



Article

Linking N₂O Emission with AOB and *nirK*-Denitrifier in Paddy Fields of Karst and Non-Karst Areas

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Abstract

Denitrification and nitrification are two pivotal microbial processes relating to N₂O emissions. However, the difference in N₂O emission fluxes and N₂O-producing bacteria between a karst (KA) and non-karst area (NKA) remains unclear. The objective of this study is to compare the differences in soil N₂O emissions, nitrifying bacteria, and denitrifying bacteria during the growth period of rice in KA and NKA, and to explore the mechanisms by which microorganisms and environmental factors drive N₂O emissions. Here, N₂O emission fluxes of paddy fields were collected using the static dark chamber and measured using gas chromatography at KA and NKA in the Maocun Karst Experimental Site in Guilin, China. The nitrifying bacteria (ammonia-oxidizing bacteria, AOB) and denitrifying bacteria (*nirK*-denitrifier) were determined using real-time PCR and high-throughput sequencing, respectively. Results showed that during the rice growth period, the N₂O emission fluxes in KA was generally lower than that in NKA, with cumulative N₂O emissions of −0.054 and 0.229 kg·hm^{−2} in KA and NKA, respectively. The absolute abundance of AOB in KA (8.91×10^6 – 2.68×10^7 copies·g^{−1}) was significantly higher than that in NKA (1.57×10^6 – 6.48×10^6 copies·g^{−1}), while the absolute abundance of *nirK*-denitrifier had no significant difference between the two areas. The composition and diversity of AOB and *nirK*-denitrifier differed significantly between KA and NKA. Results from partial least squares structural equation modeling (PLS-SEM) indicated that soil properties, carbon sources, and nitrogen sources had positive effects on AOB and *nirK*-denitrifier, while *nirK*-denitrifier had a negative effect on N₂O emissions. Partial least squares regression (PLSR) predictions revealed that NO₃[−]-N, SOC, TN, Mg²⁺, Ca²⁺, and pH were the most important factors influencing N₂O emission fluxes. This study highlights the critical role of the typical characteristics of KA soils in reducing N₂O emissions from paddy fields by driving the evolution of AOB and *nirK*-denitrifier.

Keywords: karst; paddy field; N₂O; AOB; *nirK*-denitrifier; Ca²⁺; pH



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1. Introduction

Nitrous oxide (N_2O) is the third greenhouse gas (GHG) after carbon dioxide (CO_2) and methane (CH_4) [1,2]. Soils contribute about 60% of global N_2O emissions [3], while agricultural soils account for nearly 84% of them [4]. Thus, studying the processes and mechanisms of N_2O emissions from farmland soils is of great importance.

Rice agriculture is predominantly in China and Southeast-Asian countries, supporting over 60% of the global population [5]. Long-term flooded cultivation has formed a specific soil type—paddy soil [5,6]. However, increasing rice demand and extensive nitrogen (N) fertilizer application [7] have made rice paddy a major source of N_2O emissions [8,9]. Therefore, investigating the processes and mechanisms of N_2O emissions from paddy soils is crucial for mitigating global climate change.

Nitrification is one of the core processes contributing to N_2O production [10–13]. The N-fertilizer and native N in soils provide available substrates for nitrification, initiating N mineralization [10,13] and thereby triggering N_2O emissions [14]. Soil organic carbon (SOC) is a key soil component, which directly influences soil physicochemical properties such as pH [15], which are major factors determining the potential for N_2O emissions [16]. In alkaline soils with high nitrification rates [12], N_2O emissions decrease with increasing SOC [12] and pH [17]. For instance, in neutral paddy soils, biochar addition can reduce N_2O emissions by increasing pH [18]. However, some studies report that cumulative N_2O emissions increase significantly with rising pH under aerobic conditions [12]. In summary, the nitrification controlled by soil physicochemical properties (such as pH, SOC, and N content) is a key process in N_2O emissions from agricultural soils, driven by the combined effects of N-fertilizer input and soil background N mineralization [19,20].

Ammonia-oxidizing bacteria (AOB) are important bacteria dominating the nitrification process [21,22] and are sensitively regulated by soil physicochemical properties. Studies show that AOB contribute to about 85% of nitrification-related N_2O emissions, which vary with soil pH [23]. In flooded and alkaline paddy soils [24] with high N [13,25], oxygenated zones near rice roots promote nitrification primarily mediated by AOB due to rhizosphere respiration, leading to N_2O emissions. Fertilizer-derived N_2O emissions are positively correlated with AOB abundance and negatively correlated with soil pH [14]. In acidic paddy soils, AOB are the primary ammonia-oxidizing microorganisms in both abundance and function [26] with autotrophic nitrification being dominant [27]. At different depths of soil profiles, AOB abundance is positively correlated with N_2O emissions [28]. Thus, alternating wet–dry conditions can change soil properties and oxygen availability, leading to variations in AOB abundance and community structure, thereby altering nitrification activity [26]. SOC, as a carbon and energy source for bacteria, also influences N_2O emissions [20]. These findings indicate that soil physicochemical properties regulate N_2O emissions during nitrification by influencing AOB community.

Denitrification and dissimilatory NO_3^- reduction to NH_4^+ are the other core processes contributing to N_2O emissions in agricultural soils [12,29], which is affected by environmental parameters, functional microbe composition, and key enzyme activities [19,20,29]. The flooded rice paddy creates anaerobic conditions that facilitate N_2O emissions via denitrification [24]. Biochar addition significantly reduces N_2O emissions from denitrification in acidic paddy soils [30]. Therefore, regulating N_2O emissions from denitrification through exogenous amendments is important for optimizing N-cycle management.

Denitrifying bacteria are key microbial groups in the denitrification process [21,22]. In agricultural soils, fertilizer application [31] and straw return [32] significantly alter soil physicochemical properties, carbon, and nitrogen levels (e.g., pH and SOC), strongly influencing the growth, population structure, composition, and diversity of *nirS*- and *nirK*-denitrifier [33,34], thereby changing N_2O emissions [14]. Flooded paddy fields host

diverse bacteria adapted to anaerobic conditions, particularly *nirK*-denitrifier [35], which is influenced by the changes in soil environment, such as water regime and root growth, thereby altering denitrification and N₂O emissions. Insufficient nutrients, for example dissolved organic carbon (DOC), could reduce *nirK*-denitrifier abundance, limit denitrification, and further decrease N₂O emissions [36]. The activity, rather than the abundance of *nirK*-denitrifier is a key factor determining N₂O emission potential [16], suggesting that their function is more important than their abundance.

In KA, intense dissolution of carbonates (mainly limestone and dolomite) results in soils with higher exchangeable Ca²⁺ and Mg²⁺ concentrations and pH [37,38]. SOC and total N (TN) in KA are also significantly higher [39,40], where Ca²⁺ and Mg²⁺ promote strong immobilization of organic matter, dissolved N, and phosphorus [39], changing soil N mineralization and nitrification [41] and potentially affecting N₂O emissions. It has been reported that denitrification controls N₂O emissions under high soil moisture conditions (70% water-filled pore space) in KA [20]. Thus, N₂O emissions from paddy soils with variable moisture conditions (flooding early and drainage later) may fluctuate significantly in KA. In summary, the unique physicochemical environment of paddy fields in KA likely influences the N-cycle by suppressing N mineralization and nitrification, ultimately regulating N₂O emissions.

Compared to NKA, the distinct soil properties of KA support unique microbial communities that have adapted over time to form stable assemblages. These microbes exhibit differences in C and nutrient utilization efficiency, leading to variations in microbial function [37]. In KA, vegetation drives the evolution of *nirK*-denitrifier by altering the rhizosphere environment [42], with dominant *nirK*-denitrifier such as *Bradyrhizobium* showing positive correlations with SOC and TN [43]. It has been reported that KA forest soils were inhabited significantly by more *nirK*-denitrifier such as Bacillales compared to NKA [39], while pH and nutrients are key factors influencing denitrification and N₂O emissions. However, the coupling relationships between soil physicochemical properties, AOB/*nirK*-denitrifier, and N₂O emissions during the rice growth period in KA and NKA remain unexplored.

Based on the above research, we hypothesize that significant differences in soil physicochemical factors (e.g., pH, SOC, and Ca²⁺) between KA and NKA drive notable changes in the community structure, composition, and diversity of AOB and *nirK*-denitrifier, thereby altering soil N₂O emissions. To test this hypothesis, this study was conducted at the Maocun Karst Experimental Site in Guilin, China, including KA and NKA. The following investigations were carried out: (1) measurement of soil physicochemical properties; (2) determination of N₂O emission fluxes and cumulative emissions; (3) analysis of the absolute and relative abundances of AOB and *nirK*-denitrifier communities; and (4) use of partial least squares structural equation modeling (PLS-SEM) and partial least squares regression (PLSR) to explore the underlying drivers of N₂O emission fluxes and cumulative emissions in KA and NKA.

2. Materials and Methods

2.1. Study Area and Sampling Site Description

The study area is 30 km from the Guilin city, within a subtropical monsoon climate zone. The region comprises both KA and NKA geological formations. The paddy fields in KA with an area of 127 m² (25°08'30" N, 110°31'28" E) and in NKA with an area of 140 m² (25°10'51" N, 110°31'35" E) were selected as experimental sites. Each field was divided into three replicate plots with the same area (KA: 42.20 m² and NKA: 46.67 m²) using PVC ridge. Each plot was subjected to uniform irrigation, fertilization, and management practices. The soil in KA is classified as limestone soil, while that in NKA is zonal silicate red soil. The rice growth cycle spanned 93 days for a single season. Prior to seedling

transplantation, 7.56 kg (KA) and 8.53 kg (NKA) of compound fertilizer ($\text{N-P}_2\text{O}_5\text{-K}_2\text{O}$, 18% each) was applied in each plot as basal fertilizer. On 14 July, an additional topdressing was applied at 5.70 kg (KA) and 6.40 kg (NKA) of the same compound fertilizer. The plot information can be found in reference [44].

2.2. Sample Collection and Preparation

To characterize variations in soil properties and communities of AOB and *nirK*-denitrifier during the rice growing, soil samples were collected on eight dates: 4 June (transplanting), 12 June (seedling), 26 June (tillering), 10 July (tillering), 24 July (jointing), 7 August (booting), 21 August (heading), and 4 September (maturity). Three adjacent soil samples of 0–20 cm in each plot were collected and thoroughly mixed and then transported to the laboratory quickly. Visible plant roots, debris, and other extraneous materials were removed from samples. All samples were divided into two subsamples: one was stored at $-80\text{ }^{\circ}\text{C}$ for microbial analysis, while the other was air-dried for the determination of physicochemical properties and nutrient content.

2.3. Analysis of Physicochemical Properties

Soil physicochemical properties were analyzed by routine methods [45,46]. Soil water content was determined using the oven-drying method. Soil pH was measured directly with a Leici PHS-3E pH meter (Shanghai Yidian Scientific Instrument Co., Ltd., Shanghai, China), using CO_2 -free distilled water as the extractant at a water-to-soil ratio of 2.5:1. Soil organic carbon (SOC) was analyzed by the potassium dichromate external heating method with concentrated sulfuric acid [47]. Dissolved organic carbon (DOC) was extracted using the water–soil oscillation method using an analyzer (Analytikjena C3100, Jena, Germany). Total nitrogen (TN) was determined by the Kjeldahl method with concentrated sulfuric acid digestion. Alkali-hydrolyzable nitrogen (AN) was measured using the alkali diffusion method. Ammonium nitrogen ($\text{NH}_4^+\text{-N}$) was analyzed by the Nessler's reagent colorimetric method, and nitrate nitrogen ($\text{NO}_3^-\text{-N}$) was determined using ultraviolet spectrophotometry. Total phosphorus (TP) was measured by molybdenum–antimony anti-colorimetry after digestion with concentrated H_2SO_4 and HClO_4 . Available phosphorus (AP) was analyzed using the Olsen-P method. Cation exchange capacity (CEC) was determined by the rapid EDTA-ammonium salt method using a UV-Vis spectrophotometer (METASH UV-9000S, Shanghai, China) [48].

2.4. Measurement of N_2O Emissions

A static dark chamber assembled with a length, width, and height of 50 cm was used for in situ collection of gas. The chamber consists of a box body made from PVC material with a fan for stirring the gas inside and a base made from stainless steel material. A regular grid was established in both KA and NKA to ensure that gas collection points represent the entire area variability. Each chamber was randomly allocated to each plot in the grid and placed at the center of plot. The gas sample was collected at 9:00–11:00 A.M. while collecting soil samples. The gas was collected in a 60 mL syringe at 0, 10, 20, and 30 min after covering the upper box and then transferred into gas sampling bags. The gas samples were immediately brought back to the laboratory for measurement and analysis through a gas chromatography–mass spectrometry system (Agilent 7890 B, Shanghai, China). N_2O concentrations were determined using an electron capture detector (ECD) with a detection limit of $1\text{ }\mu\text{g}\cdot\text{L}^{-1}$. A standard calibration curve was generated after every 48 samples analyzed. Each gas sample was measured twice. If the relative deviation between the two data is less than 5%, the average value is taken as the final value of the sample. If the deviation exceeds 5%, a third measurement was performed and the average of the two closest data points was used as the final value of the gas sample. Before measuring

samples of each batch, a calibration curve containing five concentration points (standard gas of N₂O at 0.2, 0.5, 1.0, 1.5, 2.0 ppm) was run, and the linear regression coefficient (R^2) of the calibration curve was all ≥ 0.995 . At the beginning and end of each batch of sample measuring, run a high-purity N₂ ($\geq 99.999\%$) as a blank to ensure that the entire analysis system was free of contamination. The measured value was only adopted when the R^2 was ≥ 0.90 . The N₂O emission fluxes (dc/dt) was calculated based on the slope derived from linear regression analysis of four consecutive sample concentration values. The N₂O emission fluxes was computed using the following formula [49]:

$$F = H \cdot \frac{MP}{R(273 + T)} \cdot \frac{dc}{dt}$$

In the formula

F represents the gas emission fluxes ($\text{mg} \cdot \text{m}^{-2} \cdot \text{h}^{-1}$);

H denotes the height of the sampling chamber (m);

M is the molar mass of the gas ($\text{g} \cdot \text{mol}^{-1}$);

P indicates the atmospheric pressure at the sampling site (Pa);

R is the universal gas constant ($8.314 \text{ Pa} \cdot \text{m}^3 \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$);

T represents the average temperature inside the chamber during sampling ($^{\circ}\text{C}$);

dc/dt refers to the gas emission fluxes ($\mu\text{L} \cdot \text{L}^{-1} \cdot \text{min}^{-1}$);

The cumulative N₂O emission is expressed in $\text{kg} \cdot \text{hm}^{-2}$.

2.5. Calculation of Global Warming Potential

The global warming potential (GWP) was calculated using the following formula [50]:

$$\text{GWP} = F_{\text{N}_2\text{O}} \times 298$$

where, expressed in terms of CO₂ emissions, the GWP of a unit mass of N₂O is 298 times that of CO₂ [2]. $F_{\text{N}_2\text{O}}$ denotes the cumulative N₂O emission ($\text{kg} \cdot \text{hm}^{-2}$).

2.6. Quantification of AOB and *nirK*-Denitrifier Abundance

The absolute abundances of AOB and *nirK*-denitrifier were determined using real-time PCR (qPCR). Genomic DNA was extracted using a commercial extraction kit. After assessing purity and concentration, the DNA was used as a template for PCR amplification with barcode-specific primers targeting the selected regions, using TaKaRa Premix Taq[®] Version 2.0 (TaKaRa Biotechnology Co., Dalian, China). The primer pairs used were as follows: AOB: amoA-F (5'-GGG GTT TCT ACT GGT GGT-3') and amoA-1R (5'-CCC CTC KGS AAA GCC TTC TTC-3') [51]; *nirK*-denitrifier: *nirK*-583F (5'-TCA TGG TGC TGC CGC GYG ANG G-3') and *nirK*-909R (5'-GAA CTT GCC GGT KGC CCA GAC A-3') [52]. The amplification program consisted of initial denaturation at 95 $^{\circ}\text{C}$ for 3 min, followed by 40 cycles of denaturation at 95 $^{\circ}\text{C}$ for 15 s, and annealing/extension at 60 $^{\circ}\text{C}$ for 30 s. PCR products were verified by agarose gel electrophoresis. The amplification efficiencies for AOB and *nirK*-denitrifier were 95% and 91%, with R^2 values of 0.933 and 0.928, respectively.

High-throughput sequencing of AOB/*nirK*-denitrifier was conducted by Guangdong Magigene Biotechnology Co., Ltd. (Foshan, China). Each soil sample was sequenced in triplicate, then used for library construction and sequencing. The amplicon libraries were sequenced on the Illumina Nova 6000 platform (San Diego, CA, USA). Data processing was performed using the mothur v.1.48.0 online software. Paired-end reads were merged using FLASH, followed by removal of low-quality bases and adapter-contaminated sequences. ASVs (Amplicon Sequence Variants) were clustered at 97% similarity using the UPARSE algorithm. Representative ASV sequences were selected, and chimeras were removed.

before data resampling. Taxonomic identification was performed by alignment against the FunGene database.

2.7. Data Analysis and Visualization

Initial data processing was performed using Microsoft Excel 2010 (Redmond, WA, USA). Significant differences in soil physicochemical properties, N₂O emission fluxes, and abundances of AOB/*nirK*-denitrifier between KA and NKA were analyzed by one-way ANOVA-LSD in SPSS 19.0 (IBM Corporation, Armonk, NY, USA). Bar charts of relative bacterial abundances and graphs of N₂O emission fluxes and cumulative emissions and their correlations were generated using Origin 9.5 (OriginLab Corporation, Northampton, MA, USA). Pearson correlation analysis in SPSS 19.0 (IBM, USA) was conducted to examine relationships between bacterial abundances and N₂O emission fluxes based on a 95% confidence interval. Bacterial diversity and heat maps illustrating correlations with soil physicochemical factors were computed and plotted using RStudio 2022.07.1+554 (Posit Software, PBC, Boston, MA, USA).

2.8. Correlation and Importance Prediction

Partial least squares structural equation modeling (PLS-SEM) and partial least squares regression (PLSR) were performed in RStudio 2024.12.1 to evaluate the correlations and relative importance of soil physicochemical factors, AOB/*nirK*-denitrifiers, and N₂O emission fluxes in KA and NKA. The 500 bootstrap resampling iterations were used to evaluate the stability of the PLS-SEM model. The robustness of the model parameters was verified by calculating the confidence interval and statistical significance of the path coefficients. At the same time, the composite reliability (CR > 0.7), average variance extraction rate (AVE > 0.5), and cross-validity of the measurement model were evaluated to ensure the reliability of construct measurement.

3. Results and Analysis

3.1. Soil Factors and Physicochemical Properties

The pH, TP, AP, and exchangeable Ca²⁺ and Mg²⁺ were significantly higher in KA than those in NKA (Table A1). More of sample's nutrients on eight dates, including SOC, TN, AN, NO₃[−]-N, and CEC (but NH₄⁺-N) in KA were significantly higher than that in NKA, indicating a generally higher nutrient level in KA (Table A1). The average values of pH, SOC, TN, NO₃[−]-N, TP, AP, CEC, exchangeable Ca²⁺, and exchangeable Mg²⁺ across the rice growth in KA remained significantly higher than those in NKA (Table A1). These results demonstrate that KA overall exhibited higher pH, nutrients, and CEC compared to NKA.

3.2. N₂O Emission Fluxes and Cumulative Emissions in KA and NKA

The N₂O emission fluxes On 4 June (seedling transplanting), 26 June (tillering stage), 3 July (tillering stage), 17 July (jointing stage), and 31 July (booting stage) in KA were significantly lower than those in NKA, while no significant differences were on the other nine dates (Figure 1). The cumulative N₂O emissions were −0.054 kg·hm^{−2} in KA and 0.229 kg·hm^{−2} in NKA throughout the rice growth period. Thus, the KA soil is a net sink for N₂O, while the NKA soil is a net source. The global warming potential (GWP) contributed by N₂O was −16.092 kg·hm^{−2} and 68.242 kg·hm^{−2} for KA and NKA paddy soils, respectively.

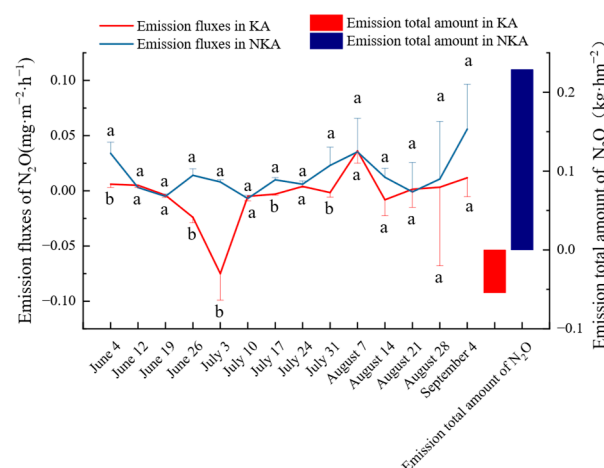


Figure 1. Emission fluxes and total amount of N_2O from paddy soil in KA and NKA. Note: Different lowercase letters represent significant differences in data at the 0.05 level.

3.3. Absolute Abundance of AOB/*nirK*-Denitrifier in KA and NKA

The absolute abundance of AOB was 8.91×10^6 – 2.68×10^7 copies·g $^{-1}$ in KA, which is higher than that in NKA (1.57×10^6 – 6.48×10^6 copies·g $^{-1}$), but there was no difference in the absolute abundance of *nirK*-denitrifier in between KA (1.30×10^7 – 3.82×10^7 copies·g $^{-1}$) and NKA (1.64×10^7 – 3.84×10^7 copies·g $^{-1}$) (Figure 2).

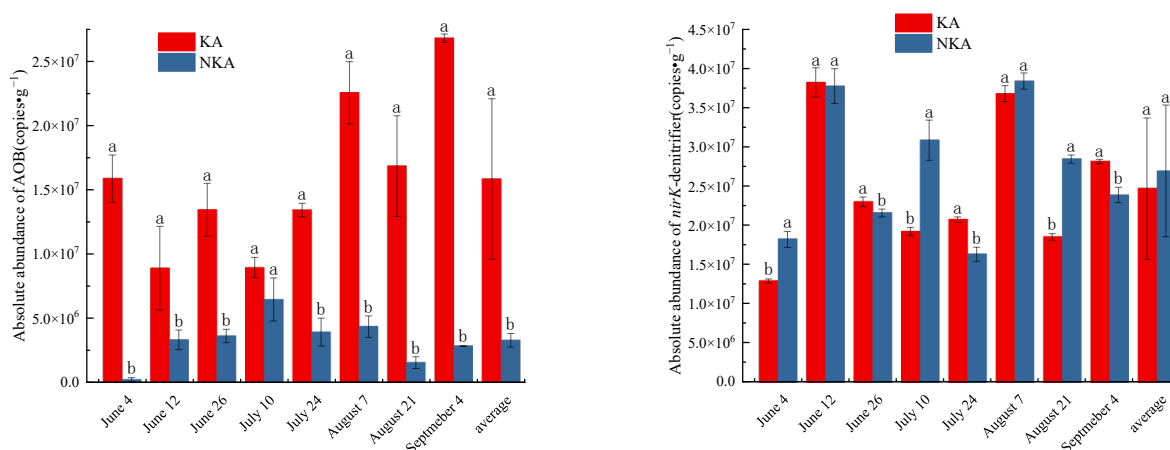


Figure 2. Absolute abundance of AOB and *nirK*-denitrifier in KA and NKA. Note: Different lowercase letters represent significant differences in data at the 0.05 level.

3.4. Community Structure of AOB in KA and NKA

The AOB at the genus level in KA was dominated by *Nitrosospira* and *Nitrosomonas*, with the relative abundances of 17.95–98.43% (average 71.48%) and 0–76.28% (average 22.04%), respectively (Figure 3). In NKA, the dominant AOB genus was the uncultured ammonia-oxidizing β -proteobacterium (ASV15), with a relative abundance of 71.62–98.74% and an average of 90.92% (Figure 3). These results indicate significant differences in the composition of AOB between KA and NKA. The relative abundance of *Nitrosospira* showed a significant positive correlation (** $p < 0.01$) with pH, TN, TP, AP, CEC, Mg^{2+} , and Ca^{2+} , and a significant positive correlation (* $p < 0.05$) with SOC (Figure 3), while the relative abundance of the uncultured ammonia-oxidizing β -proteobacterium (ASV15) was significantly negatively correlated ($p < 0.05$) with the aforementioned soil factors (Figure 3). The relative abundance of *Nitrosomonas* exhibited a significant negative correlation ($p < 0.05$) with $\text{NH}_4^+\text{-N}$ (Figure 3).

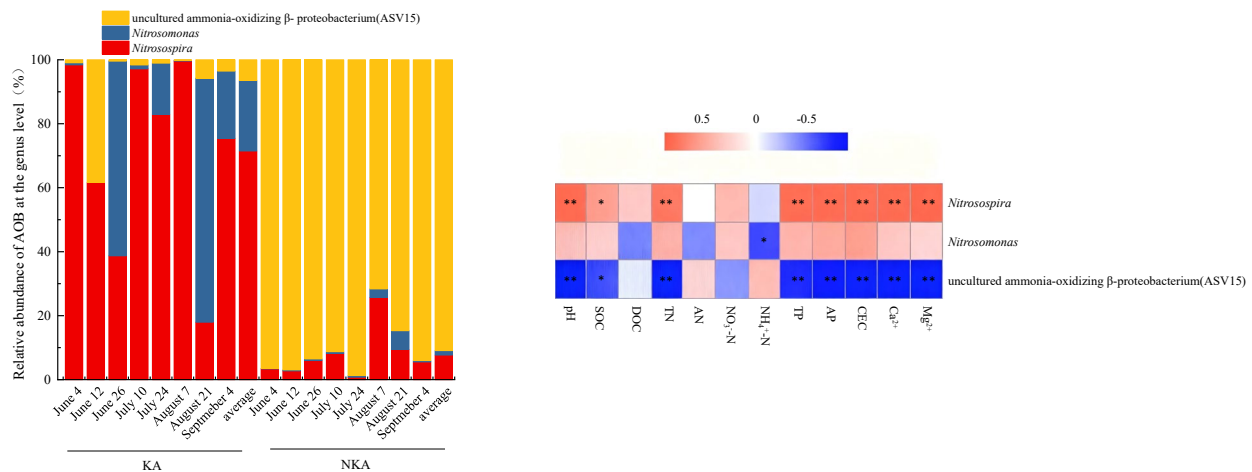


Figure 3. Relative abundance of AOB at the genus level and heat map of the correlation between the abundance of AOB and soil physicochemical factors in KA and NKA. Note: * and ** indicate significant correlations at the 0.05 and 0.01 probability levels, respectively.

The ASVs (Amplicon Sequence Variants) of AOB showed no significant differences between the two areas during the rice growth period, and exhibited considerable variability (Table A2).

3.5. Community Structure of *nirK*-Denitrifier in KA and NKA

Among the 12 predominance genera, the relative abundances of *Bradyrhizobium*, *Mesorhizobium*, *Aestuariivirga*, and *Bosea* were significantly higher in KA than those in NKA, indicating that the four genera are typical *nirK*-denitrifier in KA (Figure 4). All four belong to Hyphomicrobiales (order), and are classified into four families, Bradyrhizobiaceae, Phyllobacteriaceae, Aestuariivirgaceae, and Boseaceae, respectively. In contrast, the relative abundances of *Rhizobium* and *Ensifer* were significantly higher in NKA, identifying them as unique denitrifying genera in NKA (Figure 4). Both genera belong to the family of Rhizobiaceae within Hyphomicrobiales (order). These results demonstrate significant differences in *nirK*-denitrifier between KA and NKA. As also shown in Figure 4, the relative abundances of *Bradyrhizobium*, *Mesorhizobium*, *Aestuariivirga*, *Bosea*, *Rhodopseudomonas*, and *Bacillus* were significantly positively correlated with physicochemical factors including pH, Mg^{2+} , Ca^{2+} , CEC, TN, TP, AP, SOC (but *Aestuariivirga* and *Bosea*), and NO_3^- -N (but *Bradyrhizobium*, *Bosea*, *Bacillus*, and *Rhodopseudomonas*). Conversely, the relative abundances of four genera (*Rhizobium*, *Ensifer*, *Propylenella*, and *Hyphomicrobium*) were significant negative correlations with pH, SOC (but *Hyphomicrobium*), TN, TP (but *Propylenella*), AP (but *Hyphomicrobium*), CEC (but *Propylenella*), Ca^{2+} , and Mg^{2+} (but *Hyphomicrobium*).

The significant positive correlation between the relative abundance of *Bradyrhizobium* and N_2O emission fluxes (Figure A1) indicates that *Bradyrhizobium* may have a considerable influence on N_2O emissions.

The Chao1 index showed no significant differences between KA and NKA throughout rice growth period, while the number of ASVs in KA was significantly higher than that in NKA at all stages (Table A3). Additionally, the Shannon and Simpson indices in KA before 21 August (heading) were significantly higher than those in NKA (Table A3). These results demonstrate significant diversity differences in *nirK*-denitrifier between the two areas.

The correlation analysis between diversity indices of *nirK*-denitrifier and soil physicochemical factors is summarized in Table 1. The number of ASVs were significant positive correlations with pH, SOC, TN, NO_3^- -N, TP, AP, CEC, Ca^{2+} , and Mg^{2+} . The Chao1 index was significantly positively correlated with pH, NO_3^- -N, CEC, and Mg^{2+} . The ACE index exhibited significant positive correlations with pH, SOC, TN, CEC, and Mg^{2+} . Both

the Shannon and Simpson indices were significantly positively correlated with pH, SOC, NO_3^- -N, TP, AP, CEC, Ca^{2+} , and Mg^{2+} . These correlations indicate that the diversity of *nirK*-denitrifier in both KA and NKA is regulated by multiple key physicochemical factors, including pH, NO_3^- -N, SOC, Ca^{2+} , Mg^{2+} , and CEC. Furthermore, the distinct soil environment of KA supports a unique *nirK*-denitrifier community with higher diversity.

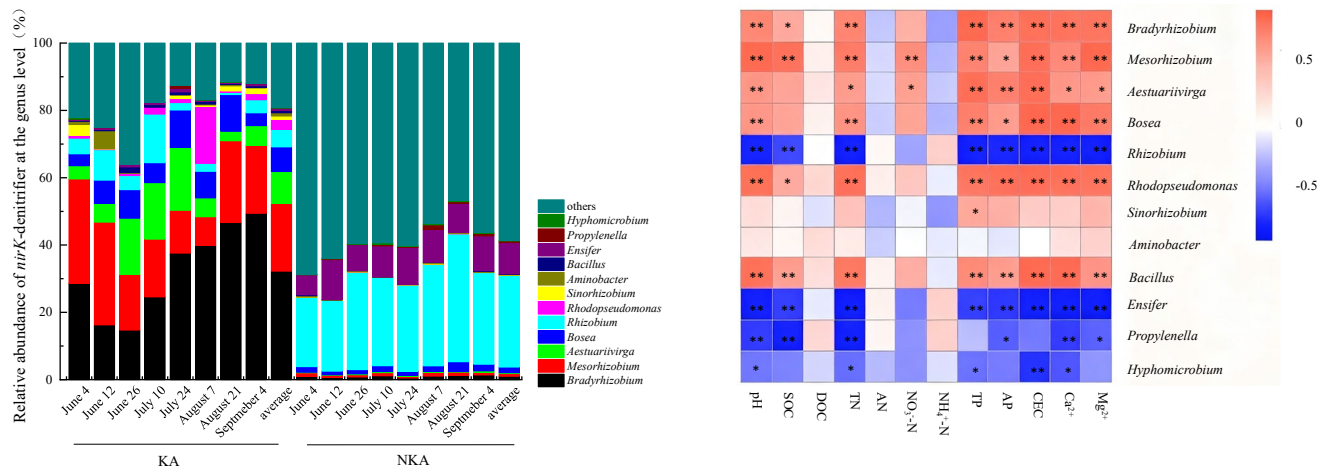


Figure 4. Relative abundance of *nirK*-denitrifier at the genus level and heat map of the correlation between the abundance of *nirK*-denitrifier and soil physicochemical factors in KA and NKA. Note: * and ** indicate significant correlations at the 0.05 and 0.01 probability levels, respectively.

Table 1. Correlation between diversity index of *nirK*-denitrifier and soil physicochemical factors.

Diversity	pH	SOC	DOC	TN	AN	NH_4^+ -N	NO_3^- -N	TP	AP	CEC	Ca^{2+}	Mg^{2+}
ASVs	0.915 **	0.749 **	−0.015	0.773 **	−0.168	−0.187	0.551 *	0.725 **	0.723 **	0.935 **	0.739 **	0.879 **
Chao1 Index	0.466 *	0.371	−0.044	0.405	−0.193	−0.161	0.518 *	0.189	0.168	0.496 *	0.249	0.506 *
ACE Index	0.522 *	0.466 *	−0.144	0.600 **	−0.197	−0.19	0.405	0.194	0.379	0.554 *	0.314	0.569 *
Shannon Index	−0.793 **	−0.568 *	−0.038	−0.389	0.25	0.209	−0.648 **	−0.792 **	−0.559 *	−0.837 **	−0.634 **	−0.733 **
Simpson Index	−0.746 **	−0.500 *	−0.036	−0.318	0.27	0.215	−0.632 **	−0.783 **	−0.514 *	−0.784 **	−0.555 *	−0.689 **

Note: * and ** indicate significant correlations at the 0.05 and 0.01 probability levels, respectively. Abbreviations: SOC: soil organic carbon; DOC: dissolved organic carbon; TN: total nitrogen; AN: available nitrogen; NH_4^+ -N: ammonium nitrogen; NO_3^- -N: nitrate nitrogen; TP: total phosphorus; AP: available phosphorus; CEC: cation exchange capacity; Ca^{2+} : exchangeable calcium ion; Mg^{2+} : exchangeable magnesium ion.

3.6. Correlation and Relative Importance of Soil Physicochemical Factors, AOB, *nirK*-Denitrifier, and N_2O Emission Fluxes in KA and NKA

The results as predicted by PLS-SEM and PLSR indicate that soil properties, carbon sources, and nitrogen sources exerted positive effects on both AOB and *nirK*-denitrifier (Figure 5). The sensitivity analysis of the PLS-SEM model showed that all critical path coefficients remained statistically significant ($p < 0.05$) in Bootstrap validation. The measurement model showed good reliability ($\text{CR} > 0.7$), convergent validity ($\text{AVE} > 0.5$), and acceptability ($\text{GoF} = 0.67 > 0.1$), confirming the robustness of the model results. AOB showed a positive correlation with N_2O emission fluxes, whereas *nirK*-denitrifier exhibited a significant negative correlation with N_2O emission fluxes (Figure 5). These correlations demonstrate that N_2O emission fluxes is directly influenced by both AOB and *nirK*-denitrifier.

Environmental factors identified as having considerable importance for N_2O emission fluxes included NO_3^- -N, SOC, TN, Mg^{2+} , Ca^{2+} , and pH. Key microbial factors included *Nitrosomonas*, ASVs of *nirK*-denitrifier, and *Mesorhizobium* (Figure 5). This indicates that both environmental factors and microbes significantly influence N_2O emissions.

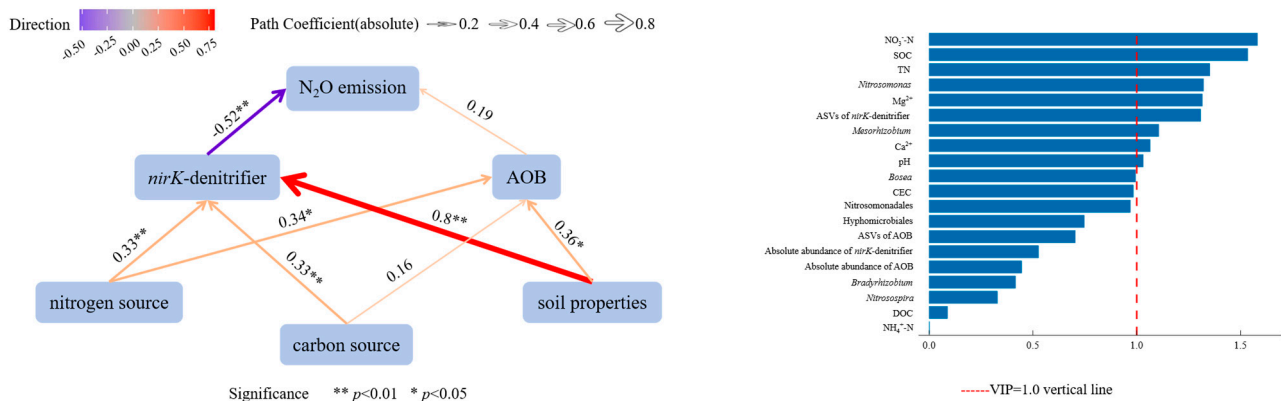


Figure 5. The correlation and significance of soil physicochemical factors, AOB, *nirK*-denitrifier, and N₂O emission fluxes in KA and NKA. Note: soil properties include pH, Ca²⁺, Mg²⁺ and CEC; carbon source includes SOC and DOC; nitrogen source includes TN, NH₄⁺-N, and NO₃⁻-N; AOB includes ASVs, absolute abundance, *Nitrosospira*, *Nitrosomonas*, and uncultured ammonia-oxidizing β -proteobacterium (ASV15); *nirK*-denitrifier includes ASVs, absolute abundance, Hyphomicrobiales, *Bradyrhizobium*, and *Bosea*.

4. Discussion

4.1. Differences in N₂O Emissions Between KA and NKA and Their Environmental Drivers

Meta-analyses of global data suggest that environmental factors such as soil carbon and nitrogen content, pH, and clay minerals are primary regulators of the relative contributions of nitrification/denitrification ratio of N₂O emissions [21,53]. In this study, KA with higher pH exhibited significantly lower N₂O emission fluxes and cumulative emissions compared to NKA. To our knowledge, this is the first report of low N₂O emissions from soil in KA during rice growth. This finding aligns with reports that N₂O emissions are more pronounced under aerobic conditions with high NO₃⁻-N [54], and that the consumption of NH₄⁺-N and accumulation of NO₃⁻-N are positively correlated with N₂O emissions in paddy soils [55]. Furthermore, incubation experiments using ¹⁵N tracing on ten soils from southern China with varying pH and SOC also demonstrated that increasing soil pH reduces N₂O emissions [56]. Elevated soil pH promotes the conversion of NO₂⁻ to NO₃⁻, thereby decreasing the concentrations of NH₂OH and NO₂⁻ (key precursors of N₂O formation) and ultimately mitigating N₂O emissions [57]. This is consistent with the higher NO₃⁻-N observed in KA in our study. Therefore, higher content of NO₃⁻-N and pH in could be an important reason of lower N₂O emissions in KA. Similarly, amending acidic soils with biochar to raise pH and increase NO₃⁻-N content could effectively reduce N₂O emissions [18].

SOC is also another major factor in regulating denitrification potential [58]. Labile carbon continuously supplied from the rhizosphere profoundly influences terrestrial nitrogen cycling [56], which may be utilized by *nirK*-denitrifier, enhancing denitrification activity and potentially increasing N₂O emissions. In our study, carbon source was identified as the second-most important factor affecting N₂O emissions. A possible explanation is that Ca²⁺ binds with DOC and humic acids to form insoluble calcium humate, altering the availability of carbon to *nirK*-denitrifier and thus influencing N₂O emission processes [59]. At the same time, nitrogen elements in karst soil are partly stabilized due to their binding with calcium minerals, which also limits the nitrogen utilization by *nirK*-denitrifier. In farmland, fertilization promotes the accumulation of organic matter, which stimulates denitrifying bacteria to reduce N₂O to N₂, leading to net absorption of N₂O [16]. Wang et al. [60] reported that applying alkaline amendments such as lime can also reduce N₂O emissions in acidic soils (pH < 5.5). Hence, in this study, the lower N₂O emissions in

KA are associated with their alkaline environment and high Ca^{2+} content. In agricultural production, soil amendments such as adding lime, SOC, and adjusting pH (such as biochar) can be used to reduce N loss and N_2O emissions in acidic soils.

The Mg^{2+} content in KA was significantly higher than that in NKA. Mg^{2+} showed a significant negative correlation with the Shannon and Simpson indices of *nirK*-denitrifier, and PLS-SEM predictions indicated that Mg^{2+} significantly influences *nirK*-denitrifier, suggesting a key role for Mg^{2+} in shaping *nirK*-denitrifier assemblages. Studies have shown that Mg-modified biochar (BCMg) reduces N_2O emissions by increasing pH and decreasing the activity of N_2O -producing bacteria in coastal saline soils [61]. Additionally, short-term incubation experiments demonstrated that Mg-fertilizer can mitigate N_2O production in agricultural soils [62]. These findings imply that Mg^{2+} may influence N_2O emissions by altering the community structure and function of *nirK*-denitrifier. The mechanism by which Mg affects *nirK*-denitrifier to reduce N_2O emissions in KA deserves further investigation. The above research also suggests that we can reduce N_2O emissions by applying Mg-fertilizer in NKA.

Meta-analyses indicate that increased N-fertilizer application significantly enhances soil N_2O emissions [57,63], though native soil nitrogen (rather than fertilizer-derived nitrogen) is the main contributor to N_2O emissions, accounting for 67% of total emissions [56]. In upland soils of the North China Plain, a significant linear relationship exists between the available nitrogen and N_2O emissions ($p < 0.01$) [64]. In our study, nitrogen sources influenced N_2O emissions by affecting *nirK*-denitrifier. KA had higher TN and NO_3^- -N, while the NKA had higher NH_4^+ -N. NO_3^- -N and TN were among the most important factors affecting N_2O emissions. High NO_3^- -N content provides substrate for AOB while also reducing N_2O emissions [57]. Thus, N_2O emissions in KA may be regulated by NO_3^- -N dynamics, suggesting different soil N_2O emission mechanisms between KA and NKA. Future studies could use ^{15}N stable isotope tracing to test this hypothesis. In summary, soil factors indirectly drive N_2O emissions by influencing the core processes of nitrification and denitrification [12]. And Ca^{2+} may combine with organic nitrogen to affect the nitrogen availability and change the concentration of NO_3^- -N and NH_4^+ -N, thus affecting the activities of AOB and *nirK*-denitrifier. Understanding N_2O emissions therefore requires integrating soil environmental factors with microbial processes.

4.2. Dominant AOB Communities in KA and Their Underlying Causes

Microbially mediated nitrification and denitrification are core processes governing N_2O emissions in paddy soils [12,65]. In this study, the absolute abundance of AOB was significantly higher in KA than in NKA. Specifically, the relative abundance of *Nitrosospira* (71.48%) was significantly higher in KA compared to NKA (7.71%). Ke et al. [66] reported that *Nitrosospira* prefers low NH_4^+ -N environments, which aligns with our finding of higher relative abundance of *Nitrosospira* in the low NH_4^+ -N in KA than the high NH_4^+ -N in NKA. This is further supported by studies showing a significant positive correlation between TN and the relative abundance of *Nitrosospira* [67], and that N-fertilization enhances the competitive advantage of *Nitrosospira* Cluster 3 [68]. In summary, the neutral alkaline KA had a significantly higher relative abundance of *Nitrosospira* than the acidic NKA, indicating that *Nitrosospira* is a typical and dominant nitrifier in KA, representing a key distinction from NKA.

Nitrosomonas was the second dominant genus in KA, with an average relative abundance of 22.04% (compared to 1.37% in NKA). Studies have identified *Nitrosomonas* as the third dominant genus in KA soils under four vegetations [69]. The relative abundance of *Nitrosomonas* was significantly negatively correlated with NH_4^+ -N (Figure 3), suggesting that the low NH_4^+ -N content in KA may be a limiting factor for this genus. Research

indicates that the ammonia oxidation rate of *N. europaea* (a common species of *Nitrosomonas*) peaks at pH 6.7–7.0 and is not strictly dependent on NH_4^+ -N availability [70], a condition consistent with the neutral alkaline pH and low NH_4^+ -N environment of KA in this study.

Aggregate size significantly influences the relative abundance of *Nitrospira* [71]. Huang et al. [72] found that *Nitrospira* is primarily concentrated in micro-aggregates, whereas Jiang et al. [73] reported a higher proportion of *Nitrospira* in macro-aggregates. Additionally, Hou et al. [74] noted that *Nitrosomonas* is relatively more abundant in micro-aggregates. Future research should therefore explore the niche differentiation of AOB populations in aggregates and their underlying mechanisms in KA and NKA.

4.3. Relationship Between AOB and N_2O Emissions

From a global perspective, nitrification contributes more to N_2O emissions on average than denitrification, though the relative contributions vary across terrestrial ecosystems [21]. In this study, the absolute abundance of AOB was significantly higher in KA and positively correlated with N_2O emission fluxes (Figure A2), suggesting that AOB may substantially contribute to N_2O emissions in KA paddy soils. Similarly, in KA forest soils of Guizhou, China, autotrophic nitrification pathways (ammonia oxidation, nitrifier denitrification, and nitrification-coupled denitrification) account for over 70% of total N_2O emission [75]; the abundance of AOB, organic nitrogen mineralization, and total autotrophic nitrification rates are positively correlated with N_2O emissions [10]. Thus, nitrification is likely the dominant process driving N_2O emissions in KA. In this study, *Nitrosomonas* had a high importance value for N_2O emissions, indicating a potential intrinsic link between its relative abundance and N_2O emissions. However, studies show that *Nitrosomonas europaea* can perform denitrification using NH_4^+ or H_2 as electron donors and ON_2^- as an electron acceptor, producing N_2O and leading to N loss [76,77]. Notably, *N. europaea* produces N_2O only under O_2 -limited conditions [76]. Other reports indicate that *N. eutropha* can utilize N_2O in anaerobic ammonia oxidation [78]. These findings highlight the functional diversity of *Nitrosomonas*, which complicates its contribution to N_2O emissions. Environmental factors such as pH, O_2 , and NH_4^+ -N likely play decisive roles in driving the nitrifying/denitrifying functions of this genus. Future research should focus on the functional diversity of *Nitrosomonas* in KA and NKA and its actual contribution to N_2O emissions.

Meta-analyses suggest that the relative abundance ratio of AOB and ammonia-oxidizing archaea to denitrifiers is a key driver of N_2O emissions [21]. However, another meta-analysis of 101 global field monitoring datasets showed no correlation between soil N_2O emissions and AOB abundance [64]. These contrasting results may imply that interactions between AOB and other microbes in specific ecosystems are also critical in determining N_2O emission contributions. Therefore, clarifying the assembly mechanisms of specific microbial communities and identifying key ecological factors influencing N_2O emissions in KA are particularly important.

4.4. Differences in *nirK*-Denitrifier Community Structure Between KA and NKA and Their Causes

Meta-analyses of global data indicate that N-fertilizer application significantly increases the absolute abundance of *nirK*-denitrifiers in agricultural soils [57,58], primarily because N-fertilizers provide a sufficient substrate for them.

In this study, the diversities of *nirK*-denitrifier differed significantly between KA and NKA (Table A3). Moreover, ASVs of *nirK*-denitrifier and multiple diversity indices correlated with various soil physicochemical factors, including pH, SOC, TN, NO_3^- -N, Ca^{2+} , and Mg^{2+} (Table 1), indicating that the distinct soil environment of KA supports a unique and more diverse *nirK*-denitrifier. No correlation was between the absolute abundance of *nirK*-denitrifiers and soil physicochemical factors in either area or overall.

However, PLS-SEM predictions showed that soil properties, nitrogen sources, carbon sources, and phosphorus sources positively affect *nirK*-denitrifier (Figure 5), suggesting that *nirK*-denitrifiers are regulated by multiple interconnected environmental factors rather than single factor [79].

In KA, *Bradyrhizobium* was the most abundant *nirK*-type denitrifier and showed a significant positive correlation with N_2O emission fluxes (Figure A1). Studies indicate that *Bradyrhizobium* possesses N-fixing [80] and P-solubilizing abilities [81]. *Bradyrhizobium* is sensitive to warming, which reduces its relative abundance, while N-fertilizer increases its relative abundance under warmed conditions [82]. *Bradyrhizobium* has also been reported as a *nirS*-type denitrifier [83]. These results highlight the genetic and functional diversity of *Bradyrhizobium*, suggesting that its ecological role may contribute significantly to N_2O emissions in KA. *Aestuariivirga* was the third-most dominant denitrifying genus in KA. Reportedly, *Aestuariivirga* litoralis shares high DNA sequence similarity with bacteria of Rhizobiales [84], implying that *Aestuariivirga* may also have N-fixing capabilities.

4.5. Relationship Between *nirK*-Denitrifiers and N_2O Emissions

In this study, ASVs of *nirK*-denitrifier and *Mesorhizobium* had high importance values for N_2O emissions, and *nirK*-denitrifier overall had a negative effect on N_2O emissions, suggesting that *Mesorhizobium* may contribute little to N_2O emissions. *Mesorhizobium* was the second dominant denitrifier in KA, with a significantly higher relative abundance than in NKA. Research shows that *Mesorhizobium* also has N-fixing [85] and P-solubilizing abilities [81], and increasing NH_4^+ -N can enhance its abundance [81]. In this study, NH_4^+ -N was generally low in KA, so NH_4^+ -N fertilizer might increase the relative abundance of *Mesorhizobium*. Some strains of *Mesorhizobium* harbor N_2O reductase (*nosZ*), which reduces N_2O to N_2 and thus reduces N_2O emissions [86]. Moreover, the reduction in N_2O by *Mesorhizobium* may be enhanced by the neutral alkaline pH in KA, resulting in negative N_2O emissions. These results indicate that the unique neutral alkaline and Ca-rich environment of KA supports *nirK*-denitrifiers with diverse N metabolic functions, resulting in negative N_2O emissions. However, whether *Mesorhizobium* can truly reduce N_2O emissions in KA still needs to be verified by adding different *Mesorhizobium* strains. Recently, studies using isotope tracing show that fungal-mediated denitrification is the largest contributor of N_2O emissions (51–63%) compared to bacterial and chemical denitrification in paddy soils [54]. Therefore, future research should investigate the contributions of fungi to N_2O emissions in KA and NKA [16].

In summary, the possible reasons for the net absorption of N_2O in KA is that the alkaline and Ca-rich environment suppress the production of N_2O and a portion of N_2O is reduced by microorganisms. The above findings suggest that we can reduce soil N_2O emissions by adding lime, Mg-fertilizer, SOC, NO_3^- -N, pH adjusters, and specific microorganisms in NKA paddy soils, which is important for agricultural emission reduction practices under global climate change.

5. Conclusions

- (1) The cumulative N_2O emissions were $-0.054 \text{ kg} \cdot \text{hm}^{-2}$ in KA and $0.229 \text{ kg} \cdot \text{hm}^{-2}$ in the NKA throughout the rice growth period, respectively, indicating that karst rice fields are reservoirs of N_2O .
- (2) The absolute abundance of AOB was significantly higher in KA than that in NKA, whereas the absolute abundance of *nirK*-denitrifier did not differ significantly between the two areas.
- (3) The dominant AOB in KA were *Nitrosospora* and *Nitrosomonas*, while the dominant AOB in NKA was an uncultured ammonia-oxidizing β -proteobacterium. The domi-

nant *nirK*-denitrifiers in KA were *Bradyrhizobium*, *Mesorhizobium*, *Aestuariivirga*, and *Bosea*, whereas *Rhizobium* and *Ensifer* were dominant in NKA.

- (4) Soil properties, nitrogen sources, and carbon sources had positive effects on AOB, while soil properties, nitrogen sources, and phosphorus sources positively affected AOB. The *nirK*-denitrifiers had a negative effect on N₂O emission fluxes. Environmental factors with high importance for N₂O emission fluxes included NO₃[−]-N, SOC, TN, Mg²⁺, Ca²⁺, and pH, and key microbial factors were *Nitrosomonas*, ASVs of *nirK*-denitrifiers, and *Mesorhizobium*, indicating that these factors significantly influence N₂O emissions.

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Conflicts of Interest: The authors declare no conflicts of interest.

Appendix A

Table A1. Characteristics of soil physicochemical factors in KA and NKA during the rice growing.

	Areas	4 June	12 June	26 June	10 July	24 July	7 August	21 August	4 September	Average
pH	KA	7.50 ± 0.04 a	7.26 ± 0.61 a	7.39 ± 0.13 a	7.57 ± 0.29 a	7.09 ± 0.11 a	7.38 ± 0.14 a	7.50 ± 0.21 a	7.31 ± 0.25 a	7.38 ± 0.16 a
	NKA	5.01 ± 0.26 b	4.90 ± 0.23 b	6.23 ± 0.5 b	4.82 ± 0.11 b	4.78 ± 0.06 b	4.88 ± 0.27 b	4.74 ± 0.14 b	4.80 ± 0.31 b	5.02 ± 0.50 b
SOC (g/kg)	KA	15.42 ± 0.37 a	14.43 ± 1.10 a	14.79 ± 0.90 a	14.52 ± 1.72 a	13.27 ± 1.76 a	13.48 ± 0.42 a	14.11 ± 0.95 a	14.48 ± 2.09 a	14.31 ± 0.69 a
	NKA	13.78 ± 1.01 b	14.05 ± 1.27 a	12.07 ± 1.56 b	12.59 ± 0.37 b	10.19 ± 1.42 b	10.01 ± 0.94 b	11.14 ± 0.73 b	9.74 ± 3.39 b	11.70 ± 1.69 b
DOC (mg/kg)	KA	0.23 ± 0.03 a	1.06 ± 0.1 a	0.21 ± 0.04 b	0.70 ± 0.02 a	0.90 ± 0.13 a	0.56 ± 0.09 a	0.24 ± 0.10 a	0.25 ± 0.06 a	0.52 ± 0.34 a
	NKA	0.38 ± 0.07 b	0.32 ± 0.08 b	1.65 ± 0.15 a	0.38 ± 0.03 b	0.21 ± 0.06 b	0.44 ± 0.21 a	0.17 ± 0.04 a	0.26 ± 0.09 a	0.48 ± 0.48 a
TN (g/kg)	KA	1.90 ± 0.03 a	1.71 ± 0.03 a	1.91 ± 0.05 a	1.84 ± 0.03 a	1.69 ± 0.03 a	1.91 ± 0.31 a	1.97 ± 0.00 a	1.91 ± 0.10 a	1.86 ± 0.10 a
	NKA	1.68 ± 0.03 b	1.57 ± 0.02 b	1.72 ± 0.03 b	1.67 ± 0.06 b	1.52 ± 0.14 a	1.55 ± 0.04 a	1.36 ± 0.04 b	1.17 ± 0.02 b	1.53 ± 0.19 b
AN (mg/kg)	KA	55.49 ± 5.67 b	91.43 ± 2.04 a	56.43 ± 10.30 b	98.89 ± 2.13 a	91.48 ± 9.15 a	83.96 ± 18.16 a	64.58 ± 2.01 a	69.03 ± 3.83 a	76.41 ± 17.10 a
	NKA	93.25 ± 8.11 a	92.57 ± 1.47 a	158.05 ± 7.10 a	78.57 ± 5.33 b	65.33 ± 1.58 b	71.60 ± 7.16 a	56.47 ± 2.01 b	53.53 ± 1.76 b	83.67 ± 33.51 a
NH ₄ ⁺ -N (mg/kg)	KA	11.55 ± 0.37 b	32.41 ± 0.15 b	13.61 ± 0.31 b	27.82 ± 1.38 b	26.5 ± 0.45 b	22.2 ± 0.17 b	13.76 ± 0.45 b	13.81 ± 0.15 b	20.21 ± 8.03 b
	NKA	46.67 ± 0.60 a	55.18 ± 1.32 a	88.60 ± 4.00 a	50.56 ± 0.55 b	47.63 ± 0.27 b	32.21 ± 0.32 a	25.76 ± 0.44 a	24.77 ± 0.36 a	40.40 ± 12.51 a
NO ₃ [−] -N (mg/kg)	KA	39.30 ± 0.77 a	54.29 ± 10.65 a	45.64 ± 1.11 a	49.96 ± 6.95 a	35.76 ± 13.72 a	26.43 ± 2.33 a	36.44 ± 0.41 a	40.79 ± 0.17 a	41.08 ± 8.80 a
	NKA	25.99 ± 1.18 b	22.87 ± 1.09 b	8.83 ± 0.62 b	27.49 ± 1.79 b	30.25 ± 1.64 b	9.61 ± 0.95 b	31.77 ± 0.82 b	29.02 ± 0.52 b	23.23 ± 9.06 b
TP (g/kg)	KA	0.32 ± 0.08 a	0.21 ± 0.01 a	0.15 ± 0.03 a	0.18 ± 0.03 a	0.33 ± 0.04 a	0.21 ± 0.04 a	0.24 ± 0.03 a	0.26 ± 0.03 a	0.24 ± 0.06 a
	NKA	0.08 ± 0.04 b	0.14 ± 0.01 b	0.07 ± 0.01 b	0.08 ± 0.02 b	0.13 ± 0.04 b	0.11 ± 0.02 b	0.12 ± 0.01 b	0.13 ± 0.02 b	0.11 ± 0.03 b
AP (mg/kg)	KA	30.53 ± 3.09 a	12.82 ± 1.05 a	27.76 ± 1.53 a	20.09 ± 0.17 a	24.14 ± 2.46 a	42.38 ± 6.79 a	23.47 ± 1.87 a	33.73 ± 6.10 a	26.87 ± 8.98 a
	NKA	13.05 ± 1.53 b	7.60 ± 0.55 b	15.99 ± 0.72 b	15.89 ± 0.24 b	14.60 ± 0.10 b	12.88 ± 3.44 b	11.60 ± 3.78 b	12.59 ± 1.04 b	13.03 ± 2.70 b
CEC (cmol/kg)	KA	6.51 ± 2.40 a	6.47 ± 1.25 a	6.97 ± 1.39 a	7.30 ± 0.77 a	7.87 ± 1.29 a	6.86 ± 0.99 a	8.34 ± 0.18 a	6.98 ± 2.49 a	7.16 ± 0.65 a
	NKA	4.14 ± 0.25 a	3.97 ± 0.35 b	4.58 ± 0.09 b	3.92 ± 0.82 b	3.55 ± 0.66 b	4.05 ± 0.27 b	2.84 ± 0.67 b	3.58 ± 0.13 a	3.83 ± 0.51 b
Ca ²⁺ (cmol/kg)	KA	4.83 ± 0.16 a	4.73 ± 0.33 a	5.02 ± 0.14 a	4.63 ± 0.19 a	4.89 ± 0.25 a	5.08 ± 0.27 a	4.96 ± 0.17 a	4.64 ± 0.29 a	4.85 ± 0.17 a
	NKA	2.43 ± 0.22 b	2.44 ± 0.3 b	2.41 ± 0.03 b	2.30 ± 0.01 b	1.87 ± 0.32 b	2.20 ± 0.16 b	2.34 ± 0.22 b	2.08 ± 0.03 b	2.26 ± 0.20 b
Mg ²⁺ (cmol/kg)	KA	0.26 ± 0.02 a	0.24 ± 0.01 a	0.23 ± 0.00 a	0.23 ± 0.01 a	0.24 ± 0.00 a	0.24 ± 0.00 a	0.24 ± 0.00 a	0.23 ± 0.01 a	0.24 ± 0.01 a
	NKA	0.21 ± 0.00 b	0.22 ± 0.00 b	0.22 ± 0.00 b	0.21 ± 0.01 b	0.15 ± 0.02 b	0.17 ± 0.00 b	0.20 ± 0.01 b	0.16 ± 0.00 b	0.19 ± 0.03 b

Note: Values in the table represent (mean ± standard deviation). Lowercase letters following numbers indicate significant differences between KA and NKA on the same sampling date at the 0.05 probability level. Abbreviations: SOC, soil organic carbon; DOC, dissolved organic carbon; TN, total nitrogen; AN, available nitrogen; NH₄⁺-N, ammonium nitrogen; NO₃[−]-N, nitrate nitrogen; TP, total phosphorus; AP, available phosphorus; CEC, cation exchange capacity; Ca²⁺, exchangeable calcium ion; Mg²⁺, exchangeable magnesium ion.

Table A2. Diversity index of AOB in KA and NKA.

Diversity	Areas	4 June	12 June	26 June	10 July	24 July	7 August	21 August	4 September	Average
ASVs	KA	268 ± 134 a	536 ± 592 a	268 ± 101 a	416 ± 424 a	475 ± 233 a	25 ± 23 a	28 ± 19 a	78 ± 43 a	262 ± 203 a
	NKA	212 ± 356 a	177 ± 200 a	936 ± 40 a	83 ± 61 a	288 ± 270 a	2039 ± 3243 a	52 ± 26 a	132 ± 153 a	490 ± 687 a

Note: Values in the table represent (mean ± standard deviation). Lowercase letters following numbers indicate significant differences between KA and NKA on the same sampling date at the 0.05 probability level.

Table A3. Diversity indices of nirK-denitrifier in KA and NKA.

Diversity	Areas	4 June	12 June	26 June	10 July	24 July	7 August	21 August	4 September	Average
Chao1 Index	KA	9661 ± 2658 a	5916 ± 1475 a	9990 ± 2730 a	8417 ± 2614 a	6964 ± 2732 a	7501 ± 1210 a	6999 ± 2269 a	5462 ± 2007 a	7614 ± 1639 a
	NKA	1988 ± 1230 b	2461 ± 2050 a	2105 ± 864 b	1642 ± 550 b	2176 ± 371 b	2207 ± 1117 b	1447 ± 583 b	1318 ± 695 b	1918 ± 404 b
ACE Index	KA	43.27 ± 5.72 a	70.01 ± 52.05 a	68.04 ± 16.88 a	42.88 ± 6.04 a	42.80 ± 4.93 a	48.56 ± 7.51 a	70.48 ± 32.23 a	46.05 ± 17.83 a	54.01 ± 12.99 a
	NKA	52.67 ± 17.47 a	40.06 ± 1.42 a	40.46 ± 4.46 a	39.31 ± 8.00 a	49.44 ± 22.15 a	41.92 ± 6.42 a	34.87 ± 9.42 a	47.50 ± 30.64 a	43.28 ± 5.98 a
Shannon Index	KA	47.19 ± 2.85 a	53.98 ± 16.25 a	87.36 ± 24.70 a	47.77 ± 9.54 a	50.68 ± 3.65 a	50.77 ± 5.37 a	66.42 ± 23.25 a	50.28 ± 21.76 a	56.81 ± 13.76 a
	NKA	51.21 ± 11.81 a	43.77 ± 3.16 a	43.86 ± 5.34 b	43.65 ± 7.80 a	53.52 ± 24.72 a	45.06 ± 5.97 a	35.45 ± 10.42 a	37.72 ± 13.76 a	44.28 ± 6.05 a
Simpson Index	KA	2.19 ± 0.20 a	2.36 ± 0.19 a	2.29 ± 0.15 a	2.41 ± 0.07 a	2.40 ± 0.47 a	2.46 ± 0.23 a	1.95 ± 0.12 a	1.73 ± 0.69 a	2.22 ± 0.26 a
	NKA	1.32 ± 0.10 b	1.41 ± 0.29 b	1.58 ± 0.12 b	1.61 ± 0.18 b	1.41 ± 0.43 b	1.70 ± 0.18 b	1.79 ± 0.12 a	1.68 ± 0.43 a	1.56 ± 0.17 b
Chao1 Index	KA	0.79 ± 0.05 a	0.85 ± 0.04 a	0.82 ± 0.04 a	0.87 ± 0.01 a	0.83 ± 0.08 a	0.86 ± 0.04 a	0.74 ± 0.06 a	0.62 ± 0.25 a	0.80 ± 0.08 a
	NKA	0.52 ± 0.05 b	0.56 ± 0.12 b	0.61 ± 0.03 b	0.63 ± 0.06 b	0.55 ± 0.19 b	0.67 ± 0.03 b	0.69 ± 0.02 a	0.65 ± 0.11 a	0.61 ± 0.06 b

Note: Values in the table represent (mean ± standard deviation). Lowercase letters following numbers indicate significant differences between KA and NKA on the same sampling date at the 0.05 probability level.

Appendix B

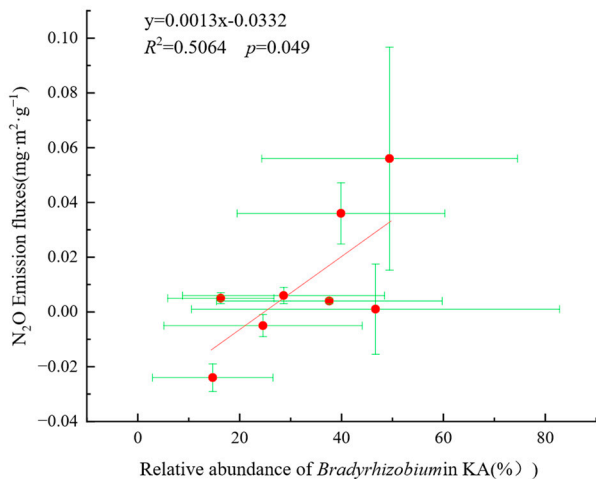


Figure A1. Correlation between relative abundance of Bradyrhizobium and N₂O emission fluxes in KA. Note: the red dots represent scatter points, and the green lines represent error bars.

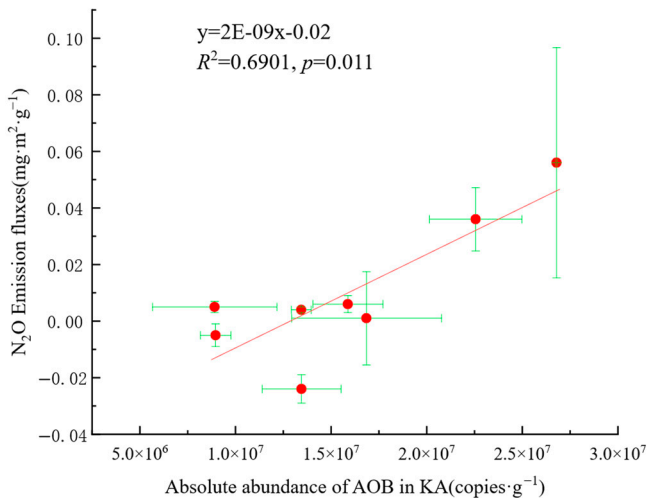


Figure A2. Correlation between absolute abundance of AOB and N₂O emission fluxes in KA. Note: the red dots represent scatter points, and the green lines represent error bars.

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