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Research article

K fertilizer alleviates N_2O emissions by regulating the abundance of nitrifying and denitrifying microbial communities in the soil-plant system

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ABSTRACT

Potassium (K) fertilizer additions can result in high crop yields of good quality and low nitrogen (N) loss; however, the interaction between K and N fertilizer and its effect on N_2O emissions and associated microbes remain unclear. We investigated this in a pot experiment with six fertilizer treatments involving K and two sources of N, using agricultural soil from the suburbs of Wuhan, central China. The aim was to determine the effects of the interaction between K and different forms of N on the N_2O flux and the abundance of nitrifying and denitrifying microbial communities, using static chamber-gas chromatography and high-throughput sequencing methods. Compared with no fertilizer control (CK), the addition of nitrate fertilizer (NN) or ammonia fertilizer (AN) or K fertilizer significantly increased N_2O emissions. However, the combined application (NNK) of K and NN significantly reduced the average N_2O emissions by 28.3%, while the combined application (ANK) of K and AN increased N_2O emissions by 22.7%. The abundance of nitrifying genes *amoA* in ammonia oxidizing archaea (AOA) and ammonia oxidizing bacteria (AOB) changed in response to N and/or K fertilization, but the denitrifying genes *nar*G, *nir*K and *nor*l were strongly correlated with N_2O emissions. This suggests that N or K fertilizer and their interaction affect N_2O emissions mainly by altering the abundance of functional genes of denitrifying microbes in the soil-plant system. The genera *Paracoccus, Rubrivivax and Geobacter* as well as *Streptomyces* and *Hyphomicrobium* play an important role in N_2O emissions during denitrification with the combined application of N and K.

1. Introduction

Nitrogen (N) plays an important role in plant growth and quality development. However, the efficiency of N use by agricultural crops is generally poor. Globally, about 50% of the N fertilizer that is used in agriculture is not absorbed by plants (Coskun et al., 2017), but is rather lost to the environment in the form of ammonia (NH_3), nitrate (NO_3^-), nitrogen oxides (NO_x), etc., resulting in serious environmental damage and climate change (Hawthorne et al., 2017; Li et al., 2020a). It has been reported that approximately 3.9 Tg (10^{12}g) N yr^{-1} N_2O emissions are induced by direct and indirect use of N fertilizer in global arable systems (Li et al., 2020b). N_2O emissions from agricultural soils in developing countries account for about 60% of the global anthropogenic N_2O emissions, due to their higher consumption of fertilizer N and low N use efficiency (Kahrl et al., 2010). Therefore, it is necessary to determine the optimal N management practices and mechanisms to reduce N_2O emissions in farmland.

Nitrification is a central process of the N cycle. The oxidation of ammonia (NH_3), which is the rate-limiting step of nitrification, plays a major role in N_2O production (Zhong et al., 2016; Di et al., 2009; Kuypers et al., 2018). Recent studies have shown that ammonia oxidation is mostly performed by ammonia-oxidizing bacteria (AOB) and archaea (AOA) (Coskun et al., 2017), and controlled by ammonia monooxygenase gene (*amo*A) (Rotthauwe et al., 1997; Kuypers et al., 2018). The abundance and community composition of soil AOB was often more sensitive to the application of mineral and organic fertilizers than AOA (Ai et al., 2013; Lage et al., 2010; Zhong et al., 2016; Tao et al., 2017). However, some studies have found the abundance of some AOA

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group increased under NP fertilization (Yang et al., 2020b) or when ammonia was supplied by mineralization of organic N derived from manure (Offre et al., 2009). It remained unclear which groups of the ammonia oxidizer microflora (AOB, AOA or both) were contributing to the nitrification process (Coskun et al., 2017).

Denitrification is a multistep reduction process that converts NO_3^- into N_2, in which N_2O was produced as an intermediate precursor (Sun et al., 2018; Li et al., 2020b; Wang et al., 2020). Due to its major contribution to N_2O emissions, many researcher have conducted extensive studies on the effect of fertilizer application on N_2O related enzymes: nitrate reductase (*NAR*), nitrite reductase (*NIR*), nitric oxide reductase (*NOR*), and nitrous oxide reductase (*NOS*) (Wang et al., 2018), as well as functional genes, such as *nir*K, *nir*S and *nos*Z (Kuypers et al., 2018; Tang et al., 2020). However, published data show that the response of the abundance and composition of denitrifiers to fertilization has not always been consistent; some studies have shown that fertilization application promotes denitrifiers abundance (Duan et al., 2017; Wang et al., 2017), or alternatively that it reduced (Huang et al., 2020) or has little effect on the abundance of denitrifying microbes (Szukics et al., 2009). It is still not clear which group of denitrifiers, nirK- or nirS-type denitrifier or both, play a major role in N_2O production after fertilizer application (Kuypers et al., 2018; Wang et al., 2017). It has been reported that *nosZ* clade II rather than clade I determines N_2O *in situ* emissions with different fertilizer types (Xu et al., 2020). However, our knowledge of how the N_2O-reducing microbial group, including the *nosZI* and *nosZII* clades, responds to fertilization remains limited (Jones et al., 2013), due to differences in soil properties and their interactions with fertilizers in different regions (Wang et al., 2018; Escanhoela et al., 2019).

Potassium (K) is the main mineral nutrient, and is key for the growth of plants (Li et al., 2019; Hou et al., 2019; Fontana et al., 2020). It plays a vital role in the improvement of crop yield and quality. As a result, global demand for K fertilizer will increase and is expected to reach about 45.7 million metric tons in 2023 (https://www.statista.com/statistics/439006/total-demand-for-potash-fertilizer-worldwide-prediction/). Long-term large-scale application of K fertilizer may have a great impact on the process of soil N transformation and global N_2O emissions (Wang et al., 2020; Li et al., 2020b), because the addition of K^+ can change soil N availability directly through competition with NH_4^+ for soil fixation sites (Nieder et al., 2011) or indirectly through plant N uptake (Li et al., 2019; Hou et al., 2019) and the activity of microorganisms involved in N transformation by ion channel proteins and osmotic pressure (Wang et al., 2020). However, few studies highlight the effects of K fertilizer on N_2O production and its related microorganisms. Previous studies, such as Li et al. (2020), showed that potassium salts such as KCl were associated with a substantial increase in N_2O emissions derived from nitrification or denitrification. Similarly, Wang et al. (2020) reported that adding K^+ increased nitrate removal from groundwater by enhancing heterotrophic denitrification, but they did not provide N_2O emission data. However, some researchers (Hahn et al., 1942; Golden et al., 1981) have reported that the addition of K fertilizer effectively suppresses soil nitrification and reduces N_2O emissions, and they found that nitrite formation but not nitrate formation was inhibited by K fertilizer, based on the numbers of *Nitrosomonas* sp. and *Nitrobacter* sp. The effects of K fertilizer on N_2O production and microbial mechanisms driving these effects remain unclear.

Cotton (*Gossypium hirsutum* L.) is an important economic crop in the world and requires a great demand for K fertilizer (Yang et al., 2014). Soil K deficiency in many areas has become a key factor limiting cotton yield and reducing N loss as N_2O emissions (Li et al., 2020b). However, few studies focused on the effects of K on soil N_2O emission and its associated mechanism in a cotton field. Recent advances in high-throughput sequencing methods (Rahmann et al., 2013) now allow us to examine the effects of K fertilization on the key functional genes, enzymes and microbial communities related to soil N_2O production in the soil–cotton system. In this study, we investigated the community structures and abundance of functional genes of nitrifying (*amoA* in AOA and AOB) and denitrifying microbes (*nar*G, *nar*H, *nar*I, *nir*K, *nir*S, *nor*B and *nos*Z), as well as their relationship with soil properties and N_2O flux, and investigated their response to K and combined N and K additions in cotton soils. We hypothesized that the application of K fertilizer exerted a positive effect on the diversity and abundance of nitrifying and denitrifying microbes associated with N_2O emissions, and K and N fertilizer combinations alleviated the promotion of N fertilizer on N_2O production in the soil-cotton system.

2. Materials and methods

2.1. Experimental design

The soil used was a yellow brown soil, equivalent to Dystrochrepts in the US classification, collected from the 0–15 cm layer of agricultural fields in the suburbs of Wuhan City, Hubei Province, Central China (114°27′57″ E, 30°42′27″ N). The region has a humid subtropical monsoon climate with an average annual temperature of 15.8–17.5 °C and average annual precipitation of 1269 mm (Zhang et al., 2017). Soil samples were air-dried and the visible stones and plant residues were removed before the samples were passed through a 2 mm sieve. The physical and chemical properties of the soil used in this experiment were: pH, 7.44; soil organic matter, 6.95 g kg^{-1}; alkaline N, 23.3 mg kg^{-1}; available phosphorous (AP), 18.6 mg kg^{-1}; and available potassium (AK), 64.2 mg kg^{-1}.

Fifty-four pots (12.4 L), each containing 10 kg of dry soil, were prepared as follows. Six treatments with nine replicates were set up: no fertilizer (CK), nitrate-source N fertilizer treatment (NN, 100 mg N kg^{-1}), ammonia-source N fertilizer treatment (AN, 100 mg N kg^{-1}), K treatment (K, 35 mg K kg^{-1}), combined NN and K treatment (NNK, 100 mg N kg^{-1} + 35 mg K kg^{-1}) and combined AN and K treatment (ANK, 100 mg N kg^{-1} + 35 mg K kg^{-1}). Germinated cotton (*Gossypium* sp. cv. Eza 10) seeds were sowed into a seedbed on April 3, 2019, and were then transplanted into the prepared pots when they had produced two expanded leaves (April 28). Nitrogen was added in the form of NH_4Cl and $Ca(NO_3)_2$, P was added in the form of NaH_2PO_4, and K as K_2SO_4. The N fertilizer was applied in three doses, 50% as basal fertilizer (May 20), 30% during the budding period (June 20) and 20% during the boll period (July 25). The K fertilizer was applied as a basal fertilizer. The same amount of P (50 mg kg^{-1}) was applied to each pot. The prepared fertilizer and deionized water were evenly injected into the soil by syringe in 30 times with a depth of 5 cm and a spacing of 4 cm. After fertilizer treatments the 54 pots were randomly placed in the greenhouse. During the whole experiment, soil moisture of each pot was maintained at 65–70% of the water filled pore space by using a moisture meter (YZQ-310, China).

2.2. Soil collection and preparation

Soil samples were collected at the seedling stage, flourishing stage and harvest stage. Three replicates of each treatment were installed with a permanent base for collecting gas samples during the test and soil samples after harvest, and the remaining 6 replicates were used to collect soil samplings at seedling stage and flourishing stage. A mixed soil sample from the 0–10 cm depth, formed by combining five soil subsamples collected at a distance of 10 cm from the plant stem using a 2.5 cm-diameter auger, was taken from each pot. Three mixed soil samples were collected from each treatment. Each mixed soil sample was divided into two parts: one was frozen immediately on dry ice for high-throughput sequencing analysis of soil microorganisms (Personal Biotechnology Co., Ltd., Shanghai, China), and the other was taken back to the laboratory, where visible plant residues and roots were removed before being passed through a 2-mm sieve. Subsamples were air-dried for pH, soil organic carbon (SOC), total nitrogen (TN), AK and AP determination. Subsamples were stored at 4 °C in a refrigerator whilst

awaiting determination of moisture content and mineral N (NH_4^+ and NO_3^-), dissolved organic nitrogen (DON).

2.3. Measurement of N_2O fluxes

N_2O fluxes were determined using a closed chamber method (Li et al., 2014). The closed chamber (2.65 L) consisted of a permanent base (bottom part) and top (lid with a rubber septum for gas sampling). A base for each chamber was placed into the soil to a depth of 8 cm prior to the experiment and remained in place throughout the study. During the monitoring period, gas samples (20 mL) were collected with a syringe from the headspace of the chambers at 0, 10, 20 and 30 min after chamber closure and immediately transferred to 12 mL pre-evacuated vials (Exetainer, Labco Ltd.). Gas samples were collected mostly between 09:00 to 11:30 every other week from May to August 2019. N_2O concentration in the gas samples were analyzed by using gas chromatography (GC) (Agilent 7890 B, USA). The N_2O flux (Eq. (1)) and total N_2O emission (Eq. (2)) were calculated as follows (Li, 2014):

$$F = K_{N_2O} \times \frac{V}{A} \times \frac{\Delta c}{\Delta t} \times \frac{273}{T} \quad (1)$$

where F is the N_2O flux (ug N_2O–N m^{-2} h^{-1}); K_{N_2O} is 1.25 (ug N uL^{-1}) according to the ideal gas law, where $K_{N_2O} = (P \times m)/(R \times T_0)$, and P is air pressure (at 1 atm), m is molecular mass (28 g mol^{-1}), R is gas constant (0.0821 L atm K^{-1} mol^{-1}) and T_0 is 273 K; T is the air temperature within the chamber (K); V (L) and A (m^2) are the volume and bottom area of the chamber respectively; and Δc (uL L^{-1}) is the change in N_2O concentration in the chamber during the period Δt (h^{-1}).

$$F_{N_2O} = \sum_{i=1}^{n} (F_i \times D_i) \quad (2)$$

where D_i is the number of days; F_i is the measured flux in the ith sampling interval, and n is the number of sampling intervals.

2.4. Soil chemical analysis

Soil chemical properties were measured following Rukun (1999). Soil pH was measured in a 1:5 soil/water mixture using a DMP-2 mv/pH detector (Quark Ltd, Nanjing, China). The NH_4^+ and NO_3^- in the soil were extracted with 0.5 M K_2SO_4 and measured using an auto analyzer (Flowsys, Systea, Italy). Total N concentrations in soil were determined by the combustion method, using a Finnigan FlashEA 1112 elemental analyzer (Thermo Scientific, Bremen, Germany). Soil AP was extracted with a 0.5 M $NaHCO_3$ solutions, colored with molybdenum-antimony solution, and measured by spectrophotometry (NanoDrop 8000). Soil AK was determined by the molybdate blue and flame photometry method. DOC was extracted using 2 M KCl in a mix of 1:5 soil-to-water and then measured with a TOC-VCPH/CPN analyzer (Shimadzu Scientific Instruments, Japan). DON was obtained by subtracting soil mineral N from DON. SOC was determined using the K_2CrO_7–H_2SO_4 oxidation method.

Nitrification intensity (NI) and denitrification intensity (DI) were determined using the aerobic and anaerobic incubation methods, respectively (Tang et al., 2016). For NI, pre-incubated 10 g moist soil samples that had been passed through a 2 mm sieve were placed in a 100 mL polyethylene bottle and then incubated at 20 °C in the dark for 14 days. For DI, a thin flooded layer was created on the surface of the soil sample in the bottle, then the bottle was placed in a vacuum dryer to form a vacuum and filled with nitrogen; this was repeated several times until there was no oxygen in the desiccator anymore. The NI and DI were calculated as the change in NO_3^--N between the initial samples and the incubated samples (ug NO_3–N g^{-1} dry soil d^{-1}).

2.5. DNA extraction, high-throughput sequencing and bioinformatics analysis

The total microbial DNA of soil samples was extracted using a DNeasy powerSoil DNA Isolation Kit (QIAGEN, Inc., Netherlands) according to the manufacturer's instructions. The quantity and quality of extracted DNA were measured at 260/280 nm using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis, respectively. Extracted microbial DNA was processed to construct metagenome shotgun sequencing libraries with insert sizes of 400 bp, using an Illumina TruSeq Nano DNA LT Library Preparation Kit. Each library was sequenced using the Illumina HiSeq X-ten platform (Illumina, USA) with the PE150 strategy at Personal Biotechnology Co., Ltd. (Shanghai, China) (Walters et al., 2016; Saenz et al., 2019).

Raw DNA sequencing reads were processed to obtain quality-filtered reads for further analysis. Sequencing adapters and low quality reads were removed using Cutadapt (v1.2.1) (Rahmann et al., 2013) and a sliding-window algorithm. The reads were aligned to the host genome using BWA (http://bio-bwa.sourceforge.net/) (Li and Durbin, 2009) to remove host contamination. The double-ended sequence was assembled to construct the metagenome for each sample by IDBA-UD (Iterative De Bruijn graph Assembler for sequencing data with highly Uneven Depth) (Peng et al., 2012). All coding regions (CDS) of metagenomic scaffolds longer than 300 bp were predicted by MetaGeneMark (http://exon.gatech.edu/GeneMark/metagenome) (Zhu et al., 2010). CDS sequences of all samples were clustered by CD-HIT (Fu et al., 2012) at 90% protein sequence identity, to obtain a non-redundant gene catalog. Gene abundance in each sample was estimated by soap. coverage (http://soap.genomics.org.cn/) based on the number of aligned reads. The lowest common ancestor taxonomy of the non-redundant genes was obtained by aligning them against the NCBI-NT database using BLASTN (e value < 0.001). Similarly, the functional profiles of the non-redundant genes were obtained by annotating them against the GO, KEGG, EggNOG and CAZy databases, respectively, using the DIAMOND (Buchfink et al., 2015) alignment algorithm. Based on the taxonomic and functional profiles of non-redundant genes, LEfSe (Linear discriminant analysis effect size) was performed to reveal differentially abundant taxa and functions across groups using the default parameters (Segata et al., 2011). Beta diversity analysis was undertaken to investigate the compositional and functional variation of microbial communities across samples using Bray-Curtis distance metrics (Bray and Curtis, 1957).

2.6. Statistical analysis

All data were analyzed using SPSS 16.0 (SPSS Inc., USA). One-way analysis of variance (ANOVA) and Tukey's test was used to compare the difference in measured parameters between the experimental treatments. All results are presented as the mean ± standard error for three replicates. Spearman's correlations and redundancy analysis (RDA) were used to determine the relationships between gene abundance, nitrifying and denitrifying microbe genera abundance and soil properties.

3. Results

3.1. Soil properties

Soil properties differed significantly between the different fertilization treatments at harvest time (Table S1 and Fig. S1, S2). Compared with CK, NN application decreased SOC, the C/N ratio, NI, DI, but AN application increased DON, AK and NI. K fertilizer application markedly reduced soil pH and significantly increased concentrations of SOC and NH_4^+. NNK application resulted in considerably lower concentrations of TN, AP and NH_4^+ than the treatment with NN fertilizer alone. However,

ANK application significantly increased C/N ratio and concentrations of SOC, AP and NO_3^- compared with the application of AN fertilizer alone.

3.2. N_2O emissions

Fig. S1 shows the dynamic change in soil N_2O flux rate over the whole growth season in soils treated with N and K fertilizers. In all fertilizer treatments, the shape of N_2O emission rates was comparable, initially increasing and then decreasing, peaking in early July. The difference of N_2O emission rates under different fertilization treatments mainly occurred in the middle and late stages of cotton growth (Fig. S1). The cumulative emission of N_2O in the soils treated with NN and AN fertilizer were 0.59 and 0.71 mg kg^{-1} dry soil, respectively (Table 1, $p < 0.01$); these figures were significantly higher than those in the soils treated with K fertilizer, which produced 0.35 mg N_2O kg^{-1} dry soil. There was a significant interaction between N fertilizer and K fertilizer with respect to N_2O emissions (Fig. S1, $p < 0.01$). NNK addition significantly reduced soil N_2O emissions: by 28.3% compared to the NN fertilizer treatment. ANK addition, however, increased soil N_2O emissions: by 22.7% compared to the AN fertilizer treatment.

3.3. Relative abundance of communities and functional genes of nitrifying microbes

Sixteen genera of nitrifying microbes were observed in the soil during this study (Fig. 1 and Fig. S3), of which the predominant communities were *Nitrospira, Nitrososphaera, Nitrosomonas* and *Nitrosospira*. Fertilizer treatments altered the diversity of nitrifying microbes at the genus level (Fig. S5) and resulted in significant differences in the abundance of different genera (Fig. 1). Compared with the CK, NN addition decreased the relative abundance of most genera, including *Nitrospina*, *Candidatus Kuenenia*, *Nitrococcus*, *Nitrospira* and *Nitrososphaera*, while AN fertilizer increased the relative abundance of most genera, including *Nitrotoga*, *Candidatus Jettenia* and *Candidatus Scalindua*. AN fertilizer, compared to NN fertilizer, was associated with higher abundance of most nitrifying microbe genera, with the exception of *Nitrolancea* and *Nitrobacter*. In contrast to N fertilizer, K fertilizer addition resulted in a greater number of genera with higher relative abundance compared to the CK treatment. The NNK treatment significantly increased the relative abundance of *Candidatus Kuenenia and Nitrososphaera* compared with the NN treatment, while ANK treatment induced most genera with lower relative abundance compared to AK treatment, except for *Nitrolancea*, *Nitrobacter, Nitrospina* and *Candidatus Jettenia*.

The relative abundance of *amoA* of AOB was higher than that of AOA in the soil, but the *amoA* gene of AOA was more sensitive to N and K fertilizer applications than that of AOB (Fig. 2). NN addition markedly decreased the relative abundance of *amoA* of AOA and AOB compared with the CK treatment, while AN applications only significantly decreased the relative abundance of *amoA* of AOA but had no significant effect on the relative abundance of *amoA* of AOB. In contrast to CK, K fertilizer application significantly increased the relative abundance of *amoA* of AOA but had no significant effect on *amoA* of AOB. The NNK treatment had a higher relative abundance of *amoA* of AOA than that in the NN treatment, while the ANK treatment had a lower relative abundance of *amoA* of AOA than that in the AN treatment.

Table 1
Cumulative N_2O emissions (mg kg^{-1} dry soil) in the soils treated with different fertilizer treatments over the whole plant growth periods. Different letters indicated significant differences among the treatments resulting from the Tukey's test, n = 3, mean ± SD. ($P < 0.05$).

Treatments	N_2O emissions (mg kg^{-1} dry soil)	Increase to control (%)	N_2O emission factor (%)
CK	0.26 ± 0.02 f	–	–
NN	0.59 ± 0.03 c	123.9 ± 11.09 c	0.33 ± 0.03 c
AN	0.71 ± 0.03 b	167.2 ± 25.76 b	0.44 ± 0.02 b
K	0.35 ± 0.02 e	34.2 ± 3.45 e	–
NNK	0.43 ± 0.02 d	62.0 ± 12.23 d	0.16 ± 0.04 d
ANK	0.87 ± 0.05 a	228.9 ± 21.98a	0.61 ± 0.06 a

Note: N_2O emission factor=(N_2O emission in N treatment-N_2O emission in no N treatment)/N application rate × 100%.

3.4. Relative abundance of communities and functional genes of denitrifying microbes

In total, 63 genera of denitrifying microbes were observed (data not shown) and the top 20 in terms of relative abundance are shown in Fig. S4. The diversity and abundance of denitrifying microbes were significantly changed under N and K fertilizer treatments (Fig. S5 and Fig. 3). In contrast to AN, NN addition resulted in more genera with a higher relative abundance than the CK treatment. K fertilizer application significantly increased the relative abundance of *Thauera*, *Geobacter*, *Comamonas and Pseudomonas*, and resulted in decreased relative abundance of a few genera. Compared with the NN or AK treatment alone, NNK or ANK treatment decreased the relative abundance of most genera of denitrifying microbes, and the decreasing effect of ANK was greater than that of NNK.

Six functional genes, *narG*, *narH*, *narl*, *norB*, *nirK* and *nosZ* involved in denitrification were monitored in the soil (Fig. 4). The order of relative abundance of these was *norB* > *nirK* > *narG* > *nosZ* > *narH* > *narl*, and there was a significant response to fertilization ($p < 0.01$). In comparison with CK, both NN and AN fertilizer additions significantly increased the relative abundances of *narG, narH, narl* and *nirK* genes but significantly decreased the relative abundances of *norB* and *nosZ* genes. Treatments with K fertilizer decreased the relative abundances of *norB* and *nirK* but increased *narG, narH*, *narl* and *nosZ* genes when compared with the CK treatment with N fertilizer alone.

3.5. Correlations between soil properties and microbial responses

The relationships between soil properties and microbial responses under different N and K fertilizer treatments determined by redundancy analysis (RDA) are shown in Fig. 5 and Tables S2–S5. The soil properties measured in this paper explained 26.89%, 79.62%, 75.43%, and 82.33% of the variation in NI and DI (Fig. 5a), functional genes (Fig. 5b), community structure of nitrifying microbes (Fig. 5c) and community structure of denitrifying microbes (Fig. 5d), respectively. Soil pH was the predominant factor affecting NI, followed by DON and AP, which were significantly and negatively correlated with NI ($p < 0.05$, Table S2 and Fig. 5a). The predominant factors with respect to DI were soil pH and AK content, and they were positively correlated ($p < 0.05$, Table S2 and Fig. 5a). Gene AOA was negatively correlated with soil pH and AP concentrations, while AOB was negatively correlated with AP ($p < 0.05$). *NarG* was negatively correlated with soil pH, but *nirK* and *NorB* were positively correlated with soil pH, AK and DON concentration ($p < 0.05$) and negatively correlated with SOC. For *nosZ*, NO_3^- concentration was the predominant factor, with a significant positive correlation between them ($R^2 = 0.899$, $p < 0.01$), followed by AK, SOC and C/N concentration. Under N and K fertilization, the abundance of most nitrifying microbes genera was not correlated with soil AK concentration, but exhibited significant correlations with the concentrations of NH_4^+ and SOC induced by fertilization (Fig. 5c). For the abundance of denitrifying microbes, some genera presented significant positive correlations with soil AK concentration, but most genera were negatively correlated with pH ($p < 0.05$) and positively correlated with NH_4^+ ($p < 0.05$, Fig. 5d).

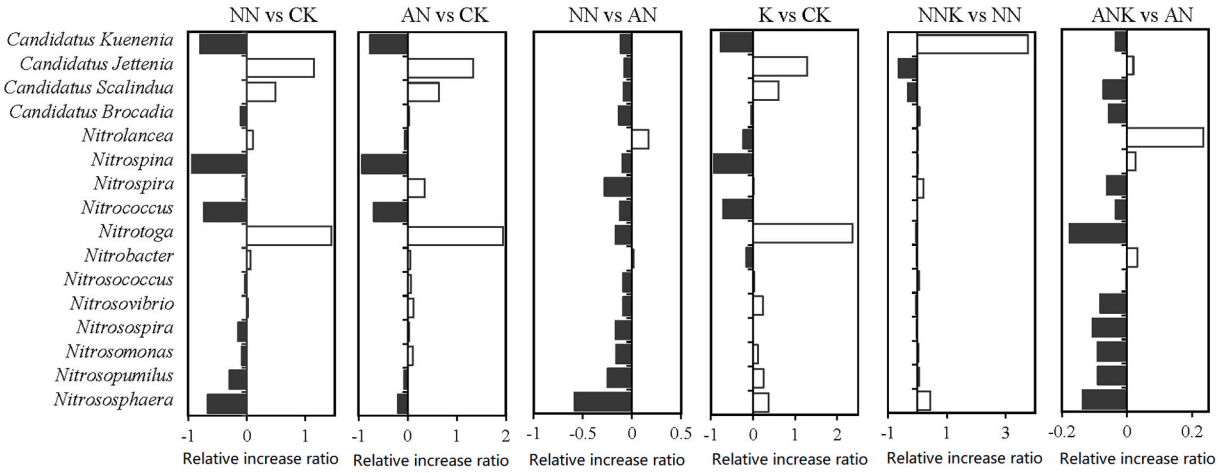

Fig. 1. The relative increase ratio in the relative abundance of most nitrifying microbe genera in NN vs CK, AN vs CK, NN vs AN, K vs CK, NNK vs NN, and ANK vs AN at harvest. CK, no fertilizer control; NN, nitrate-source nitrogen fertilizer addition; AN, ammonia-source nitrogen fertilizer addition; K, potassium fertilizer addition; NNK, combined NN and potassium fertilizer addition; ANK, combined AN and potassium fertilizer addition.

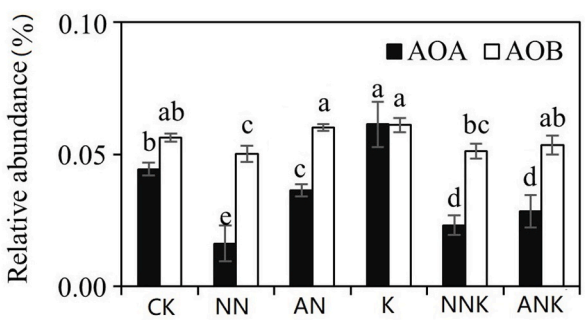

Fig. 2. The relative abundance of AOA and AOB in soils under different fertilizer treatments at harvest. CK, no fertilizer control; NN, nitrate-source nitrogen fertilizer addition; AN, ammonia-source nitrogen fertilizer addition; K, potassium fertilizer addition; NNK, combined NN and potassium fertilizer addition; ANK, combined AN and potassium fertilizer addition. Vertical bars are standard errors of the mean (n = 3). Different letters above the bars indicate significant differences ($p < 0.05$) between the treatments.

4. Discussion

4.1. Effects of N and K addition on N_2O emissions

N fertilization significantly increases N_2O emissions from farmland ecosystems (Huang et al., 2014; Wang et al., 2016; Hawthorne et al., 2017), a fact also confirmed in this study. Compared with no N application, N fertilizer additions increased N_2O emissions by 123–167%, accounting for 0.59–0.71% of the amount of N applied; this is in line with the global estimates of N_2O emissions resulting from N fertilizer applications, i.e. from 0.25% to 2.25% (Smith et al., 1998). In addition, we found lower N_2O emissions with the NO_3-based fertilizer than with the NH_4-based fertilizer. Volpi et al. (2017) reported similar results, namely that the cumulative N_2O emissions from ammonium fertilizer were higher than those from nitrate. By way of contrast, Huang et al. (2014) observed that NN application resulted in higher N_2O emissions than AN application in a paddy field. The inconsistency in N_2O emissions under NN and AN additions may depend on the strength and contribution of nitrification and denitrification under aerobic and anaerobic conditions. The soil in this study may be dominated by nitrification, under which AN application resulted in higher N_2O emission than NN application due to the stimulation of nitrification by AN (Volpi et al., 2017; Vermoesen et al., 1996). In contrast, if the soil was dominated by denitrification, NN application may produce more N_2O emissions than AN applications because of the promotion of denitrification (Huang et al., 2014).

The application of K fertilizer markedly increased the cumulative N_2O emissions, which was consistent with the previous results of long-term application of K fertilizer on N_2O emission in low fertility soil (Zhai et al., 2011; Meng et al., 2005). Reductions in soil O_2 and pH are likely to be the dominant drivers of N_2O production after K fertilizer application (Bar-Yosef and Ben Asher, 2013; Li et al., 2020b; Wang et al., 2020), because K fertilizer can result in a significant increase in the root respiration rate and secretion of organic acids (Fontana et al., 2020; Liu et al., 2020), especially in nutrient deficient or poor soils, resulting in different intensities of nitrification and denitrification and their relative contribution to N_2O emissions (Christensen, 1985; Li et al., 2020a). The increase in soil K^+ associated with K fertilizer application can also contribute to more N_2O emissions as a result of denitrification, because many denitrifiers respond positively to K, so their activity may increase with additions of K^+ (Wang et al., 2020).

There was a significant interaction between potassium and nitrogen types on N_2O emission. Potassium addition reduced the stimulation of nitrate-based fertilizers on N_2O emission, but increased the effect of ammonia-based fertilizers. The different effects of K fertilizer may be due to the changes of the main controlling factors of N_2O production in the two systems. In soils with combined application of K fertilizer and nitrate nitrogen fertilizer, the decrease in soil AN may become a determining factor for N_2O emissions, because of enhanced N absorption by plants when K is added (Qiao et al., 2014) resulting in a decrease of N_2O emission. However, in the treatment combining AN and K fertilizer, the increase of N_2O emission may be related to the increase in NH_4^+ nitrification, because K fertilizer increases soil NH_4^+ content via the NH_4^+ desorption of soil colloids by K^+ (Nieder et al., 2011).

4.2. Effects of N and K addition on nitrifiers

Fertilizer applications, especially inorganic fertilizers, change the soil microbial community structure (Tang et al., 2016; Wang et al., 2017; Yang et al., 2020b), including the overall and ammonia-oxidizing bacterial, fungal, and ammonia-oxidizing archaeal groups. As expected, the microbial community structure involved in nitrification changed with the N or K fertilizer treatments (Fig. 3 and S). The relative abundance of each genus was significantly different in response to N sources and K fertilizer. Nitrate-source fertilizer application significantly decreased the relative abundance of most nitrifying microbe genera, as in previous studies (Tang et al., 2016; Volpi et al., 2017), while ammonia-source N fertilizer and/or K fertilizer application significantly enhanced them.

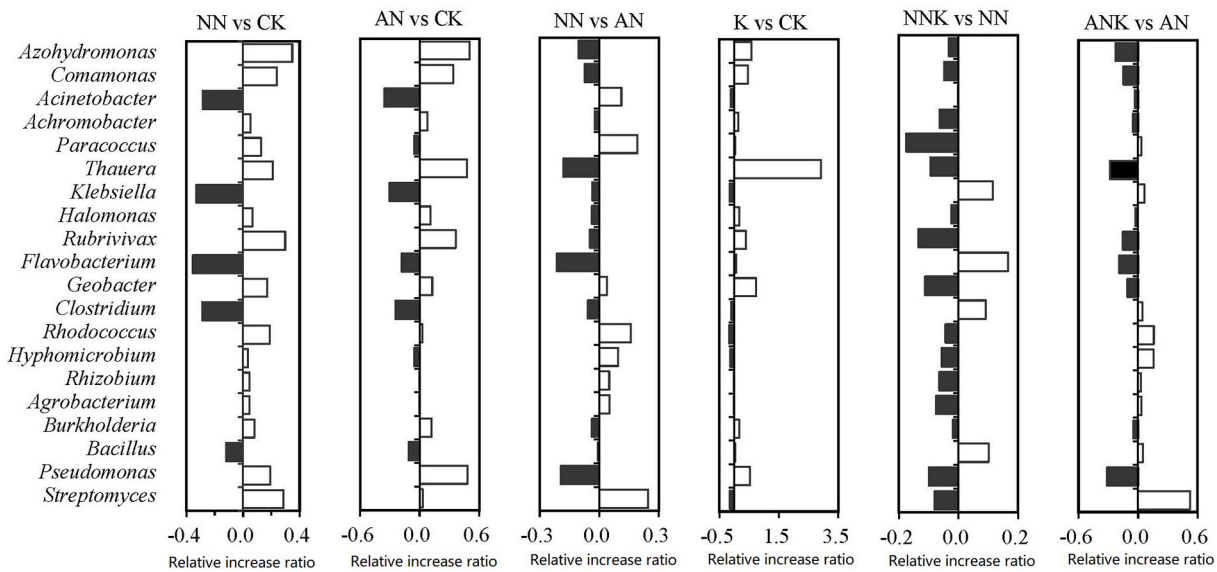

Fig. 3. The relative increase ratio in the relative abundance of most denitrifying microbe genera (top 20) in NN vs CK, AN vs CK, NN vs AN, K vs CK, NNK vs NN, and ANK vs AN at harvest. CK, no fertilizer control; NN, nitrate-source nitrogen fertilizer addition; AN, ammonia-source nitrogen fertilizer addition; K, potassium fertilizer addition; NNK, combined NN and potassium fertilizer addition; ANK, combined AN and potassium fertilizer addition.

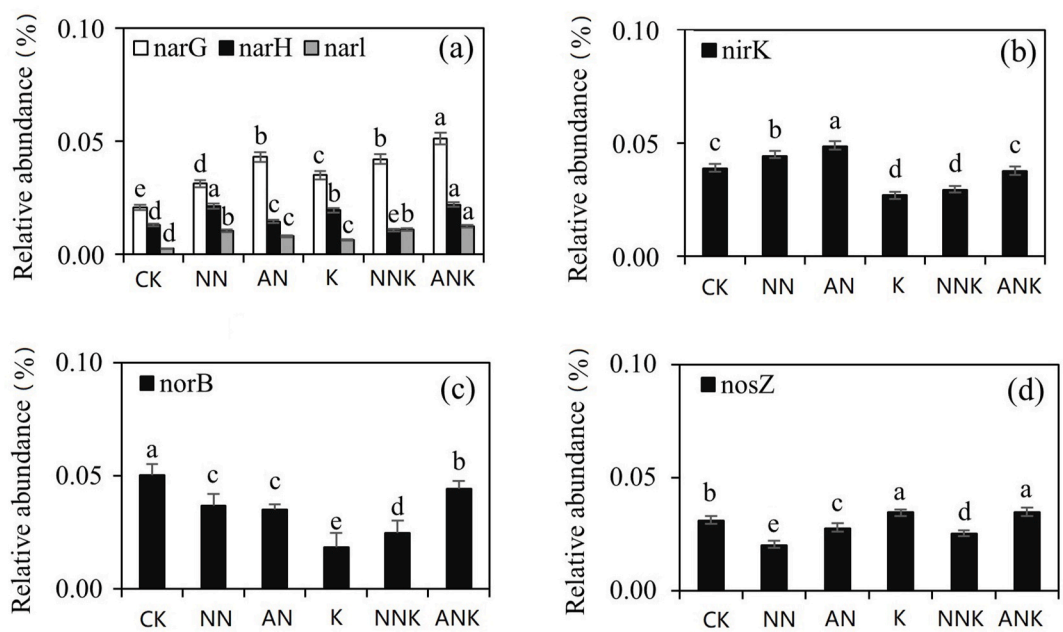

Fig. 4. The relative abundance of *narG, narH, narI, nirK, norB and nosZ* in soils under different fertilizer treatments at harvest. CK, no fertilizer control; NN, nitrate-source nitrogen fertilizer addition; AN, ammonia-source nitrogen fertilizer addition; K, potassium fertilizer addition; NNK, combined NN and potassium fertilizer addition; ANK, combined AN and potassium fertilizer addition. Vertical bars are standard errors of the mean (n = 3). Different letters above the bars indicate significant differences ($p < 0.05$) between the treatments.

These observations suggested that nitrate-source N fertilizer could inhibit the growth of nitrifying microbes (Yang et al., 2020b), especially *Nitrososphaera,* commonly known as ammonia-oxidizing archaea, and *Nitrosomonas* and *Nitrosospira*, commonly acting as ammonia-oxidizing bacteria; however, ammonia-source N fertilizer and K fertilizer selectively stimulated some nitrifying genera, including *Nitrospira*, *Nitrosomonas* and *Nitrososphacra*, a result also found in other experiments (Huang et al., 2014; Wang et al., 2020). This study further confirmed that nitrification could be inhibited by nitrate-source N fertilizer, but promoted by ammonia-source N fertilizer and K fertilizer.

In order to clarify the effect of inorganic fertilizer on nitrification further, we investigated the relative abundance of the functional genes of AOA and AOB that operate during the ammonia oxidizing stage of nitrification. Our results showed that the abundance of AOA was sensitive to N application, although AOB had higher abundance than AOA in this fertilized field soil. Yang et al. (2020b) also found that AOA was very sensitive to change in N availability in managed grassland, especially at low N levels. However, many previous studies, such as Di et al. (2009), Zhong et al. (2016), and Tao et al. (2017), reported that AOB rather than AOA drove nitrification, and the abundance and activity of AOA did not increase with the application of an ammonia substrate, even in soils with a large amount of AOA. Ai et al. (2013) also showed that higher N supply through N fertilization was not favorable to AOA and the capacity of AOA to reach maximum ammonia oxidation rates

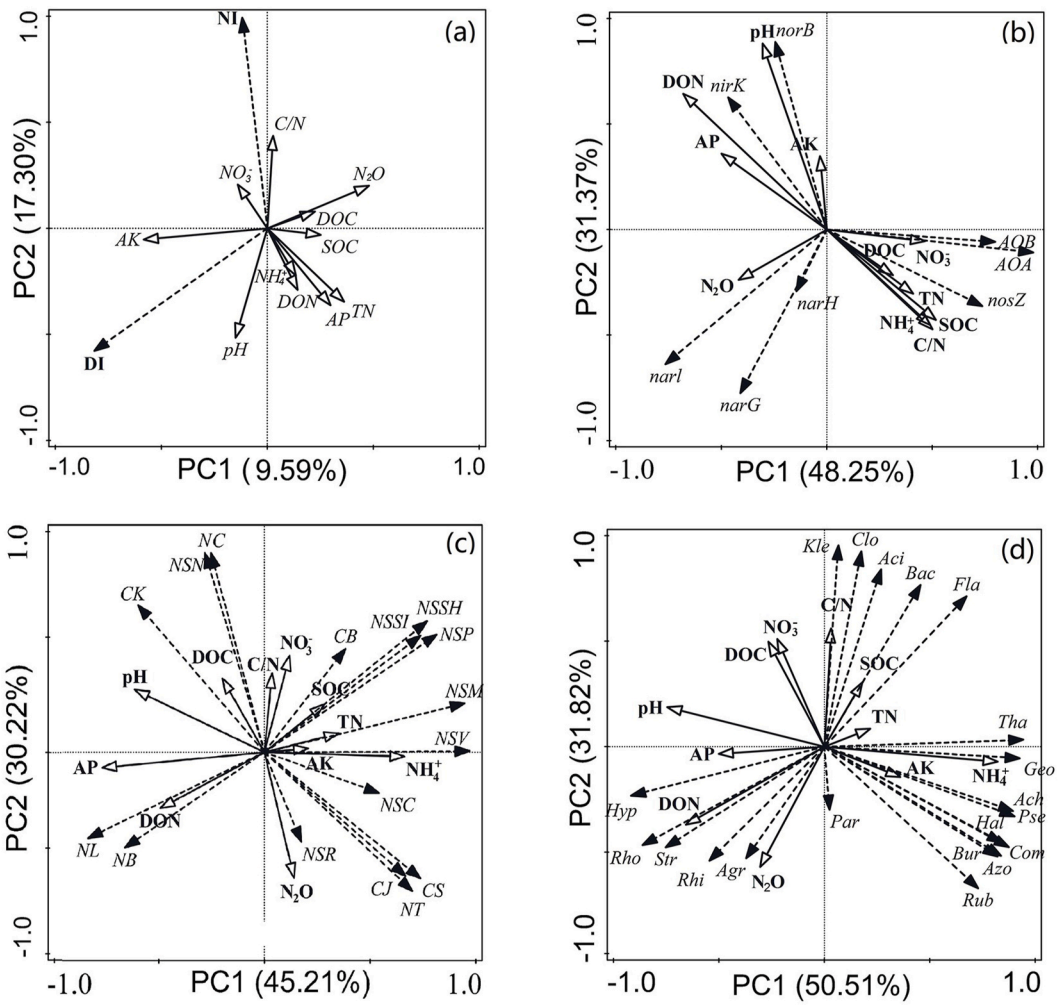

Fig. 5. Redundancy analysis of the linkages between soil properties and nitrification intensity (NI) and denitrification intensity (DI) (a), functional genes (b), nitrifying community (c), and denitrifying community (d), under different fertilizer treatments at harvest. NSSH, *Nitrososphaera*; NSP, *Nitrosopumilus*; NSM, *Nitrosomonas*; NSSI, *Nitrosospira*; NSV, *Nitrosovibrio*; NSC, *Nitrosococcus*; NB, *Nitrobacter*; NT, *Nitrotoga*; NC, *Nitrococcus*; NSR, *Nitrospira*; NSN, *Nitrospina*; NL, *Nitrolancea*; CB, *Candidatus Brocadia*; CS, *Candidatus Scalindua*; CJ, *Candidatus Jettenia*; CK, *Candidatus Kuenenia*. *Azo*, *Azohydromonas*; *Com*, *Comamonas*; *Aci, Acinetobacter*; *Ach*, *Achromobacter*; *Par*, *Paracoccus*; *Tha*, *Thauera; Kle*, *Klebsiella; Hal*, *Halomonas*; *Rub*, *Rubrivivax*; *Fla*, *Flavobacterium; Geo*, *Geobacter*; *Clo*, *Clostridium; Rho*, *Rhodococcus*; *Hyp*, *Hyphomicrobium*; *Rhi*, *Rhizobium; Agr*, *Agrobacterium*; *Bur*, *Burkholderia*; *Bac*, *Bacillus*; *Pse*, *Pseudomonas*; *Str*, *Streptomyces*.

occurs under low nutrient concentrations because of its high substrate affinity to nutrients (Yang et al., 2020b; Valentine, 2007). The higher variation of AOA than AOB under fertilizer application in this experiment might be explained by the low N availability in the soil at the time of harvest, in which some AOA groups such as *Nitrososphaera* taxa (Fig. 1S) adapted to lower N levels were induced and promoted to grow.

Fewer studies have examined how ammonia-oxidizing microbes and the genes of AOB and AOA in soils response to K compared to N addition (Li et al., 2020b; Wang et al., 2020). AOB did not change in response to K fertilization, but the relative abundance of AOA exerted a dramatic change with K application, indicating that K addition mainly induced AOA rather than AOB to perform ammonia oxidation. More interestingly, the abundance of AOA increased dramatically when K fertilization was applied alone or in combination with NN fertilization, but decreased significantly when K was applied in combination with AN fertilization. This suggests that the regulation mechanism and main driving factors of K fertilizer application on the AOA ammonia oxidation process vary with different forms and amounts of N supply. Changes of soil K concentration did not relate to AOA under K fertilization, implying that K^+ ions brought by K fertilizer did not directly affect the relative abundance or activity of AOA. Many recent studies have found that the addition of organic matter improves the population richness of AOA (Huang et al., 2014; Riya et al., 2015), and low pH environmental conditions are also considered favorable to AOA (Tao et al., 2017; Xiao et al., 2017). Soil pH and SOC availability might introduce changes of AOA abundance, especially in soils with K application alone. This is because soil organic carbon often significantly increases and soil pH significantly decreases due to the secretion of organic aids exudates and hydrogen ions from the roots after K fertilizer additions (Teixeira et al., 2011; Fontana et al., 2020; Liu et al., 2020). Besides, the abundance of AOA related significantly and negatively with soil AP after K application (Table 2S), which was consistent with the result of Tang et al. (2016) in fertilized grassland ecosystems. Soil AP might also become a limiting factor affecting AOA abundance by affecting the energy efficiency of microbial biomass (Lage et al., 2010). Additionally, soil AP associated with N mineralization (Tang et al., 2016), especially when the concentration of AP in soil is greater than 20 mg kg^{-1}, which likely triggered a cascading declining effect on AOA abundance (Yang et al., 2020b). The specific mechanism, especially for the unique effects of K application combined with NN and AN on AOA, requires further study.

4.3. Effects of N and K addition on denitrifiers

Fertilizer applications also have profound effects on the structural

and physiological properties of denitrifying microbes, by affecting cellular homeostasis, charge balance, cytoplasmic membranes, nucleic acids, ribosomes, proteins and enzymes (Duan et al., 2018; Riya et al., 2015). As with previous studies, N addition significantly increased the abundance of denitrifying microbes (Tang et al., 2016; Riya et al., 2015). In addition, we found that nitrate-source fertilizer had a stronger effect on denitrifying microbial abundance than ammonia-source fertilizer, with greater numbers of high abundance denitrifying genera, probably due to more accessibility to NO_3^- ions from nitrate-source fertilizer for denitrifiers (Omirou et al., 2020). Compared to CK, K fertilizer had a significant increasing effect on the relative abundance of denitrifying genera, especially for the genera *Thauera* and *Geobacter* with higher relative abundance (Wang et al., 2020). This could be related to the decreased soil pH with K fertilization or the affinity for K of these denitrifiers (Epstein, 2003; Wang et al., 2020); this is supported by our statistical analysis, showing that pH was negatively correlated with *Thauera* and *Geobacter* abundance and AK concentration was positively correlated with *Pseudomonas*. Wang et al. (2020) also observed that denitrifying microbes were sensitive to K concentration but each genus has different optimal K concentrations, so the relative abundance of *Thauera* could be promoted by K^+ up to 230 mg-K/L. However, interestingly, we found that the K fertilizer produced a decreasing effect in the relative abundance of most denitrifying microbes in soil under N supply. This implied that the main driving factors affecting the abundance of denitrifying microbial abundance might change when K was applied under different N supply or in soils with different N content. In the soil with N fertilizer application, the decrease in N availability due to the increase of plant N uptake by K fertilizer (Hou et al., 2019) likely became the main factor affecting denitrifying microbial abundance.

Furthermore, our studies indicated that the abundance of four functional denitrification genes, *narG*, *nirK*, *norB* and *nosZ*, varied greatly in response to N and K fertilizer. Among them, the *narG* gene, which acts as the largest structural gene encoding nitrate reductase (*Nar*), was significantly induced by N additions; this is consistent with previous studies (Tang et al., 2016; Ma et al., 2016). Based on the fact that NO_3^- promotes *Nar*, we expected that ammonia-source N fertilizer application might result in a lower abundance of *narG* than nitrate-source N fertilizer. However, the results were just the opposite. This reflects the fact that nitrate reductase may be not only driven by N substrate but may also be jointly regulated by other soil factors such as SOC, TN or AP concentration, which often change synergistically after N fertilizer addition. K addition also significantly increased the abundance of *narG*, and this effect was more obvious when combined with N fertilizer; this is inconsistent with the above conclusion that K fertilizer inhibits denitrification intensity. Some studies have suggested that nitrate reductase is not a unique step in denitrification (Kuypers et al., 2018; Yang et al., 2020a), so the coding gene of nitrate reductase cannot accurately reflect the characteristics of denitrifying microbes. This study also supports this conclusion to some extent.

The nirK and *nirS* genes, encoding nitrite reductase, are responsible for the limiting step in the denitrification process to convert nitrite into nitrogen oxide (Duan et al., 2018). It has been suggested that these two genes are influenced by a variety of environmental factors, such as carbon substrate availability, moisture and pH, and possibly exhibit niche differentiation in relation to environmental change (Yang et al., 2020a). In this experiment, only the *nirK* gene (not *nirS*) was recorded in all treatments, implying that *nirK*-type bacteria were favored and are better suited to the local soil environment than *nirS*-type bacteria; this may indicate a survival selection mechanism of nitrite reducers (Tang et al., 2016). In addition, the abundance of *nirK* significantly increased after N additions but significantly decreased after K additions; indeed, Tang et al. (2016) and Ma et al. (2016) reported that the abundance of *nirK* or *nirS* changed after N and P additions. This suggests that *nirK*-type bacteria may thrive in different habitats in response to soil nutrient change. The decrease in the abundance of *nirK* with K fertilization may be related to the decrease in substrate N concentration or pH (Cuhel et al., 2010; Bar-Yosef and Ben Asher, 2013; Qiao et al., 2014), since K addition promotes the absorption of N by plants and the release of acidic substances into the soil (Teixeira et al., 2011). This hypothesis is also supported by our PCA analysis results, showing that the abundance of *nirK* was positively affected by DON and pH.

The *norB* and *nosZ* genes are directly involved in N_2O emissions. *NorB* produces N_2O while *nosZ* is responsible for N_2O reduction (Sullivan et al., 2013; Pomowski et al., 2011). According to previous research, the abundance of both *norB* and *nosZ* is affected by nutrient additions (Omirou et al., 2020). In the current study, the two genes exhibited different responses to N and K fertilization. N fertilization limited the abundance of *norB* and *nosZ*, which is consistent with a previous study (Tang et al., 2016); however, interestingly, the addition of K fertilizer resulted in a significant decrease in *norB* but a significant increase in *nosZ*. Some studies such as Cuhel et al. (2010), Epstein (2003) and Huang et al. (2014) have shown that the change in soil pH caused by N or K fertilization may be the primary factor leading to changes in the abundance of the *norB* or *nosZ*-type bacteria. Indeed, we found that there was a significant positive correlation between the abundance of *norB*--type bacteria and soil pH, which also explained why the lower pH caused by K fertilizer compared to N fertilizer application resulted in lower abundance of *norB*. However, no significant correlation between the abundance of *nosZ*-type bacteria and pH value was observed. The results suggest that *nosZ*-type bacteria were not sensitive to the change in soil pH from 7.66 to 7.90 in the current experiment, and other factors, such as concentrations of NO_3^- and SOC and C/N ratios, possibly limited the abundance of *nosZ*-type bacteria, as indicated by their significant correlations with the abundance of *nosZ* (Fig. 4). As reported, the growth of denitrifying microbes can be limited by SOC and NO_3^- or C/N ratios, because the denitrification in which they participate requires SOC as an electron supplier and NO_3^- as an electron acceptor (Li et al., 2020a; Yang et al., 2020a). We assumed that K application may have resulted in higher SOC concentrations in soils than other fertilizer treatments (Bar-Yosef and Ben Asher, 2013) thus creating a more suitable environment for *nosZ*-type denitrifiers.

Numerous studies have highlighted the relationship between N loss caused by leaching or gas emissions and related functional gene groups (Tang et al., 2016; Wang et al., 2019). For example, soil containing higher *amoA/narG* may have a high potential for N loss through NO_3^- leaching (Tang et al., 2016). This relationship was also verified in some treatments in this study. Compared with the single N application, the combination of N and K had higher *amoA/narG* and lower N leaching (data not shown). Besides, agricultural soil with high ratios of *norB/nosZ* gene copies usually has high N_2O emissions (Tang et al., 2016; Wang et al., 2019). Indeed, we observed that compared with other treatments, the soil receiving K had a lower *norB/nosZ* ratio, and correspondingly lower N_2O emissions, while N addition was associated with higher ratios of *norB/nosZ*, which ultimately resulted in more N_2O emissions, especially when adding NN fertilizer. These results imply that the change in N_2O emission or N leaching loss can be explained from the molecular perspective on the basis of functional gene groups.

5. Conclusion

Our results indicated that the addition of N and K fertilizers significantly promoted N_2O emissions from farmland soil. Combined application of K fertilizer with AN fertilizer furtherly resulted in a significant increase in N_2O emissions, which may be due to the increase in the abundance of *norB*-type denitrifiers, especially the genera *Streptomyces* and *Hyphomicrobium,* and/or biochemical pathways promoting the transformation of nitrite to N_2O. However, combined application of K fertilizer and NN fertilizer can alleviate N_2O emissions, mainly due to the decreased abundance of *nirK* and *norB*-type denitrifiers, especially the genera *Paracoccus*, *Rubrivivax* and *Geobacter*. Our work highlights the important role of the appropriate application of N and K fertilizers, which may produce different results in reducing or promoting N_2O

emissions from farmland. Therefore, it is suggested that NN fertilizer should be the first choice for farmland without considering nitrogen form, and K fertilizer could be increased when soil AK is insufficient, which is beneficial to the environment and crop yield. In fields where AN fertilizer must be fertilized, K fertilizer should be applied as little possible or not at all.

Author contribution

Zhiguo Li: Funding acquisition, Writing – review & editing; Linyang Li: Writing – original draft; Shujie Xia: Investigation, Data curation; Runhua Zhang: Visualization, Writing – review & editing; Runqin Zhang, Investigation, Data curation, Methodology; Peng Chen: Writing – review & editing; Yi Liu: Writing – review & editing; Junfeng Pan: Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2021.112579.

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