Isotope signatures of \( N_2O \) emitted from vegetable soil: Ammonia oxidation drives \( N_2O \) production in \( NH_4^+ \)-fertilized soil of North China

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Nitrous oxide (\( N_2O \)) is a potent greenhouse gas. In North China, vegetable fields are amended with high levels of N fertilizer and irrigation water, which causes massive \( N_2O \) flux. The aim of this study was to determine the contribution of microbial processes to \( N_2O \) production and characterize isotopic signature effects on \( N_2O \) source partitioning. We conducted a microcosm study that combined naturally abundant isotopologues and gas inhibitor techniques to analyze \( N_2O \) flux and its isotopomer signatures \([\delta^{15}N_{bulk}, \delta^{18}O, \text{ and } SP \text{ (intramolecular } ^{15}N \text{ site preference)}]\) that emitted from vegetable soil after the addition of \( NH_4^+ \) fertilizers. The results show that ammonia oxidation is the predominant process under high water content (70% water-filled pore space), and nitrifier denitrification contribution increases with increasing N content. \( \delta^{15}N_{bulk} \) and \( \delta^{18}O \) of \( N_2O \) may not provide information about microbial processes due to great shifts in precursor signatures and atom exchange, especially for soil treated with \( NH_4^+ \) fertilizer. SP and associated two end-member mixing model are useful to distinguish \( N_2O \) source and contribution. Further work is needed to explore isotopomer signature stability to improve \( N_2O \) microbial process identification.
Several validated strategies can be utilized to measure $\text{N}_2\text{O}$ production and source partitioning, including acetylene ($\text{C}_2\text{H}_2$) inhibition method, single-label $^{15}\text{N}$ method, dual-label $^{15}\text{N}$-$^{18}\text{O}$ isotope method, and natural abundance isotope technique. Here, we combined the acetylene inhibition method and the natural abundance isotope technique to investigate $\text{N}_2\text{O}$ flux and production processes in vegetable soil. We also evaluated the reliability of isotopic signatures [i.e., $\delta^{15}\text{N}$, $\delta^{18}\text{O}$ and SP (intramolecular $^{15}\text{N}$ site preference)] in $\text{N}_2\text{O}$ source identification by comparing the results from the two approaches, and provide observations for the related field study.

Here, we report an incubation experiment to determine the effects of fertilizer content in a Chinese cabbage field on $\text{N}_2\text{O}$ emissions. The aim was to explore $\text{N}_2\text{O}$ emissions and sources in vegetable production, which has not been sufficiently elucidated. The results from this study can facilitate the design of reasonable agricultural mitigation strategies to alleviate the global greenhouse effect.

### Methods

#### Soil sampling.

Soil (0–20 cm depth) was collected randomly on October 20, 2014, from 10 spots in a field that was planted with Chinese cabbage at the environmental research station of the Chinese Academy of Agricultural Sciences, Shunyi District, Beijing, China (40°15′N, 116°55′E). The field had been treated with approximately 400 kg·N·ha$^{-1}$ (equals 150 mg·N·kg$^{-1}$ dry soil) of ($\text{NH}_4$)$_2\text{SO}_4$ for two years. The soil was classified as calcareous Fluvo-aquic according to the Food and Agriculture Organization (FAO). Soil properties at this site were 28.7% sand, 64.2% silt, 7.1% clay, 1.40 g cm$^{-3}$ bulk density, 1.2 g kg$^{-1}$ total N, 13.5 g kg$^{-1}$ organic C, and pH 7.4 (1:2.5, soil/water). Fresh soil was sampled randomly, homogenized, visible roots and other residues were removed, sieved to 2 mm, and refrigerated at 4°C until use within three days. Soil samples were air-dried for 24 h one day before the start of incubation to eliminate residual $\text{N}$16. The soil contained approximately 1.3 mg NH$_4$$^-$·N per kg dry soil and 30 mg NO$_3$$^-$·N per kg dry soil before incubation, which was quite low and had little influence on the fertilizer level.

#### Experimental setup.

A soil microcosm setup was established using the gas inhibitor method to simulate different $\text{N}_2\text{O}$ pathways. On day 0, soil was amended with ($\text{NH}_4$)$_2\text{SO}_4$ and deionized water, and homogenized very well to attain 100 (low-dose fertilizer application, group A) and 300 (high-dose fertilizer application, group B) mg·N·kg$^{-1}$ dry soil fertilizer levels and water content of 70%WFPS (water-filled pore space, the initial water content was measured in advance). For each gas treatment, there were six 500-ml glass jars equipped with gas-tight lids and three-way stopcocks. Three of these were for gas analysis, and three were used for soil sampling.

To quantify the contributions of AN, HN, DD, and ND processes in $\text{N}_2\text{O}$ emission, six gases were chosen as inhibitors and injected into the jars (Table 1): (i) CK (atmosphere), (ii) N (pure N$_2$, purity 99.995%), (iii) O (pure O$_2$, purity 99.99%), (iv) LA (air + 0.1% v/v C$_2$H$_2$, purity 99.99%), (v) HA (pure N$_2$ + 10% C$_2$H$_2$), and (vi) OA (pure O$_2$ + 0.1% C$_2$H$_2$). After loading 100 g of soil to the jars, they were sealed, vacuumed thoroughly, purged three times with the corresponding pure gas, injected with pure or mixed gas (pure gas was injected first, then withdrawn, and replaced with C$_2$H$_2$), and then incubated in the dark at 25°C. During the incubation period, soil water content was held constant by checking every 2–3 days using the gravimetric method. The treatments are named as CK, N, O, LA, HA and OA according to relevant gas inhibitor, and followed by -A or -B which represented the same as before sampling. Before measuring the LA, HA, and OA treatments, acetylene was removed with sulfuric acid and potassium permanganate according to the protocol of Malone et al.17. Then, 10 g of soil was sampled immediately after gas collection from three soil-tested jars for mineral NH$_4$$^+$·N and NO$_3$$^-$·N extractions using 50 ml of 2 M KCl, and then stored at −20°C until colorimetric analysis with a continuous flow analyzer (Futura, Alliance Instruments, France). Ammonium and nitrate were measured using the indophenol blue and sulfanilamide-naphthylethylenediamine methods18, respectively.

#### Determination of $\text{N}_2\text{O}$ flux and isotopic signatures of $\text{N}_2\text{O}$.

We measured $\text{N}_2\text{O}$ concentrations using an isotope ratio mass spectrometer (IRMS, Isoprime100, Isoprime, Cheadle, UK). Peak area $m/z$ 44 was used to determine $\text{N}_2\text{O}$ concentration with the help of instant atmosphere $\text{N}_2\text{O}$ peak area and the published global average $\text{N}_2\text{O}$ concentration (327 ppbv). Pure $\text{N}_2$ (99.995%) and $\text{N}_2\text{O}$ (99.999%) were used as carrier and reference gases.

| Treatment | AN | HN | DD | ND | Other |
|-----------|----|----|----|----|--------|
| CK        | +  | +  | +  | +  | +      |
| N         | −  | −  | −  | +  | −      |
| O         | +  | +  | −  | −  | +      |
| LA        | −  | +  | −  | −  | +      |
| HA        | −  | −  | −  | +  | −      |
| OA        | −  | −  | −  | −  | −      |

Table 1. Inhibitors used and their effects on $\text{N}_2\text{O}$ production processes. “+” indicates that the process occurs, “−” indicates that the process is blocked (based on previous work30,42) and slightly modified.
gases, respectively. Calibration was conducted by measuring standards of USGS32 and USGS34. Typical analytical precision was 0.5, 0.9, and 0.6‰ for \(\delta^{15}N_{\text{bulk}}, \delta^{15}N_{\alpha},\) and \(\delta^{18}O,\) respectively. The detection limit for \(\text{N}_2\text{O}-\text{N}\) was 500 ppbv. Daily \(\text{N}_2\text{O}\) flux (\(\mu\text{g} \cdot \text{N} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}\)) was calculated as follows:

\[
F_{\text{N}_2\text{O}} = \frac{\Delta C \times V}{\Delta T 	imes m} = \frac{\rho \times \text{ppbv} 	imes V}{\Delta T 	imes m \times 1000 \times 2} \times \frac{273}{273 + T} \times 24
\]

where \(F_{\text{N}_2\text{O}} (\mu\text{g} \cdot \text{N} \cdot \text{kg}^{-1} \cdot \text{d}^{-1})\) is \(\text{N}_2\text{O}\) flux; \(\Delta C (\text{mg} \cdot \text{m}^{-3} \cdot \text{h}^{-1})\) denotes the rate of increase of \(\text{N}_2\text{O}\) concentration inside the jar within 2 h (10.00–12.00 hr); \(\rho (1.964 \text{ kg} \cdot \text{m}^{-3})\) is \(\text{N}_2\text{O}\) density at 101.325 kPa and 273 K; \(V (\text{ml})\) refers to the headspace volume in the jar (417 and 400 ml for 100 and 300 mg·N·kg\(^{-1}\) dry soil, respectively); \(m (\text{g})\) denotes the mass of converted dry soil; \(T (°C)\) is 25 °C; and 24 is the number of hours within one day. Cumulative \(\text{N}_2\text{O}\) flux during the experimental period was estimated by averaging the fluxes of two successive determinations, multiplying that average flux by the length of the period between the measurements, and adding that amount to the previous cumulative total. \(\text{N}_2\text{O}\) isotopomer signatures were determined using the IRMS described above. The isotopic compositions of \(15\text{N}\) and \(18\text{O}\) in \(\text{N}_2\text{O}\) were expressed in \(\delta\) notation with respect to the atmospheric \(\text{N}_2\) and Vienna standard mean ocean water (V-SMOW), respectively.

Some Equations describing isotopomer ratios of a sample (\(R_{\text{sample}}\)) that deviate from \(15\text{N}/14\text{N}\) and \(18\text{O}/16\text{O}\) ratios of the standard materials (\(R_{\text{standard}}\)) are shown below.

\[
\delta^{15}N = 15^{i}R_{\text{sample}}/15^{i}R_{\text{standard}} - 1 \quad (i = \text{bulk or } \alpha)
\]

\[
\delta^{18}O = 18^{e}R_{\text{sample}}/18^{e}R_{\text{standard}} - 1
\]

\[
\delta^{15}N_{\text{SP}} = \delta^{15}N_{\alpha} - \delta^{15}N_{\beta} = 2 (\delta^{15}N_{\alpha} - \delta^{15}N_{\text{bulk}})
\]

**Statistical analysis.** The possible \(\text{N}_2\text{O}\) production pathways are shown in Table 1. \(\text{N}_2\text{O}\) fluxes from these processes were calculated as follows:

\[
\text{N}_2\text{O}_{\text{AN}} = \text{O} - \text{OA},
\]

\[
\text{N}_2\text{O}_{\text{HN}} = \text{LA} - \text{N},
\]

\[
\text{N}_2\text{O}_{\text{DD}} = \text{LA} - \text{OA},
\]

\[
\text{N}_2\text{O}_{\text{ND}} = \text{CK} - (\text{LA} + \text{AN})
\]

Here, CK, N, O, HA, LA, and OA represent the cumulative \(\text{N}_2\text{O}\) of their respective gas treatments.

Statistical analyses were performed using Microsoft Excel 2010 and SAS version 9.2 software packages. Significant differences were determined using one-way analysis of variance (ANOVA) and the least significant difference (LSD) test at a 5% level.

**Results**

**\(\text{N}_2\text{O}\) emissions in soil microcosms.** Figure 1 presents the time series of \(\text{N}_2\text{O}\) flux that results from treatment with different gas inhibitors in the low-dose fertilizer group A and the high-dose fertilizer group B. In group A, the peak \(\text{N}_2\text{O}\) flux occurred immediately after the start of incubation. The greatest flux was measured within 24 h for the HA-A treatment (193.33 \(\mu\text{g} \cdot \text{N} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}\)), which was approximately 12.01 times higher than the lowest
The weighted average flux of all treatments from group B were significantly higher than those from group A (P = 0.001), which indicated that NH4+-N breakdown might be initiated by other N2O production processes or by natural degradation rather than by nitrification. NH4+-N breakdown in group B treatments also was observed. The period of rapid reduction lasted for 48 h, or one day longer than in group A, which was consistent with the peak N2O flux observed in both groups. The weighted average NH4+-N contents of treatments in group B were significantly different from those in group A (P < 0.01), and they were all substantially depleted (90–98%) at the end of the incubation period.

Contributions of N2O production processes. In group A, the relative contribution of the AN process increased continuously during the first 5 days and then decreased to 33% at the end of the experiment, which produced 232.23 μg N·kg⁻¹ N2O and accounted for 51% of the total N2O produced (Fig. 2a). N2O emissions from N-B peaked on the first day (38.10 μg N·kg⁻¹), which accounted for 32% of the total N2O emission on the first day, and then leveled off at 23–27% of the daily total N2O emission before declining to approximately 15% of the daily total N2O emission during the last two days of the experiment. By contrast, the contribution of DD to N2O emissions was weak in the beginning, increased from the third day, and peaked on the last day to account for 52% of total N2O emissions, with a weighted average of 18% of total N2O emission during the whole period of the experiment. HN remained a weak contributor of 2–8% of N2O emission during 21 days, and contributed 5% of the total N2O emission.

In group B (Fig. 2b), AN contributed 58% of N2O on day 1, and was a significant contributor during the entire experimental period. The cumulative N2O emissions (258.07 μg N·kg⁻¹) produced by ND in group B was significantly higher than that in group A (P < 0.001) and contributed a higher percentage of the total N2O compared with that in group A. The daily contribution from DD increased from day 5 and reached the maximum of 41% on the last day of the experiment, which was lower than the observed maximum in group A. However, the cumulative N2O emissions produced by AN, HN, ND, and DD processes in soil treated with high-dose fertilizer were significantly higher than those produced in soil treated with low-dose fertilizer (P < 0.001). In both group A and B, the AN process was dominant compared with other pathways, followed by the ND process, and then the DD process. Although the DD process only contributed a small proportion the total N2O, this contribution increased significantly during the later period of the experiment.

Variation of mineral N in tested soil. The soil concentrations of NH4+-N and NO3−-N were analyzed to evaluate possible N2O production processes (Fig. 3). The results showed that soil NH4+-N was rapidly nitrified to NO3−-N after the start of incubation. In the CK-A and O-A treatments, NH4+-N drastically decreased by 73% within 24 h, which indicated that NN and ND might be the main processes in these treatments. By contrast, NH4+-N decreased slowly in the N-A treatment, with a reduction of 51% over the entire incubation period, which suggests that NH4+-N breakdown might be initiated by other N2O production processes or by natural degradation rather than by nitrification. NH4+-N breakdown in group B treatments also was observed. The period of rapid reduction lasted for 48 h, or one day longer than in group A, which was consistent with the peak N2O flux observed in both groups. The weighted average NH4+-N contents of treatments in group B were significantly different from those in group A (P < 0.01), and they were all substantially depleted (90–98%) at the end of the incubation period.

Figure 2. Relative contribution of individual processes to N2O production in group A (a) and B (b) during the incubation period.
experiment, except for N contents (59–63%) in both groups. NO$_3^-$-N contents increased sharply within 2 days (group A) and 3 days (group B) in all treatments except for LA-A, N-A, and N-B treatments, then increased gradually until reaching the maximum concentrations on day 21 (Fig. 3c,d). In groups A and B, NO$_3^-$-N accumulated rapidly in the O (53.95 and 116.92 mg·kg$^{-1}$) and CK (57.12 and 104.97 mg·kg$^{-1}$) treatments during 48 h and 72 h, respectively, which were consistent with the reduction of NH$_4^+$-N, and indicated that the AN process was predominant in the CK and O treatments. In the LA and OA treatments, the cumulative NO$_3^-$-N contents were between the levels of O and N, which suggests that nitrification consumes NO$_3^-$-N to some extent. In the N treatment, the cumulative NO$_3^-$-N content was less than that in other treatments in both groups, which suggests that DD was the sole process occurring in this tested soil. The variation in N levels indicated that NH$_4^+$-N transformation to NO$_3^-$-N via N$_2$O production (NN, ND, and DD processes) occurred in all treatments of both groups except for the HA treatment.

Measurement of denitrification products ratio N$_2$O/(N$_2$O + N$_2$). The pathway of N$_2$O reduction to N$_2$ is important for understanding N$_2$O consumption in agricultural soil, and it is a possible target for mitigation of N$_2$O emissions. The combination of C$_2$H$_2$ and N$_2$ (v/v = 1:9, HA) inhibits N$_2$O reduction to N$_2$; therefore, we analyzed N$_2$O fluxes produced in CK and HA treatments to estimate the contents of potential denitrification products (N$_2$O + N$_2$) and the ratio of N$_2$O/(N$_2$O + N$_2$). The results showed that both N$_2$O and (N$_2$O + N$_2$) emissions were significantly higher (P = 0.01 and 0.04, respectively) in group B (weighted average daily fluxes of 52.41 and 60.5 μg·N·kg$^{-1}$·d$^{-1}$, respectively) than in group A (weighted average daily fluxes of 26.66 and 50.15 μg·N·kg$^{-1}$·d$^{-1}$, respectively). The weighted average ratios [N$_2$O/(N$_2$O + N$_2$)] were 0.44 and 0.87 in group A and group B, respectively, which were significantly different (P = 0.002). The ratio of 0.87 indicates that only 13% of N$_2$O was reduced to N$_2$, and a substantial percentage of the remainder was lost. N$_2$O emissions showed a linear and positive relationship with total denitrification emissions (N$_2$O + N$_2$) in low-dose (P < 0.001, R$^2$ = 0.99) and high-dose (P = 0.005, R$^2$ = 0.99) fertilizer-amended soil. In group A, the N$_2$O and (N$_2$O + N$_2$) contents were significantly correlated with the ratio of N$_2$O/(N$_2$O + N$_2$) (P = 0.009, R$^2$ = 0.81; P < 0.001, R$^2$ = 0.80, respectively). These relationships were not robust in group B (P = 0.007, R$^2$ = 0.22; P = 0.005, R$^2$ = 0.16, respectively).

Measured isotopic signatures of N$_2$O in soil microcosms. We explored the isotopomer signature profiles during the first week of the incubation period because N$_2$O isotopic composition is influenced primarily by the peak flux, and in agronomic applications the peak generally occurs within one week after use$^{19}$. We found that $^{15}$N$_{bulk}$ of N$_2$O increased during the first week in both groups (Fig. 4a,b), which ranged from $-65.85\%$o (the lowest) on the first day to $-27.43\%$o (the highest) on day 7. This is in agreement with previous studies, which report that
δ\textsuperscript{15}N\textsubscript{bulk} of \textsubscript{N}_2\textsubscript{O} usually increases after urea\textsuperscript{20} or ammonia fertilizer\textsuperscript{21} application. The weighted average of δ\textsuperscript{15}N\textsubscript{bulk} of each treatment in group B was lower than those of each treatment in group A, and this is consistent with previous studies\textsuperscript{19,20} showing that \textsubscript{N}_2\textsubscript{O} emissions increase as fertilizer content increases, whereas the δ\textsuperscript{15}N\textsubscript{bulk} value was reduced as the fertilizer content increased.

The time gradient (Δδ\textsuperscript{15}N\textsubscript{bulk}/ΔT) of 15N\textsubscript{bulk} was analyzed because the changes in 15N\textsubscript{bulk} after addition of NH\textsubscript{4}\textsuperscript{+} might complicate the use of 15N\textsubscript{bulk} to identify \textsubscript{N}_2\textsubscript{O} source partitioning. In both groups A and B, the 15N\textsubscript{bulk} of the CK and O treatments had relatively high time gradients ranging from 3.36 to 3.46‰ d\textsuperscript{−1}, which were consistent with the reduction of NH\textsubscript{4}\textsuperscript{+}-N concentration in the tested soil. Accordingly, we assumed that the highest gradient should be from the O treatment rather than the CK treatment. The reason for this bias might be the reduction of N\textsubscript{2}O to N\textsubscript{2} in the CK treatment, which generally leads to an increase in the value of δ\textsuperscript{15}N\textsubscript{bulk}\textsuperscript{15,19}. The minimum time gradient for appearance of δ\textsuperscript{15}N\textsubscript{bulk} came from the N in both groups A (0.71‰ d\textsuperscript{−1}) and B (0.55‰ d\textsuperscript{−1}) This might due to the relatively low fractions of consumed NH\textsubscript{4}\textsuperscript{+} because, theoretically, nitrification would not occur. The observed slight increase in δ\textsuperscript{15}N\textsubscript{bulk} of OA suggested the occurrence of weak HN. A comparison of temporal parameters indicates that shifting of δ\textsuperscript{15}N\textsubscript{bulk} is related to the fraction of NH\textsubscript{4}\textsuperscript{+} consumption (e.g., nitrification requires more NH\textsubscript{4}\textsuperscript{+}-N, whereas denitrification does not). These considerations helped us to identify the candidate processes that occurred in these treatments.

Figure 4. Time series of δ\textsuperscript{15}N\textsubscript{bulk}, δ\textsuperscript{18}O, and SP of \textsubscript{N}_2\textsubscript{O} in different treatments in group A (a,c,e) and B (b,d,f). Error bars represent standard deviation of the mean (n = 3).
It is more complicated to describe the variation of $^{18} \text{O}-\text{N}_2\text{O}$ than that of $^{15} \text{N}_{\text{bulk}}$, because the O atom readily exchanges with O$_2$, H$_2$O, and substrate NO$_3^->$. In this study, neither a stable variation nor significant differences ($P > 0.05$) between groups A and B were observed (Fig. 4c,d). Most $^{18} \text{O}$ values were lower than measured instant values of $^{18} \text{O}-\text{H}_2\text{O}$ ($-1.4\%$) and $^{18} \text{O}-\text{O}_2$ ($19.7\%$), but were close to another incubation experiment we performed that used the same soil and fertilizer. $^{18} \text{O}$ increased rapidly during the first week in the O and OA treatments, which might be due to substantial nitrification promoting O-exchange with H$_2$O and O$_2$, and thus resulting in the enrichment of $^{18} \text{O}$ in the remaining O atom. In the CK and LA treatments, denitrification drives NO$_3^-$ consumption and enrichment of $^{18} \text{O}$ in the remaining NO$_3^-$ in the soil, which increases $^{18} \text{O}$ abundance. It was reported that cleavage of the N-O bonds during N$_2$O reduction by denitrification would lead to the accumulations of both $^{15} \text{N}_2\text{O}$ and $^{18} \text{O}$ in the residual N$_2$O. This might explain why $^{18} \text{O}$ from H$_2$O fluctuated slightly, as denitrification was believed to be the sole process and reduction was not believed to occur. However, $^{18} \text{O}$ is not suitable for N$_2$O partitioning because O atoms exchange frequently with different O sources and the O sources vary with respect to $^{18} \text{O}$.

**Evaluation of N$_2$O source partition on SP.** The weighted average SP in the CK-A and CK-B treatments were 18.58‰ and 14.71‰, respectively, which was higher than that reported for denitrification ($-10$ to 0‰) and lower than that reported for nitrification (33 to 37‰) from pure culture experiments[2$^-$]. This indicated that multiple N$_2$O processes occurred simultaneously in the CK treatment. The SP of CK and LA treatments of both groups decreased sharply on day 2, and then increased until day 7. This result might suggest that N$_2$O reduction occurred after the mixing of produced N$_2$O, which is consistent with the results of N$_2$O emission in a closed system. In the N treatment, SP increased gradually and reached 6.22‰ and 8.46‰ in group A and B, respectively, on day 7. This result indicated that fungal denitrification might occur, because its average SP value is 30.0 ± 4.8‰, according to Maeda et al.[25]. In the other treatment, SP slightly fluctuated during the first 5 days, and then decreased to 16.49‰ and 21.0‰ in group A and B, respectively, on day 7. We inferred that aerobic denitrification induced by microbes such as *Pseudomonas* spp. and *Alcaligenes* spp. occurred, thereby lowering SP values in the system. Similarly, the successive decline of SP in OA and LA treatments might be attributed to the HN process accompanied by aerobic or anaerobic denitrification, respectively. For the HA treatment, SP matched the range of −10 to 0‰ during the whole week, except for HA-B on day 1, which indicated that DD was the single process occurring. Meanwhile, no obvious increase in SP was observed, which is consistent with the conclusion that N$_2$O reduction would be induced by high C$_2$H$_4$ concentration.

In this study, the two end-members mixing model [Eq. (5)]$^{27}$ was applied to evaluate the respective contributions of pairs of processes in the CK treatment on day 1 and 7. Four cases should be considered depending on higher-SP and lower-SP combinations:$^{28}$ (i) NN$_\text{bacteria}$ and DD$_\text{bacteria}$, (ii) NN$_\text{bacteria}$ and ND$_\text{bacteria}$, (iii) DD$_\text{fungus}$ and DD$_\text{bacteria}$, and (iv) DD$_\text{fungus}$ and ND$_\text{bacteria}$. To simplify the model, we only discuss cases 1 and 2 for CK treatment. For example, the contribution of nitrification in case 1 can be expressed as shown below. The isotopomer signatures and possible contributions of processes are presented in Table 2.

$$f_{\text{NN}} = (\text{SP}_{\text{sample}} - \text{SP}_{\text{DD}})/(\text{SP}_{\text{NN}} - \text{SP}_{\text{DD}})$$  \hspace{1cm} (5)$$

In Eq. (5), $f_{\text{NN}}$ is the contribution of N$_2$O derived from nitrification; SP$_{\text{sample}}$ is the measured SP value of N$_2$O; and SP$_{\text{NN}}$ (33%), SP$_{\text{DD}}$ ($-10$ to 0‰), and SP$_{\text{ND}}$ ($-13.6$ to 5‰) represent the respective SP values of nitrification, denitrification, and nitrifier-denitrification processes reported by previous studies.$^{25,28}$

The respective contributions of NN (AN + HN), DD, and ND estimated by N$_2$O measurement were consistent with the calculated ranges based on model cases 1 and 2. N$_2$O flux from DD was relatively weak during the first week, suggesting that NN and ND were more likely to be involved in the CK treatment. This was confirmed by the comparison between the model prediction and N$_2$O measurement. We observed that ND contributions calculated from the model were higher than those of actual measurements in groups A and B on day 7, which indicates that they were overestimated by the model. This can be attributed to the observation that the contribution from DD became more prominent at later time points, which was not accounted for by the two end-members model system.

**Discussion**

Previous studies have investigated N$_2$O emission pathways in agricultural soil treated with ammonia fertilizer.$^{14,29,30}$ N$_2$O production and consumption in soil is generally mediated by microbes and microbial communities, which metabolize soil nutrients, regulate N$_2$O emissions and sources, and determine the contributions of different processes to total N$_2$O emissions in different ecosystems. The studies agree that sources of N$_2$O emissions vary as environmental factors change. In the current study, autotrophic nitrification was a prominent pathway generating N$_2$O emissions, whereas heterotrophic nitrification had a minor role in the tested soil. This result is consistent with some previous reports,$^{7,14}$ but it is not in agreement with other reports$^{29,31}$ that observed a large contribution from HN to N$_2$O emissions, especially in acidic soil or soil with a high organic carbon content. These inconsistencies may be attributed to heterogeneous soil textures, organic components, and pH. Conversely, the contribution of HN might be underestimated in the current study because C$_2$H$_4$ inhibits the HN pathway.$^{24}$

Nitrifier denitrification has been reported as another important source of N$_2$O production, and it accounts for 30–66% of N$_2$O emissions in pure cultures.$^{32}$ In the current study, the contribution from ND increased greatly with higher fertilizer content in the tested soil. This can be explained by extremely high NH$_4^+$ levels, suboxic conditions, and low organic carbon contents, which created conditions that were more favorable for ND than other processes.$^{30}$ The low contribution from denitrification observed in this study was related to the absence of initial NO$_3^-$ and the presence of only a few denitrifiers in the soil. The latter condition was verified by subsequent field study results that detected relatively few *nirS* genes but abundant *amoA* genes in the same soil used in the
current experiments. The contribution of DD continuously increased and became the main process during the later experimental period due to transformation of NH$_4^+$ to NO$_3^-$. However, the DD contribution was less than that of AN because the total N$_2$O flux decreased greatly during the later period. This result indicates that DD has a weak contribution at 70% WFPS. Although this result is not consistent with previous studies, it is supported by several recent analyses of global trends in the $^{15}$N, $^{18}$O, and SP signatures of N$_2$O, which suggest that ammoniacal N fertilizers and nitrification are the principal sources responsible for the rise in atmospheric N$_2$O.$^{9,33}$

Other pathways in this study refer to dissimilatory nitrate reduction to ammonium (DNRA), chemodenitrification, and chemonitrification. DNRA has been reported to significantly contribute to N$_2$O emissions under certain conditions.$^{34,35}$ It requires more energy to reduce NO$_3^-$ to NH$_4^+$ and is favorable for conditions with high C:NO$_3^-$ ratio; therefore, it is reasonable to hypothesize that DNRA contributes weakly to N$_2$O emissions in our local vegetable soil. Although we cannot track chemodenitrification and chemonitrification patterns in our data, the existence of these processes should not be ignored. Future work should investigate these unusual N$_2$O production pathways.

The final step in denitrification is the conversion of by-product N$_2$O to N$_2$ by nitrous oxide reductase. This step is critical for evaluating N$_2$O consumption and to understand nitrogen accumulation in soil and emission to the atmosphere. The ratio of denitrification products [N$_2$/([N$_2$O+N$_2$])] was used to evaluate the degree of N$_2$O conversion to N$_2$, which ranged from 0 (all N$_2$O was reduced to N$_2$) to 1 (N$_2$O was the sole terminal denitrification product)$^{36}$. Our results show that the denitrification product ratio in soil with high NH$_4^+$ fertilizer content is significantly higher than that in soil with low NH$_4^+$ fertilizer content ($P < 0.001$), which indicates that the higher the fertilizer content, the larger the ratio of denitrification products and loss of N. It results in more N$_2$O gas and NO$_3^-$ production which emit to atmosphere, immobilize in soil, diffuse into groundwater, and cause severe environmental pollution. The soil is a source rather than a sink in this case. Previous work showed that high N$_2$O production was generated primarily by NH$_4^+$ oxidation pathways under low O$_2$ availability$^4$, which is also consistent with our results. This suggests that N$_2$O emissions could be reduced by avoiding high ammonium concentrations in the soil, or by selecting a different type of fertilizer to mitigate N$_2$O emissions, especially in vegetable fields that require high water content (which causes low oxygen concentration) and fertilizer content.

Some studies reported that $\delta^{15}$N$_\text{bulk}$ is a good indicator for distinguishing nitrification and denitrification because nitrification more strongly depletes $^{15}$N compared with denitrification.$^{26,37}$ Other authors contend that $\delta^{15}$N$_\text{bulk}$ can be affected by NH$_4^+$ and NO$_3^-$ origins, microsite heterogeneity, and reductant.$^{31}$ Here, we observed that $\delta^{15}$N$_\text{bulk}$ level increased after NH$_4^+$ application due to isotope fractionation during nitrification, which enriches the remaining $^{15}$N of N$_2$O. Therefore, $\Delta^{15}$N (the difference between $^{15}$N of the substrate and the product) was proposed to differentiate among N$_2$O sources in N-fertilized agricultural soil, and approximate ranges of different pathways were reported.$^{18,37}$ In the current study, data for $\Delta^{15}$N varied greatly and was not constant over time due to $\delta^{15}$N$_\text{bulk}$ of the precursor (NH$_4^+$). Therefore, we cannot use this parameter to identify N$_2$O sources. We analyzed an alternative time course of $\delta^{15}$N$_\text{bulk}$ and found that it did provide some information regarding possible processes. However, we do not recommend $\delta^{15}$N$_\text{bulk}$ as a powerful indicator for N$_2$O source partitioning, especially under the conditions of NH$_4^+$ fertilizer application. The possible process stated in this paper was deduced based on whether a process occurs or not and by estimating possible sources in advance. Currently, there is a lack of

### Table 2. Isotopomer signatures of N$_2$O and contributions of different pathways on N$_2$O production

- **NN**: Nitrification, **HN**: Heterotrophic nitrification, **DD**: Denitrification, **ND**: Nitrifier denitrification
- Contributions of different N$_2$O production pathways were based on case 3 of the two end-members mixing model. nd, not determined with this method.

| CK | Case 1 | Case 2 | Case 1 | Case 2 |
|----|--------|--------|--------|--------|
| A  | $-52.44$ | $-28.20$ | $-45.40$ | $-13.86$ |
| B  | $-60.10$ | $-33.28$ | $-41.27$ | $-31.91$ |

| N  | Case 1 | Case 2 | Case 1 | Case 2 |
|----|--------|--------|--------|--------|
| A  | $-47.17$ | $-42.18$ | $-25.81$ | $-17.80$ |
| B  | $-47.81$ | $-43.76$ | $-22.81$ | $-25.61$ |

| O  | Case 1 | Case 2 | Case 1 | Case 2 |
|----|--------|--------|--------|--------|
| A  | $-61.13$ | $-37.47$ | $-51.50$ | $18.36$ |
| B  | $-65.85$ | $-42.34$ | $-66.57$ | $20.82$ |

| HA | Case 1 | Case 2 | Case 1 | Case 2 |
|----|--------|--------|--------|--------|
| A  | $-42.29$ | $-27.43$ | $-19.47$ | $-24.91$ |
| B  | $-53.64$ | $-45.30$ | $-26.44$ | $-28.51$ |

| LA | Case 1 | Case 2 | Case 1 | Case 2 |
|----|--------|--------|--------|--------|
| A  | $-55.99$ | $-23.41$ | $-32.62$ | $-5.99$ |
| B  | $-57.10$ | $-35.55$ | $-27.92$ | $-11.39$ |

| OA | Case 1 | Case 2 | Case 1 | Case 2 |
|----|--------|--------|--------|--------|
| A  | $-53.53$ | $-42.96$ | $-57.05$ | $2.62$ |
| B  | $-54.02$ | $-49.86$ | $-38.53$ | $5.54$ |

|贡献 | N$_2$O | AN | HN | DD |
|------|-------|-----|-----|-----|
| NN:  | 100   |     |     |     |
| ND:  | 50–62 |     |     |     |
| DD:  | 38–50 |     |     |     |
| ND:  | 32–60 |     |     |     |

$^{\alpha}$15N$_\text{bulk}$ and found that it did provide some information regarding possible sources in advance.
robust evidence that identifies a distinct range of $\delta^{15}$N bulk associated with different microbial sources. Therefore, N₂O source partitioning based solely on $\delta^{15}$N N₂O should be treated with caution, and additional studies are needed. However, it is useful to identify natural and anthropogenic N₂O sources because $\delta^{15}$N N₂O of N₂O emitted from agricultural fields is more depleted than that from N-limited soils as shown in this study.

The $\delta^{18}$O value is not only affected by reduction of N₂O to N₂ but also by exchanging of O atom with O₂, H₂O, and NO₃. Previous studies reported that 100% of N₂O-O is derived from O₂ during NH₂OH oxidation. Half of this originates from O₂ and the other half is from H₂O from the NO₃− → N₂O reaction, or completely from substrate NO₃− during denitrification of NO₃− fertilizer, or N₂O-O derived from both O₂ and NO₃− during the NO₃− to N₂O step. The contributions of H₂O-O and NO₃− O to N₂O-O also are influenced by the species present. Therefore, the original sources of O in N₂O are hidden and uncertain so that $\delta^{18}$O is questionable as a stable indicator in some cases. Although ranges of $\delta^{18}$O values for process identification are given in some studies, it remains debatable and not reliable to use only $\delta^{18}$O for source partitioning. A combination with other isotopomer signatures, such as $\delta^{15}$N or SP, might improve its accuracy. Compared with $\delta^{15}$N bulk and $\delta^{18}$O, SP is a powerful tool for N₂O source partitioning due to the minimal disturbance for samples and independence of the precursor $\delta^{15}$N composition. However, it is affected by some factors such as microbial genera, N₂O reduction to N₂, and soil heterogeneity. SP values used for source partitioning were measured from pure culture experiments in the laboratory, which significantly differ from microbial communities in natural environments. It is not possible to partition N₂O sources only by SP value in a complex ecosystem because multiple microbial processes coupled with reduction sometimes occur simultaneously in the soil matrix. Therefore, one of the great challenges is to provide an explicit scope of SP values associated with different microbial processes or their combinations. Future studies should characterize SP, generate data sets, determine standard calibrations, and link to microbial approaches, such as high-throughput sequencing technique to ascertain the relationships between relevant microbial communities and N₂O source partition in order to reduce the current discrepancies.

Using the two end-members mixing model, we estimated the approximate contributions of process pairs using the SP values derived in this study. This was a quantitative rather than qualitative analysis. The combinations of NN bacterium − ND bacterium and NN bacterium − DD bacterium gave reasonable estimates for nitrification contributions, and NN bacterium − ND bacterium was closer to the real measured proportion of N₂O. In both groups A and B, the contributions of nitrification calculated from the model NN bacterium − DD bacterium were higher than the measured values. This finding was not in agreement with a previous report that nitrification was usually overestimated by the inhibition and N₂O source partition in order to reduce the current discrepancies.

The measurements and analyses performed in this study demonstrate the possible application of a natural N₂O flux measurements in the future could help to verify, quantify, and understand microbial processes within the spatiotemporal scale of the environment.

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Author Contributions
W.Z. and Y.L. designed the experiments. W.Z., Q.L. and W.L. performed the experiments. W.Z. and C.X. performed the data analysis. W.Z. and Y.L. wrote the paper. All authors discussed the results and commented on the manuscript.

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