>4</sub><sup>+</sup>-N was always at a lower level (Figure 2E). With the increase in urea application rate, the peak value of total NH<sub>4</sub><sup>+</sup>-N and urea-derived NH<sub>4</sub><sup>+</sup>-N shifted backward, and the content of both NH<sub>4</sub><sup>+</sup>-N increased (Figure 2A,C). The addition of corn stalks significantly delayed the peak value of total NH<sub>4</sub><sup>+</sup>-N and urea-derived NH<sub>4</sub><sup>+</sup>-N, and a small addition of corn stalk had a stronger effect on reducing the content of NH<sub>4</sub><sup>+</sup>-N at the N<sub>3</sub> level (Figure 2A,C). The total NH<sub>4</sub><sup>+</sup>-N was mainly derived from urea (Figure 2A,C,E).

After the application of urea and corn stalks, the total NO<sub>3</sub><sup>-</sup>-N and urea-derived NO<sub>3</sub><sup>-</sup>-N increased rapidly, reaching the maximum on the 21st day (Figure 2B,D), while the soil-derived NO<sub>3</sub><sup>-</sup>-N remained almost unchanged (Figure 2F). With the increase in urea application rate, total NO<sub>3</sub><sup>-</sup>-N and urea-derived NO<sub>3</sub><sup>-</sup>-N increased significantly (Figure 2B,D), while the soil-derived NO<sub>3</sub><sup>-</sup>-N increased only slightly (Figure 2F). After corn stalk addition, the total NO<sub>3</sub><sup>-</sup>-N content increased slightly under all nitrogen addition levels (Figure 2B). The total NO<sub>3</sub><sup>-</sup>-N is mainly derived from urea (Figure 2B,D,F).

3.4. Abundance of Key N<sub>2</sub>O-Producing Genes

After the 21-day incubation period, at the N<sub>1</sub> 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 N<sub>2</sub> level, compared with urea alone, corn stalk application decreased the AOA amoA and nirK genes, increased the AOB amoA and nirS genes of N<sub>2</sub>S<sub>1</sub> and decreased the nirS gene of N<sub>2</sub>S<sub>2</sub>. At the N<sub>3</sub> level, compared with urea alone, corn stalk application reduced the AOA amoA and nirK genes, increased the AOB amoA genes of N<sub>3</sub>S<sub>1</sub> and N<sub>3</sub>S<sub>3</sub> and reduced the nirS gene of N<sub>3</sub>S<sub>1</sub>.

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 S<sub>1</sub> 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 S<sub>2</sub> 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 S<sub>3</sub> 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 NH$_4^+$-N (A) and NO$_3^-$-N (B), urea-derived NH$_4^+$-N (C) and NO$_3^-$-N (D), and soil-derived NH$_4^+$-N (E) and NO$_3^-$-N (F) within 21 days of incubation. Bars are ± 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 ± one standard errors of means (n = 3). Different letters indicate significant difference (p < 0.05).

3.5. Pearson Correlation Analysis of N₂O, Soil Properties and Gene Abundance

Total N₂O, urea-derived N₂O and soil-derived N₂O were all significantly positively correlated with other indexes except soil-derived NO₃⁻-N and AOB amoA gene (Table 2). Total NH₄⁺-N and urea-derived NH₄⁺-N were all significantly positively correlated with other indexes, except soil-derived NO₃⁻-N and AOB amoA gene. Total NO₃⁻-N, urea-derived NO₃⁻-N and soil-derived NH₄⁺-N were all significantly positively correlated with other indexes, except the AOB amoA gene. Soil-derived NO₃⁻-N was significantly positively correlated with total NO₃⁻-N, urea-derived NO₃⁻-N, soil-derived NH₄⁺-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 N₂O, 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 ** | 0.825 ** | 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 ** | 0.385 * | 0.385 * | 0.385 * | 0.385 * | 0.385 * | 1     |
| K      | -0.037 | -0.142 | -0.006 | 0.037 | 0.205 | 0.016 | 0.122 | 0.191 | 0.316 * | -0.023 | 0.604 ** | -0.035 | 0.686 ** | 1     |
| L      | 0.611 ** | 0.546 ** | 0.62 ** | 0.498 ** | 0.47 ** | 0.497 ** | 0.567 ** | 0.385 * | -0.027 | 0.434 ** | 0.2     | 0.2     | 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 N₂O; B: urea-derived N₂O; C: soil-derived N₂O; D: total NH₄⁺-N; E: total NO₃⁻-N; F: urea-derived NH₄⁺-N; G: urea-derived NO₃⁻-N; H: soil-derived NH₄⁺-N; I: soil-derived NO₃⁻-N; J: AOA amoA; K: AOB amoA; L: nirS; M: nirK.
3.6. Comprehensive Effects of the Combined Application of Residue and Urea on N₂O, Soil Inorganic Nitrogen and Gene Abundance

Corn stalks, urea and their interaction were significantly correlated with total N₂O, urea-derived N₂O, soil-derived N₂O, AOA amoA, AOB amoA, nirS and nirK genes (Table 3). The rate of urea application had the greatest impact on total N₂O, urea-derived N₂O, soil-derived N₂O 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 NH₄⁺-N, total NO₃⁻-N, urea-derived NH₄⁺-N and soil-derived NO₃⁻-N, and corn stalk application had the greatest impact on soil-derived NO₃⁻-N. Urea application was significantly related to total NH₄⁺-N, total NO₃⁻-N, urea-derived NH₄⁺-N, urea-derived NO₃⁻-N and soil-derived NH₄⁺-N and had the greatest impact on these measurements. The interaction between corn stalks and urea was significantly related to total NH₄⁺-N and urea-derived NH₄⁺-N.

Table 3. The response of N₂O, 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 × Urea | 10 *** | 11 *** | 8 *** | 5 * | 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 N₂O; B: urea-derived N₂O; C: soil-derived N₂O; D: total NH₄⁺-N; E: total NO₃⁻-N; F: urea-derived NH₄⁺-N; G: urea-derived NO₃⁻-N; H: soil-derived NH₄⁺-N; I: soil-derived NO₃⁻-N; J: AOA amoA; K: AOB amoA; L: nirS; M: nirK.

4. Discussion

The N₂O 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 N₂O, 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 N₂O peak was delayed, its intensity increased, and the N₂O 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 NH₄⁺-N concentration had a toxic effect on soil nitrifying bacteria [30], thereby inhibiting the appearance time of the N₂O peak, but when the amount of NH₄⁺-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 N₂O, and the faster the increase of N₂O 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, N₂O 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 N₂O was significantly positively correlated with the content of NH₄⁺-N and NO₃⁻-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 N₂O production and AOA amoA, nirS and nirK in this experiment also supports this point. AOA amoA is the key gene of N₂O production in the nitrification pathway, and nirS and nirK are the key genes of N₂O production in the denitrification pathway [4]. Among them, ammonia oxidation may be the main pathway of N₂O production. The production of N₂O was most strongly correlated with the content of NH₄⁺-N; moreover, the high sand content in the experimental soil was conducive to the production of N₂O by nitrification [34]. In addition, the presence of corn stalks and collecting N₂O samples after sealing for 24 h may have increased
oxygen consumption [34,35], thus underestimating the N₂O produced by the ammonia oxidation process. This was different from the study of Hink et al. [36], who believed that the N₂O produced by denitrification in 60% water-filled pore space could be ignored. N₂O production in the present study was mainly affected by urea-derived NH₄⁺-N and NO₃⁻-N (Table 2; Figure 2), but mainly came from the soil-derived NH₄⁺-N and NO₃⁻-N (78.6–94.6%; Table 1), which was similar to the results of previous studies [27,37,38]. It may be that NH₄⁺-N and NO₃⁻-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 N₂O. The significant positive correlation between N₂O production and AOA amoA in this study also supports this view (Table 2), because AOA produces N₂O resulting from mineralized ammonia [4,36]. However, our experiment cannot distinguish between soil-derived N₂O and corn stalk-derived N₂O.

Compared with nitrogen application alone, low nitrogen (105 kg N ha⁻¹) combined with application of corn stalks had little effect on N₂O 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 N₂O 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 N₂O. Chen et al. [33] and Shi et al. [39] believed that the production of N₂O in nitrogen-limited soil is mainly affected by AOA rather than AOB. Our research also found that the production of N₂O 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 N₂O 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 N₂O production in the denitrification pathway [4]) were least abundant in the N₃S₁ 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 N₂O 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 N₂O production, which increased with an increase in urea dosage. An increase in urea application delays the emergence of the N₂O emission peak and increases the time of N₂O generation. The production of N₂O is mainly affected by urea-derived NH₄⁺-N and NO₃⁻-N, but the main source of N₂O is soil nitrogen itself, accounting for 78.6–94.6%. Returning corn stalks to the field will reduce the production of N₂O. The N₂O production reduction effect is strongest when a large amount of urea (420 kg N 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 N₂O emission reduction effect. The combined application of corn stalks and urea mainly affects N₂O production by changing the concentration of urea-derived NH₄⁺-N and NO₃⁻-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 N₂O production and the role of functional genes related to N₂O 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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