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Ammonia-oxidation as an engine to generate nitrous oxide in an intensively managed calcareous Fluvo-aquic soil

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We combine field observations, microcosm, stoichiometry, and molecular and stable isotope techniques to quantify N₂O generation processes in an intensively managed low carbon calcareous fluvo-aquic soil. All the evidence points to ammonia oxidation and linked nitrifier denitrification (ND) being the major processes generating N₂O. When NH₄⁺-based fertilizers are applied the soil will produce high N₂O peaks which are inhibited almost completely by adding nitrification inhibitors. During ammonia oxidation with high NH₄⁺ concentrations (>80 mg N kg⁻¹) the soil matrix will actively consume oxygen and accumulate high concentrations of NO₂⁻, leading to suboxic conditions inducing ND. Calculated N₂O isotopomer data show that nitrification and ND accounted for 35–53% and 44–58% of total N₂O emissions, respectively. We propose that slowing down nitrification and avoiding high ammonium concentrations in the soil matrix are important measures to reduce N₂O emissions per unit of NH₄⁺-based N input from this type of intensively managed soil globally.

Nitrogen-fertilized intensively managed agricultural soils represent the largest global anthropogenic source of the potent greenhouse gas N₂O^{1,2}. Understanding the N₂O production processes is the prerequisite for developing targeted mitigation strategies. Most previous studies have been conducted under laboratory conditions and it is still a challenge to connect these results to real field situations^{3,4}.

N₂O emissions from soils are produced by a diversity of microorganisms⁵ through at least three main microbially-mediated processes nitrifier [nitrification (NN), nitrifier denitrification (ND) and denitrifier denitrification (DD)]^{3,6,7}, although abiotic reactions are also known to be an important N₂O source under certain circumstances⁸. The occurrence of each N₂O production process and its contribution to total N₂O emissions depend on prevailing soil conditions such as soil oxygen conditions (water status), microbially available C and N, and pH, which are governed by edaphic and climatic conditions and agricultural management practices^{6,9}. Although many approaches have been employed for source partitioning of soil N₂O emissions under laboratory conditions, none of the current methods has convincingly quantified the N₂O sources under real field situations^{6,10}. However, a combination of existing methods provides great potential to narrow the uncertainties associated with N₂O source partitioning in the field.

Our laboratory and field experiments conducted over a number of years investigating intensively managed low carbon calcareous soils support the assertion that nitrifying prokaryotes are responsible for a large share of the emitted N₂O^{11–15} in marked contrast to many European studies on agricultural soils with higher readily decomposable carbon and moisture contents and with relatively low pH values in which N₂O is produced mainly by denitrification. However, we still have no unequivocal evidence that the high peaks of N₂O emission induced by applying NH₄⁺- or urea-based fertilizer to the soils studied are derived from the first steps of nitrification (NH₄⁺ to NH₂OH to NO₂⁻) or from successive steps of ND (NO₂⁻ to NO to N₂O to N₂). Indeed, one of our earlier studies strongly suggested an important role of ND in N₂O production¹¹. We therefore hypothesize that the transition from aerobic (NN) to suboxic (ND) conditions might be largely responsible for the N₂O peaks after the application of NH₄⁺- or urea-based fertilizer.

Here, we report a series of pot incubation experiments to study the response of N₂O emission to soil water content, NH₄⁺ concentrations, and varying N and C supply. Moreover, a robotized incubation/analytical system¹⁶ was employed to study the stoichiometric relationship between N rate, gaseous dynamics (N₂O, NO, N₂, O₂ and



CO₂), and the response of these gases to the type of N and C sources. Two field experiments were conducted to investigate the isotopic composition of N₂O and the composition and abundance of the functional genes of nitrification (*amoA*) and of nitrifier denitrification or denitrification (*nirK* and *nirS*). The experiments were carried out in different years and under a range of conditions using the same soil type at two sites on the North China Plain in order to show that the phenomenon and results are congruent and support each other.

Results

N₂O emission vs. soil water content, C, and N supply. Total cumulative N₂O emissions were significantly ($P < 0.05$) related to soil water filled pore space (WFPS) in urea or urea plus straw treatments, with maximum emissions at around 70% WFPS. In the treatment without urea, total cumulative N₂O emissions were very low, and only slightly affected by soil moisture, even at high soil NO₃⁻-N status (31.8 mg N kg⁻¹) (Pot Incubation Experiment 1, *PIE1*) (Fig. 1a). These observations are very different from previous reports based on European agricultural soils with high indigenous soil NO₃⁻-N status¹⁷. We speculated that the large amount of N₂O in 70% WFPS was produced from the nitrification process in urea or urea plus straw treatments because the course of the N₂O emission was coincident with urea hydrolysis and the nitrification process¹³. Measurements of soil NH₄⁺-N and NO₃⁻-N status at the end of the incubation period partly explain the conclusion (from substrate bases as side evidence) that the occurrence of high N₂O emissions at 70% WFPS could be attributed mainly to nitrification and ND, or coupled nitrification-denitrification (CND) (see also Discussion section). The NH₄⁺ contents at higher moisture levels (90 and 110% WFPS) were significantly higher than at lower moisture levels (30, 50 and 70% WFPS), whereas their NO₃⁻ contents were significantly lower (Table S5, $P < 0.05$) irrespective of whether nitrogen and straw were added to the soil. This indicates that nitrifiers might dominate N-transformation processes when the soil water content is below 70% WFPS but denitrification might dominate N-transformation processes when the soil moisture exceeds 90% WFPS.

Cumulative N₂O emissions exhibited a significant positive linear correlation with NH₄⁺-N input rate ($R^2 = 0.9898$, $P < 0.001$) under 70% WFPS (Pot Incubation Experiment 2, *PIE2*) (Fig. 1b). NH₄⁺-N input rate almost completely explained the variation in total N₂O emissions and the emission factor (here, the slope of the linear function) was 0.57% in this incubation system, very similar to previous field experiments in the summer maize season^{13,15}. Soil NH₄⁺-N,

NO₃⁻-N and NO₂⁻-N contents at days 1, 3 and 18 during the incubation period showed that NH₄⁺-N was rapidly (within 1–3 days) nitrified to NO₂⁻-N and then NO₃⁻-N and that high NO₂⁻-N accumulated when NH₄⁺-N > 80 mg N kg⁻¹ (Table S6). The nitrification process corresponds well with the time course of N₂O emission (Fig. S2). The NO₃⁻ content at the end of incubation was closely linearly related to NH₄⁺ input rate ($R^2 = 0.9922$, $P < 0.001$) (Fig. S3). This further confirms that N₂O emissions were dominated by NH₄⁺ transformation process.

Application of a nitrification inhibitor (NI), either DMPP or DCD, reduced the cumulative N₂O emissions by 99.2% and 97.1%, respectively, compared to urea alone. They almost completely blocked N₂O emission in urea or urea plus straw treatments (Pot Incubation Experiment 3, *PIE3*) (Fig. 1c). Similar to *PIE2*, soil NH₄⁺-N was rapidly nitrified to NO₃⁻-N in urea and urea plus straw treatments and this process slowed down when using NI (Table S7). Furthermore, NI application precluded NO₂⁻-N accumulation so that no more N₂O was produced (Table S7; Fig. S4).

Stoichiometry of N transformation and gas fluxes. We performed an experiment with different urea-N concentrations using a robotized incubation/analytical system¹⁶ to investigate the relationship between the emissions of various gases and O₂ consumption (Robotized Incubation Experiment 1, *RIE1*). Cumulative N₂O emission increased exponentially ($P < 0.001$; Fig. 2a) and CO₂ production increased linearly ($P < 0.001$; Fig. 2b) with increasing concentration of urea-N. The O₂ consumption slope of the linear relationship was only 1.45 which lies between the theoretical slopes of pure nitrifier nitrification and pure nitrifier denitrification (Fig. 2c), suggesting that the N₂O production originated from a combination of these processes. The significant increase in CO₂ may be attributed to the hydrolysis of urea and the concomitant release of CO₂. The dynamics of O₂ consumption, CO₂ production, and N₂O emissions were synchronized with each other and this demonstrates well the close coupling between urea hydrolysis, nitrification, and N₂O production.

N₂O emission patterns were significantly different between high concentrations of urea-N (320 mg N kg⁻¹) and low concentrations (<80 mg N kg⁻¹) which likely reflected the different N₂O production processes involved. NO and N₂ emissions were generally low within the concentration range 0–80 mg N kg⁻¹ but gradually increased at the end of incubation in the highest urea treatment (320 mg N kg⁻¹). This can be attributed to the depletion of O₂ by nitrifiers and the induction of denitrification (Fig. S5). At the end of

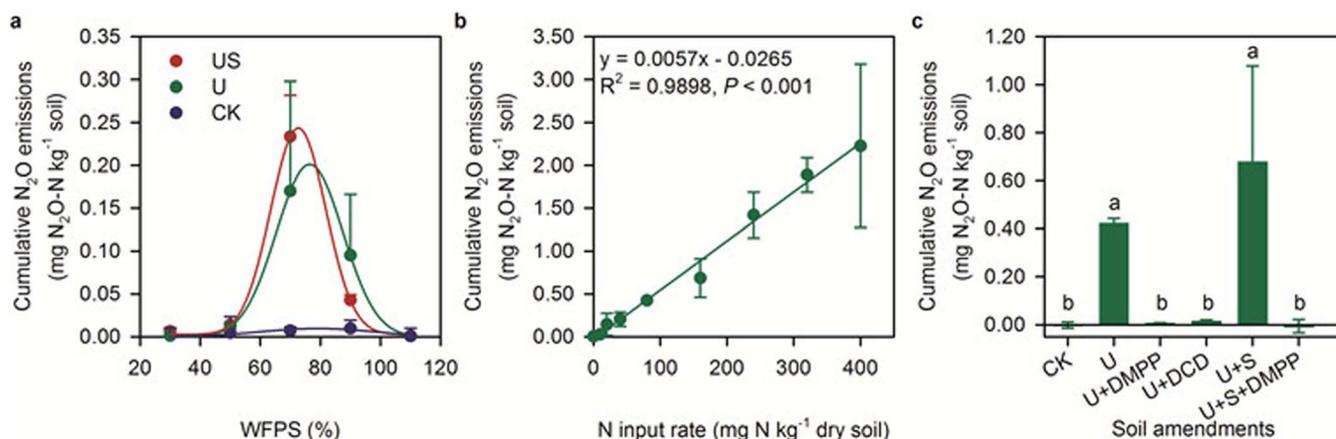


Figure 1 | Cumulative N₂O emissions in response to soil conditions in pot microcosm incubation experiments. (a), N₂O emission as a function of soil water filled pore space (WFPS). CK denotes control treatment. U denotes applied urea at a rate of 40 mg N kg⁻¹. US denotes application of urea with comminuted wheat straw. (b), N₂O emission as a function of NH₄⁺-based fertilizer (ammonium sulfate) input rate at 70% WFPS. (c), Effect of nitrogen (urea), carbon source (comminuted wheat straw), and nitrification inhibitors (DMPP and DCD) on soil N₂O emission at 70% WFPS. DMPP and DCD denote 3,4-dimethylpyrazole phosphate and dicyandiamide, respectively. Error bars in plots (a–c) are standard deviation of the mean ($n = 3$ replicates).

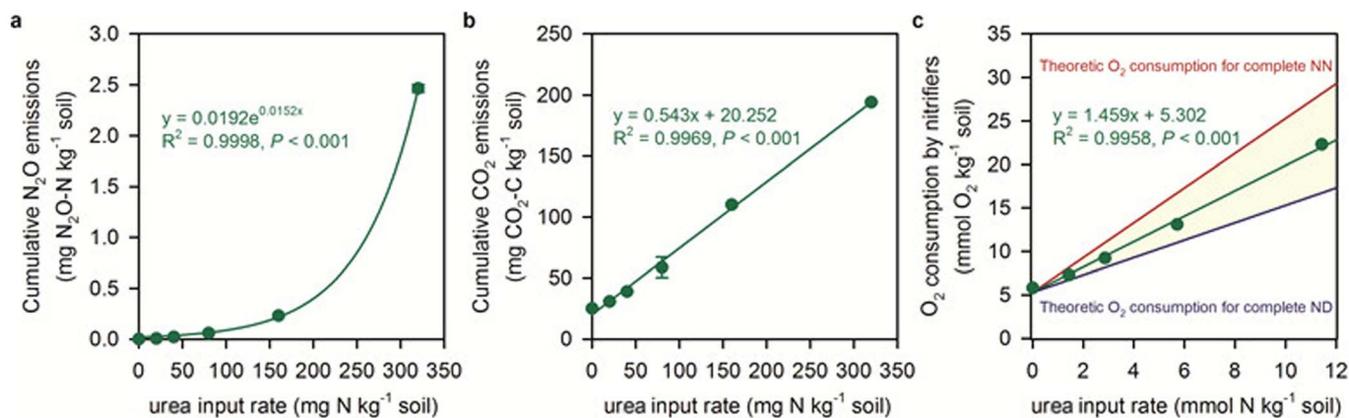


Figure 2 | Stoichiometric relationships between N_2O , CO_2 , O_2 and urea-N input rate. (a), N_2O emission as a function of urea-N input rate. (b), CO_2 emission as a function of urea-N input rate. (c), O_2 consumption as a function of urea-N input rate. In plot (c), NN and ND denote nitrifier nitrification and nitrifier denitrification, respectively. Note that O_2 consumption in the highest urea input treatment (U320) was not included due to the development of anaerobic conditions in the later stages of incubation and the units of x- and y-axis in plot (c) are different from plots (a & b).

the incubation period the O_2 concentration in the 320 mg N kg^{-1} treatment was 2.36%, low enough to trigger denitrification¹⁸. Our automated incubation experiments demonstrate that denitrification can proceed immediately once the oxygen consumption has lowered O_2 to a low level. The importance of O_2 conditions on the denitrification process outweighs the easily decomposed carbon limitation reported in a previous study¹².

To further explore the kinetics of N gas production under conditions favoring nitrification or denitrification, we performed another robotized incubation/analytical experiment (*RIE2*) with different conditions in terms of NH_4^+ -N, NO_3^- -N, organic carbon amendment, NI amendment, O_2 concentration, or combinations of these factors (Fig. 3). N_2O concentration increased to 3.9 ppm at the beginning of the incubation with NH_4^+ -N (Fig. 3b) and was blocked by simultaneous application of a nitrification inhibitor (Fig. 3c). However, there was virtually no N_2O emission when NO_3^- -N was applied alone or together with glucose (Fig. 3d,e). In contrast,

both the N_2O concentration (from 61.1 to 1307.5 ppm) and the NO concentration (from 16.0 to 1834.9 ppm) increased sharply and showed clear peaks at the initial stage of incubation when NO_3^- -N was applied together with glucose under anaerobic conditions (Fig. 3f). NO and N_2 emissions were not detected in the other treatments except when NO_3^- -N was applied together with glucose under anaerobic conditions, in which NO, N_2O , and N_2 production patterns typical of denitrification occurred. Under aerobic conditions, NO, N_2O , and N_2 emissions were not elevated even when glucose was applied with NO_3^- -based fertilizer and this supports our conclusion from *RIE1* that oxygen is the dominant factor inhibiting denitrification rather than carbon limitation. We therefore conclude that 70% WFPS was too low to trigger denitrification in our soil. However, if we withdraw O_2 from the system the typical denitrification process will proceed. At 70% WFPS aerobic nitrification dominated soil N transformations and N_2O was the major gaseous product.

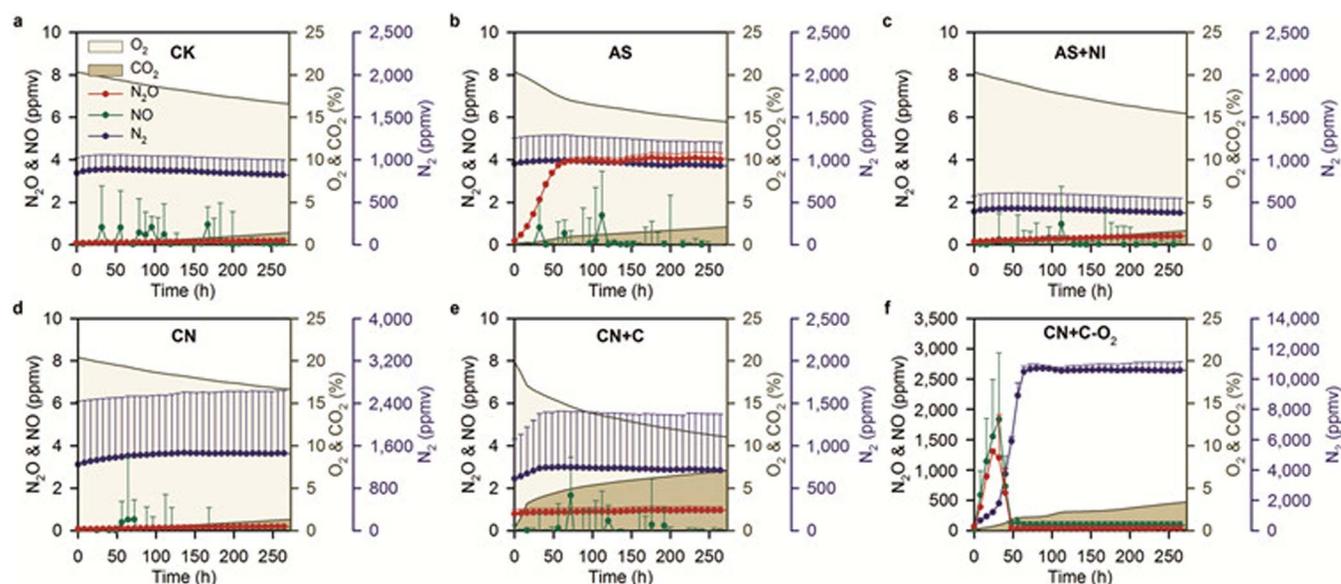


Figure 3 | Kinetics of gaseous N_2O , NO, N_2 , O_2 , and CO_2 in robotized analytical/incubation systems. (a), Control treatment (CK). (b), Ammonium sulfate (AS). (c), AS with nitrification inhibitor (AS + NI) 3,4-dimethylpyrazole phosphate (DMPP). (d), Calcium nitrate (CN). (e), CN with glucose (CN + C). (f), without oxygen from the system of treatment CN + C (CN + C- O_2). Soils were incubated at 70% WFPS. Error bars in plots (a–f) are standard deviation of the mean ($n = 4$ replicates). Note that the right and left y axis scales have large differences. In some cases the error bars are smaller than the symbols.



Isotopic fingerprints of soil-derived N₂O. N₂O generated by nitrification is more depleted in ¹⁵N and ¹⁸O in relation to precursor N compounds compared with N₂O derived from denitrification¹⁹. Furthermore, the distinction in ¹⁵N site preference (SP: difference in ¹⁵N between the central and peripheral N positions of the asymmetric N₂O molecule) between nitrification and denitrification differentiates these two N₂O sources²⁰. We established a field experiment to study the N isotopic characteristics of soil N₂O derived from three treatments, namely fertilized with (NH₄)₂SO₄ (AS), Ca(NO₃)₂ (CN) or Ca(NO₃)₂ plus compaction of the soil, glucose addition, and increased irrigation (creating soil environmental conditions favoring denitrification, CN + C). The N₂O emission patterns were detailed in a previous paper¹³ and are summarized in Fig. S6. The characteristics of $\delta^{18}\text{O}$ -N₂O, $\delta^{15}\text{N}$ -N₂O and $\Delta\delta^{15}\text{N}$ -N₂O are detailed in Fig. 4a,b,c.

In the AS, $\delta^{18}\text{O}$ -N₂O (referenced to standard mean ocean water, SMOW) increased with time after fertilization (27.9 to 36.4‰). The time gradient of $\delta^{18}\text{O}$ was 2.2‰ d⁻¹. In the CN, $\delta^{18}\text{O}$ of N₂O increased during the first three days after fertilization (36.6 to 45.0‰) with a time gradient of 4.2‰ d⁻¹ and levelled off on the fifth day with a $\delta^{18}\text{O}$ of 44.2‰. In the CN + C, $\delta^{18}\text{O}$ of N₂O increased from 26.6 to 43.4‰ during the first three days after fertilization (the data on the fifth day were lost) with a time gradient of 8.4‰ d⁻¹ (Fig. 4a). The rapid enrichment of ¹⁸O may be attributed to the intensive consumption of NO₃⁻ and the subsequent enrichment of ¹⁸O in the remainder of the NO₃⁻ in the soil as shown by the high N fertilizer N₂O loss. In this treatment N₂O emission accounted for 52.9% of the input N during the 5 days following fertilization (Fig. 4f)²¹. Although the overlaps of $\delta^{18}\text{O}$ from the soil favoring denitrification with the other two treatments and the complicated O sources of N₂O derived from nitrification and denitrification make it difficult to partition N₂O sources based solely on $\delta^{18}\text{O}$ ^{22,23}, our $\delta^{18}\text{O}$ for N₂O in the AS was within the upper end of the reported $\delta^{18}\text{O}$ for

N₂O produced in aerobic environments (13 to 35‰)²⁴, suggesting that nitrification was the predominant N₂O source.

The $\delta^{15}\text{N}$ (referenced to standard atmospheric N₂) of N₂O increased with time after fertilization in all three treatments. In the AS, $\delta^{15}\text{N}$ increased from -33.5 to -7.5‰. The time gradient of $\delta^{15}\text{N}$ was 6.5‰ d⁻¹ and the calculated $\Delta\delta^{15}\text{N}$ of N₂O on the first sampling day was -58.7‰ (= difference between $\delta^{15}\text{N}$ of N₂O and $\delta^{15}\text{N}$ of AS of which the $\delta^{15}\text{N}$ was 25.2‰). In the CN ($\delta^{15}\text{N}$ = 9.0‰), $\delta^{15}\text{N}$ increased from -7.0 to 1.6‰ with a time gradient of 2.2‰ d⁻¹ and the calculated $\Delta\delta^{15}\text{N}$ on the first sampling day was -16.0‰. In the CN + C, $\delta^{15}\text{N}$ increased from -1.3 to 15.2‰ with a time gradient of 8.3‰ d⁻¹ and the calculated $\Delta\delta^{15}\text{N}$ on the first sampling day was -10.3‰ (Fig. 4b). The $\delta^{15}\text{N}$ of the third sampling event was 6.2‰ higher than that CN and we attribute this to the enrichment of ¹⁵N in the remainder of the soil NO₃⁻ by the same mechanism of enrichment as discussed for ¹⁸O above.

Our $\Delta\delta^{15}\text{N}$ values on the first sampling day in the AS agree very well with the reported $\Delta\delta^{15}\text{N}$ values for nitrification (-111 to -35‰)^{25,26}, indicating that nitrification was the main source of N₂O. The $\Delta\delta^{15}\text{N}$ values in the CN are within the range of reported $\Delta\delta^{15}\text{N}$ values for denitrification, and in the CN + C they were close to the lower end of the range (-45 to -13‰)^{25,26}, indicating that denitrification might be an important source of N₂O in the former. We attribute the relatively low enrichment factor in the latter to the reduction of N₂O resulting in $\delta^{15}\text{N}$ enrichment of the remaining N₂O. In the CN + C treatment a plot of $\delta^{18}\text{O}$ versus $\delta^{15}\text{N}^{\text{bulk}}$ of N₂O gave a slope of 0.89 revealing that N₂O reduction might have occurred to some extent (Fig. 4e)²⁷.

In the AS, SP remained constant with a mean value of 15.4‰ (ranging from 12.7 to 17.1‰). The CN SP showed an increasing trend and the mean value was 15.0‰ (ranging from 11.4 to 23.5‰). In the CN + C, SP decreased slightly over time with a mean value of -2.4‰ (ranging from -5.4 to 0.1‰) (Fig. 4c). Our SP of the

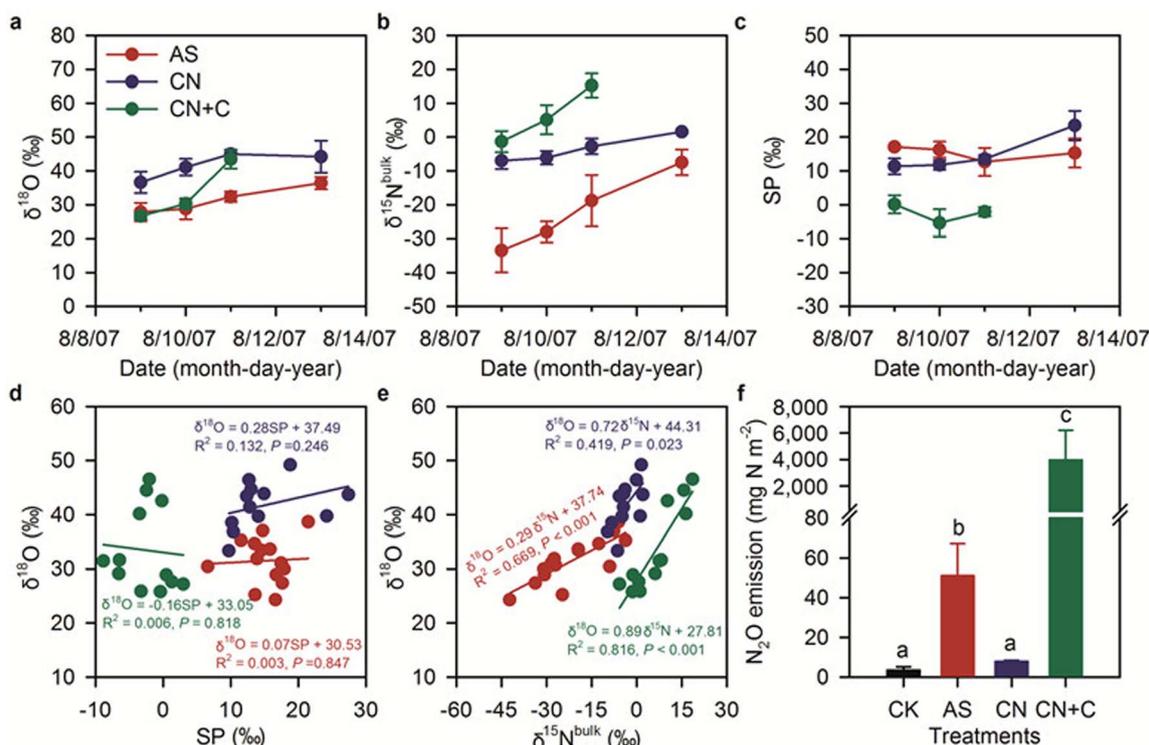


Figure 4 | Isotopic fingerprints and emissions of N₂O. (a), $\delta^{18}\text{O}$. (b), $\delta^{15}\text{N}^{\text{bulk}}$. (c), $\delta^{15}\text{N}$ site preference (SP). (d), $\delta^{18}\text{O}$ (vs. SMOW) versus SP. (e), $\delta^{18}\text{O}$ versus $\delta^{15}\text{N}^{\text{bulk}}$. (f), cumulative N₂O emissions. AS, CN, CN + C, and CK denote amendment with ammonium sulfate, calcium nitrate, and calcium nitrate + glucose + compaction of soil, and control, respectively. Error bars in plots (a), (b), (c), and (f) are standard deviation of the mean (n = 4). Note that data points are individual observations of the treatments in plots (d) and (e).



soil favoring denitrification (CN + C) was very consistent with the SP of N₂O produced by bacterial denitrification in pure culture studies (0 to -10‰)^{20,28}. In the AS, SP was lower than typical results reported for the NH₂OH-to-N₂O pathway from pure cultures of nitrifiers (33‰) but was higher than the SP of denitrifiers or nitrifier denitrification (0 to -10‰)^{20,28}. Similar results were observed in our earlier soil incubation study in which the same soil was incubated at 45% WFPS and amended with AS and the observed SP was 18.4‰¹¹. Therefore, both NH₂OH oxidization and NO₂⁻ reduction contributed substantially to the release of N₂O from the AS. Furthermore, the positive correlation between δ¹⁸O and δ¹⁵N^{bulk} with a slope of 0.29 (*P* < 0.001) of the N₂O also indicates that nitrifier denitrification was a significant N₂O source (Fig. 4e)^{29,30}. In addition, the lack of a relationship between δ¹⁸O and SP indicates a constant relative contribution of NH₂OH oxidization and NO₂⁻ reduction to the gross N₂O production in AS and CN + C (Fig. 4d) because the SP values for these two N₂O production processes are constant while their δ¹⁸O values are variable due to the different O sources and to O exchange with H₂O as well as the variable δ¹⁸O of the O sources^{31,32}.

In the present study the SP value of soil-derived N₂O in the AS treatment indicates that both NH₂OH oxidization (NN) and NO₂⁻ reduction (including ND and DD) processes contribute substantially to total N₂O production. Moreover, our molecular analysis also shows that it was nitrifiers that dominated NH₄⁺ transformation (see paragraph below). Evidently, the additional N₂O derived from NO₂⁻ reduction in the AS treatment compared to the CN treatment can be attributed to the ND process. Therefore, we calculate the fertilizer-induced N₂O generating processes in the AS treatment according to the following equations:

$$F_{AS} = F_{AS(\text{Total})} - F_{CK} = F_{AS(\text{NN})} + F_{AS(\text{ND})} + F_{AS(\text{DD})} \quad (1)$$

$$F_{AS(\text{DD})} \approx F_{CN(\text{Total})} - F_{CK} \quad (2)$$

$$F_{AS(\text{NN})} = F_{AS(\text{Total} - \text{NN})} - F_{CK(\text{NN})} \quad (3)$$

$$F_{CK(\text{NN})} \approx F_{CN(\text{NN})} \quad (4)$$

$$F_{AS(\text{Total} - \text{NN})} = F_{AS(\text{Total})} \times f_{\text{NN}}; F_{CN(\text{NN})} = F_{CN} \times f_{\text{NN}} \quad (5)$$

$$f_{\text{NN}} = (\text{SP}_{\text{bulk}} - \text{SP}_{\text{denit}}) / (\text{SP}_{\text{nit}} - \text{SP}_{\text{denit}}) \quad (6)$$

where *F* is cumulative N₂O emissions, AS is ammonium sulfate, CN is calcium nitrate, Total is the gross N₂O including both fertilizer derived and background emissions, CK is the control treatment, NN is nitrifier nitrification, ND is nitrifier denitrification, DD is denitrifier denitrification, and Total-NN is NN produced N₂O emissions including both fertilizer and background derived. *f*_{NN} is the fraction of N₂O derived from the NN process, SP_{bulk} is the measured SP value of N₂O, SP_{nit} and SP_{denit} are the SP values for nitrification and denitrification processes, respectively. There are two assumptions needed to proceed to the source partitioning. First, the DD derived N₂O was equivalent to Ca(NO₃)₂ fertilizer derived N₂O in the treatment receiving CN only. This assumption is reasonable because most of the applied NH₄⁺ was transformed into NO₃⁻ as shown in our microcosm studies, and denitrification thus contributed to the total N₂O emission of the AS treatment. Second, that NN process derived N₂O in the control was equivalent to that of NN in the treatment receiving Ca(NO₃)₂ only. Then we adopt flux-weighted SP value and the above mass-balance equation with two end-members to calculate the relative contribution of NN, ND, and DD to the gross (NH₄)₂SO₄ and Ca(NO₃)₂ derived N₂O of the fertilizer treatments, respectively. In the two end-members equation we adopt 33‰ as the SP for nitrification and 0‰ or -10‰ for denitrification in two rounds of calculation^{19,20,28}. Finally, we show that NN, ND, and DD account for

35–53%, 44–58%, and 2–9% of (NH₄)₂SO₄ derived N₂O emissions, respectively.

N₂O-generating microorganisms and functional genes. To further understand the linkage of functional genes associated with N₂O production we investigated the community structure and the abundance of the ammonia oxidizing bacteria (AOB) and denitrifiers during the crucial periods of N₂O emission. The treatments were zero-N (Control, CK), NH₄⁺-based N (AS), NH₄⁺-based N + DMPP (AS + NI), NO₃⁻-based N (CN), and NO₃⁻-based N + glucose + more water (CN + C). The N₂O emission patterns in all treatments were very similar to those previously reported^{13–15} (Fig. S7).

A high abundance of *amoA* gene copy numbers for AOB was found in AS treatment but DMPP clearly suppressed *amoA* gene copy numbers and also impacted *nirK/nirS* gene copy numbers to some extent (Fig. S8a). CN + C treatment increased *nirK/nirS* gene copy numbers considerably but effects from the CN treatment were negligible (Fig. S8b,c). Community structures of AOB in AS and AS + NI treatments in the pivotal period of N₂O emission were similar to that in the CK treatment at each sampling time (Fig. S9a). There were no changes in the *nirK* and *nirS* gene community structure under various N-fertilizer treatments during all crucial periods of N₂O emission (Fig. S9b,c).

The phylogenetic tree revealed that all AOB sequences were affiliated with *Nitrosospora* or *Nitrosospora*-like species (Fig. S10). The results indicate that *Nitrosospora* species are ubiquitous in the soil studied and this is consistent with the results of other studies conducted in this region³³ and the findings in most soil media worldwide³⁴. The dendrogram of deduced amino acids shows five major clusters of *nirK* sequences (I to V) (Fig. S11). The great majority of clones were closely related to *nirK* of *Rhizobiales* as well as clones which belonged to cluster II³⁵. However, these *nirK*-harboring denitrifiers do not belong to *Rhizobiales* because *nirK* phylogeny is incompatible with 16S rDNA phylogeny³⁶. The *nirS* tree was divided into four clusters (clusters I–IV) (Fig. S12). The major *nirS* clones were grouped in clusters I and IV and were primarily related to *nirS* of *Rhodobacteraceae* and *Rhodocyclaceae*³⁷. Only two *nirS* (102 bp) clones were grouped in cluster II; while the remaining clones belonged to cluster III closely related to the *nirS* of *Micrococcaceae* and *Burkholderiaceae*³⁸.

Conceptual model of N₂O generation and regulation. Fig. 5 presents a summary conceptual model of N₂O generation and regulation in this calcareous soil. Ammonia oxidation is the first N₂O-generating process. It is regulated by NH₄⁺-N concentration and soil moisture and temperature and can be efficiently inhibited using nitrification inhibitors. Ammonia oxidation occurs in aerobic conditions but consumes oxygen significantly and accumulates NO₂⁻ temporarily, leading to suboxic conditions that induce a secondary nitrifier-denitrification process to generate N₂O with high NH₄⁺ concentrations in the soil matrix. Denitrifier denitrification is less active under normal field conditions with high O₂ concentrations in the light textured soil which has low moisture during most of the year and low available carbon. Due to the very low denitrification activity the soil profile normally accumulates large amounts of NO₃⁻-N which presents great leaching potential to the shallow groundwater when surplus N is present in intensively managed high N-input agricultural systems^{4,13,39,40}. We conclude that N₂O emissions are due mainly to the activities of nitrifiers in this calcareous fluvo-aquic soil with 35–53 and 44–58% of N₂O produced by nitrification and nitrifier denitrification, respectively.

Discussion

Collectively, all of our evidence points to NN and ND as the dominant N₂O generation processes in this intensively managed calcareous

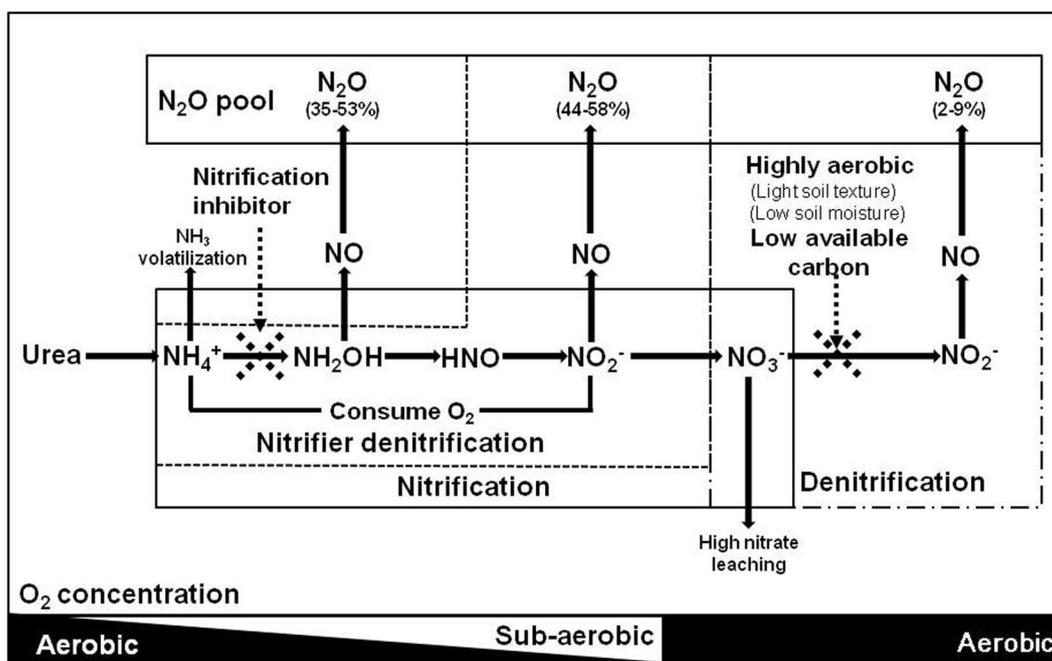


Figure 5 | Conceptual model of N₂O generation in the intensively managed calcareous fluvo-aquic soil.

fluvo-aquic soil with DD playing a minor role. Our results are further supported by a recent report showing that ammonia oxidation pathways (NN and ND) are important sources of N₂O under low oxygen availability following urea or ammonium sulfate application to loam, clay and sandy loam soils (that also contain ample nitrate) by using advanced ¹⁵N-¹⁸O isotope and NH₃ oxidation inhibition approaches⁴¹. The weak contribution of DD to N₂O emission was substantiated by the weak response of N₂O emissions to soil NO₃⁻ content in our pot incubation experiments and in the robotized incubation experiments and the very low N₂O emission factors of the soil receiving NO₃⁻ based fertilizer in the field^{13,14}. Although DD can proceed in anaerobic microsites in the soil matrix according to numerous reports^{42,43}, the weak contribution of DD to N₂O emission in the soil studied was ultimately attributable to the lack of anaerobic microsites for triggering the reduction of NO₃⁻ and thus the subsequent sequential reduction of N oxides by denitrifiers and the associated N₂O production. The weak contribution of DD to N₂O emission on the North China Plain has also been reported in a previous study and has been attributed mainly to the low content and availability of soil organic carbon for denitrifiers¹². However, our robotized incubation experiments demonstrate that DD can proceed immediately once the oxygen consumption has lowered O₂ to a low level. The importance of the soil redox conditions on the process of DD outweighs that of dissolved organic carbon (DOC) limitation as demonstrated in *RIE2* within which N₂O emission responded weakly to the simultaneous addition of NO₃⁻ and glucose under the normal oxygen levels. Therefore, the foremost influence of DOC on DD in this region might be its indirect effects on creating anaerobic soil conditions by consumption of soil O₂ and the formation of soil aggregates within which anaerobic microsites tend to result^{42,44}.

The dominant contribution of N₂O production pathways, including NN and ND, to N₂O emissions from NH₄⁺ or urea-based fertilizer was substantiated by the observed intensive and proportional response of N₂O emissions to soil NH₄⁺ and NO₂⁻ contents in our laboratory and field studies. The overwhelming importance of the NH₄⁺ oxidation pathway on N₂O emissions in the present soil has also been verified by two of our earlier ¹⁵N-labeled laboratory incubation investigations in one of which NH₄⁺ oxidation accounted for 88% of total N₂O emissions at 45% WFPS¹¹, and in the other NH₄⁺

oxidation accounted for 85 to 95% and 83 to 94% of the total N₂O emissions at 40% and 60% WFPS¹², respectively. These two studies also demonstrate that the relative contribution of nitrification and denitrification to gross N₂O emissions was quite constant across this relatively large range of water content in these soils. Surprisingly, the relative contributions of nitrification and denitrification reported in these two studies agree well with our present field study in which NH₄⁺ oxidation and DD accounted for nearly 90 and 10% of total (NH₄)₂SO₄-derived N₂O emissions, respectively. However, neither of our former ¹⁵N-labeled studies discriminated between the contributions of NN and ND to N₂O derived from NH₄⁺ oxidation.

It has long been debated how microbial community structure or abundance and ecosystem functions are coupled. Our study suggests that environmental factors (mainly soil N content) affected the abundance of AOB and denitrifiers harboring *nirK* and *nirS* genes positively and linkages occur between N₂O emissions and the abundance of active AOB and denitrifiers harboring *nirK*, and *nirS* genes rather than their community composition. There was a striking variation in the abundance of AOB and denitrifiers in our different treatments and the shifts coincided with N₂O emission patterns, suggesting that shifts in active abundance play a relatively important role in determining N₂O emissions under different N, C, and water management strategies.

The potential for nitrifiers to proceed with denitrification and associated N₂O production has been recognized for a long time^{7,45,46} and was recently verified to be a prevalent trait of the beta-proteobacterial AOB such as *Nitrosomonas europaea* and *Nitrosospora* spp. in pure culture investigations⁴⁷ and to be a significant N₂O source in soil incubation investigations^{37,48,49}. In our field study all of the AOB sequences were affiliated with *Nitrosospora* or *Nitrosospora*-like species, allowing us to elucidate the processes of N₂O production as well as the associated SP values for NN, ND, and DD.

We did not identify the response of ammonia-oxidizing archaea (AOA) to N fertilization and it has been reported that AOA are ubiquitous in soils but they do not respond to NH₄⁺ oxidation and N₂O production in intensively managed agricultural soils²¹. The contribution of archaea to the SP value of the associated N₂O is not yet clear^{21,50,51}. A recently reported SP value of N₂O from marine AOA is virtually identical to that of AOB³⁴. Up to now the



soils where AOA have a significant impact on NH_4^+ oxidization seem to be mainly confined to acid types⁵². One study conducted in our region also revealed that *Nitrosospora*-like AOB were dominant over AOA in oxidizing NH_4^{+33} .

We did not have the direct evidence to show that the NO_2^- produced by ammonia oxidization will not be utilized by denitrifiers in CND process, but is utilized instead by nitrifiers in ND. However, it produces different isotopic and isotopomeric values, which gave rise to the separation in the first place for isotopic studies. In addition, our soil molecular results showed that only the nitrifiers respond to the NH_4^+ -based fertilizer application and the denitrifiers only respond to the NO_3^- -based fertilizer together with glucose application. These results reinforce our conclusion that the reduction of NO_2^- in our soil should mainly through ND pathway while the contribution of DD pathway might be minor. Some previous studies have concluded that CND is not a separate process⁷ but instead comprises rapid denitrification of the nitrification byproduct and end product (NO_2^- & NO_3^-)⁵³. This process has been found in paddy soils⁵³, wastewater⁵⁴ and soil aggregates⁵⁵ when favorable environmental conditions for both nitrification and denitrification prevail, but the soil-climatic conditions in our study are very favorable for nitrification processes¹³.

The conventional wisdom, based on research on soils with higher readily decomposable carbon, higher moisture contents and relatively low pH values in Europe, is that denitrification by denitrifiers is the dominant soil N transformation process leading to N_2O emissions. In contrast, the soil type in our studies (calcareous fluvo-aquic soil) is widely distributed among agricultural soils globally and is frequently associated with semi-arid or sub-humid continental climates, and typically has high pH, low available C, aerobic and low moisture conditions. The dominant effect of nitrifiers and the importance of their denitrification function and the consequentially large amounts of N_2O emissions may be phenomena that are widespread globally, at least in similar edaphic-climatic conditions. Our understanding of N_2O production processes can contribute to the development of efficient mitigation strategies for these land areas, i.e. to slow down nitrification and avoid high ammonium concentrations in the soil matrix.

Methods

Study sites. Two sites were used in the present study, one located at Dongbeiwang town, Beijing (40°00' N, 116°12' E) and the other at Quzhou county, Hebei Province (36°52' N, 115°10' E), China. Fields at the two sites have typical intensively managed agricultural soils on the North China Plain where a winter wheat-summer maize rotation is the dominant crop production system and urea and NH_4^+ -based fertilizer are the most commonly applied nitrogen fertilizers. The soils are calcareous Fluvo-aquic soils (calcareous Cambisols according to the FAO Classification). Soil properties in the upper 20 cm at the Dongbeiwang and Quzhou sites were: texture 27/57/16 and 39/58/3 (sand/silt/clay, %); bulk density 1.37 and 1.34 g cm^{-3} ; CaCO_3 content 52.5 and 58.2 g kg^{-1} ; DOC 51 and 43 mg C kg^{-1} ; pH (1:2.5, soil/water) 7.9 and 8.2; EC 0.18 and 0.16 mS cm^{-1} ; total N 1.2 and 0.7 g kg^{-1} ; organic matter 21.3 and 14.7 g kg^{-1} , respectively.

Pot incubation experiments (PIE). Three PIEs were conducted to investigate the response of N_2O emission to soil water content (PIE1), to NH_4^+ input rate (PIE2), and to varying nitrogen and carbon source supply (PIE3). Surface soils (0–20 cm) used for PIEs were collected from Quzhou site at the beginning of April, 2010. In PIE1, it was a completely factorial design with three factors, namely control, urea (applied at a rate of 40 mg N kg^{-1} dry soil), urea plus straw (applied at a rate of 1.0 g C kg^{-1} dry soil), and within each factor five classes of soil water content, 30, 50, 70, 90, 110% WFPS were included (Table S1). Incubation was conducted for 15 days. In PIE2, nine classes of N input rate, 0, 10, 20, 40, 80, 160, 240, 320, 400 mg N kg^{-1} dry soil were selected and $(\text{NH}_4)_2\text{SO}_4$ was the selected N fertilizer. Incubation was conducted at a water content of 70% WFPS for 18 days. In PIE3, soils were amended with and without N fertilizer (urea, applied at a rate of 40 mg N kg^{-1} dry soil) and wheat straw (applied at a rate of 1.0 g C kg^{-1} dry soil) and nitrification inhibitors (DMPP and DCD) (Table S2). Incubation was conducted at a water content of 70% WFPS for 16 days. All incubations were conducted aerobically and in the dark at 20°C with three replicates of each treatment. N_2O emissions were measured periodically by gas chromatography (GC). Detailed information about the operation of PIEs and N_2O measurements can be found in the SI.

Robotized incubation/analytical experiment (RIE). Detailed information about the robotized incubation/analytical system has been given by Molstad *et al.*¹⁶. In brief, the robotized system comprises an incubation system and a gas analysis system. The headspace air of the incubation flasks was replaced with a mixture of helium (He) and oxygen (O_2) at the desired proportions. Gas in the headspace of each flask was sampled every 8 hours by CTC GC-PAL (delivered by Agilent) and analyzed by gas chromatography (Agilent GC 7890A) for the concentrations of N_2O , N_2 , O_2 , and CO_2 and by NO analyzer for NO. Two RIEs were conducted to investigate the stoichiometric relationship between N input rate and gaseous (N_2O , NO, N_2 , O_2 and CO_2) flux (RIE1) and the response of these gas fluxes to the type of N and C sources and inhibitors (RIE2). Soils used for RIEs were collected from the Quzhou site in April 2011. In RIE1, six classes of N input rate, 0, 20, 40, 80, 160, and 320 mg N kg^{-1} dry soil were adopted and urea was selected as N fertilizer. In RIE2, six treatments were set up, including control (CK), amended with $(\text{NH}_4)_2\text{SO}_4$ at a rate of 40 mg N kg^{-1} dry soil (AS), amended with nitrification inhibitor DMPP on the basis of AS (AS + NI), amended with $\text{Ca}(\text{NO}_3)_2$ at a rate of 40 mg N kg^{-1} dry soil (CN), amended with glucose (1.0 g C kg^{-1} dry soil) on the basis of CN (CN + C), and anaerobic incubation on the basis of CN + C (CN + C- O_2). RIEs were conducted in a thermostatic water bath at 20°C and at a soil water content of 70% WFPS with four replicates for 256 and 272 h for RIE1 and RIE2, respectively.

Isotopic fingerprint experiment. The experiment was conducted in the field at Dongbeiwang site in the maize season in 2007, based on Field study 2 reported in Ju *et al.*¹³ of which three treatments (AS, CN, and CN + C) were selected to measure the N_2O isotope signatures of soil gases (Table S3). In summary, treatment AS was a typical N fertilizer management practice for maize on the North China Plain, i.e. application of NH_4^+ -based N fertilizer ($(\text{NH}_4)_2\text{SO}_4$ in the present study, 150 kg N ha^{-1} , the same rates as other fertilizer treatments) followed by irrigation. Treatment CN was amended with $\text{Ca}(\text{NO}_3)_2$ and received the same amount of irrigation as treatment AS. Treatment CN + C was set up to create conditions favoring denitrification, i.e. applying NO_3^- -based fertilizer ($\text{Ca}(\text{NO}_3)_2$ in the present study) and glucose, compacting the soil, and increasing irrigation. Each treatment was replicated four times. The methods for collecting and storing gas samples for isotope analysis are introduced in detail in the SI. Isotopic signatures of N_2O ($\delta^{15}\text{N}^{\text{bulk}}$, $\delta^{15}\text{N}^{\text{z}}$, $\delta^{15}\text{N}^{\text{b}}$, $\delta^{18}\text{O}$) were determined by isotope ratio mass spectrometry (IRMS, Thermo-Finnigan, Bremen, Germany) and the results are expressed in δ -notation as per mil (‰) deviations from the isotopic ratios of atmospheric N_2 or standard mean ocean water (SMOW). For $\delta^{15}\text{N}^{\text{z}}$ and $\delta^{15}\text{N}^{\text{b}}$, pure N_2O was used as reference gas which was calibrated in the laboratory of N. Yoshida at the Tokyo Institute of Technology. The difference between the ^{15}N isotopomer ratios of the central and peripheral N of N_2O ($\delta^{15}\text{N}^{\text{z}}$ - $\delta^{15}\text{N}^{\text{b}}$) is referred to as ^{15}N site preference (SP). Detailed information on isotopic analysis of N_2O and calculations were given by Well *et al.*¹¹.

Microorganisms and functional gene analysis. Soils were collected from a field experiment conducted at Quzhou site in the maize season in 2009¹⁴. The field experiment consisted of five treatments (Table S4), namely (1) control treatment without N input (CK), (2) amended with NH_4^+ -based fertilizer (AS), (3) amended with nitrification inhibitor DMPP and NH_4^+ -based fertilizer (AS + NI), (4) amended with NO_3^- -based fertilizer (CN), (5) amended with glucose and NO_3^- -based fertilizer (CN + C). $(\text{NH}_4)_2\text{SO}_4$ and $\text{Ca}(\text{NO}_3)_2$ were used as the NH_4^+ - and NO_3^- -based N fertilizers (250 kg N ha^{-1}), respectively. The N fertilizers were split into two applications: 100 kg N ha^{-1} at the third-leaf stage (June 27th) and 150 kg N ha^{-1} at the tenth-leaf stage (July 29th). We directly irrigated 20 mm after each fertilization. Soil was sampled on two periods of strong N_2O emissions (July 1st and August 3rd) and two periods of low N_2O emissions (August 7th and September 26th). A DNA-based approach with real-time PCR and T-RFLP analysis was used to assess the abundance and community structures of the AOB and denitrifiers and focused on the amoA gene for AOB and the nirK/nirS gene for denitrifiers. Detailed information about microbial and functional gene analysis and N_2O emission measurements can be found in SI.

Statistical analysis. The primary data were treated using Microsoft Excel spreadsheets. The data shown in this paper were calculated by averaging three or four replicates. The cumulative N_2O , soil mineral N, gene copies and T-RFs among the different treatments were tested by analysis of variance and mean values were compared using SAS statistical software (Version 8.2; SAS Institute, Inc., Cary, NC) to calculate least significant difference (LSD) at the 5% level. Total N_2O fluxes and WFPS and NH_4^+ -N application rate were analyzed by regression using Sigmaplot 11.0 software (Systat Software Inc., Erkrath, Germany).

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Author contributions

X.T.J. designed the experiments. T.H., B.G. and X.L. carried out the experiments and performed the analyses. X.T.J., T.H., X.K.H., R.W., P.C. and L.R.B. substantially contributed to interpreting the results and writing the paper.



Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

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