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The interactive effect of temperature and fertilizer types determines the dominant microbes in nitrous oxide emissions and the dicyandiamide efficacy in a vegetable soil

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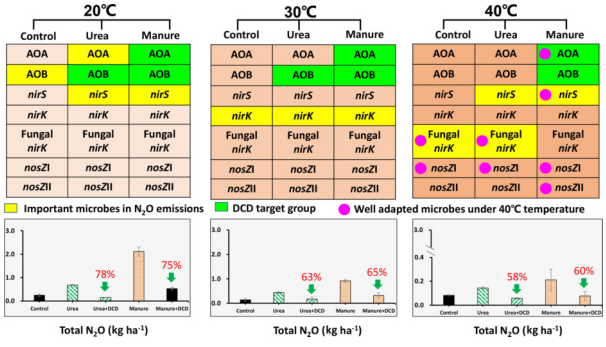
ABSTRACT

- Dicyandiamide decreased N_2O emissions even under 40°C.
- Ammonia oxidizers and *nirS* were well adapted to 40°C in manured soils.
- Fungal *nirK* tolerated high temperature better in urea than manure treatment.
- Compared to *nosZ*II, *nosZ*I adapted to all temperature regardless of fertilization.
- *nirS*-denitrifier dominated N_2O emissions at high temperature in fertilized soil.



Heat waves associated with global warming and extreme climates would arouse serious consequences on nitrogen (N) cycle. However, the responses of the functional guilds to different temperatures, especially high temperature and the cascading effect on N_2O emissions remain unclear. An incubation study was conducted to examine the effect of different temperatures (20°C, 30°C, and 40°C) and fertilizer types (urea and manure) on N_2O-producers and N_2O-reducers, as well as the efficacy of dicyandiamide (DCD) on N_2O emissions in a vegetable soil. Results showed that ammonia oxidizers and *nirS*-type denitrifiers were well adapted to high temperature (40°C) with manure application, while the fungal *nirK*-denitrifiers had better tolerance with urea application. The *nosZ* clade I microbes had a strong adaptability to various temperatures regardless of fertilization type, while the growth of *nosZ* clade II group microbes in non-fertilized soil (control) were significantly inhibited at higher temperature. The N_2O emissions were significantly decreased with increasing temperature and DCD application (up to 60%, even at 40°C). Under high temperature conditions, fungal denitrifiers play a significant role in N-limited soils (non-fertilized) while *nirS*-type denitrifiers was more important in fertilized soils in N_2O emissions, which should be specially targeted when mitigating N_2O emissions under global warming climate.

Keywords nitrogen fertilizer, microorganisms, nitrification inhibitor, bacteria, archaea, fungi

1 Introduction

Nitrous oxide (N_2O) is a powerful greenhouse gas (GHG) with a global warming potential approximately 300 times that of carbon dioxide (CO_2) on a 100-year time horizon (IPCC, 2007, 2014). Additionally, N_2O destroys the stratospheric ozone layer (Ravishankara et al., 2009). Agriculture is the main source of anthropogenic N_2O emissions because of N

fertilizer application (Kuypers et al., 2018). N_2O emissions are expected to increase continuously with the excessive use of fertilizer to meet the growing demand for food production (Thompson et al., 2019; Zhang et al., 2020).

Nitrification and subsequent denitrification are thought to be the main pathways of N_2O emissions in soil (Voigt et al., 2020). These pathways are mainly driven by functional microbes, including N_2O-producers and N_2O-reducers (Xu et al., 2020). Ammonia oxidizers (i.e., archaeal and bacterial ammonia oxidizers, respectively AOA and AOB), bacterial denitrifier (i.e., *nirS*- and *nirK*-type denitrifiers), and fungal denitrifiers (i.e., fungal *nirK*-type denitrfier) are common N_2O-producers. These functional microbes are temperature-sensitive and their relative contributions to N_2O emissions vary, depending on their inherent sensitivity of temperature, fertilizer types, and soil types (Lai et al., 2019; Mukhtar et al., 2019; Li et al., 2021; Xu et al., 2021). For example, it was reported that ammonia oxidizers and bacterial denitrifiers were all significantly inhibited under high temperatures, while fungal denitrifiers were well adapted and might be the dominant contributor of N_2O emissions in an acidic soil (Xu et al., 2017). Other studies have also indicated that temperature change may cause niche differentiation of these N_2O-producers during the production of N_2O emissions (Yin et al., 2017; Duan et al., 2019a; Lai et al., 2019). However, the responses of N_2O-producers to different temperatures and the underlying pattern, depending on fertilizer type are still poorly understood (Stein, 2020; Yin et al., 2022).

The currently known N_2O-reducers are *nosZ* clade I (*nosZ*I) and *nosZ* clade II (*nosZ*II), which are responsible for encoding the N_2O reductase, the enzyme catalyzing the last step of denitrification ($N_2O \rightarrow N_2$) (Domeignoz-Horta et al., 2018). This pathway represents the only known biological sink of N_2O emissions (Samad et al., 2016). Compared with *nosZ*I group, *nosZ*II group is newly identified and less studied (Xu et al., 2020). Recent studies have indicated that these two clades have largely different characteristics, and their sensitivities to environmental variations and agricultural practices also largely differ (Stein, 2020). It was reported that *nosZ*II group, rather than *nosZ*I group, was better adapted to warmer and drier conditions (Xu et al., 2020), and explained the variation of *in situ* N_2O emissions (Domeignoz-Horta et al., 2018). Comparably, other studies have found that the *nosZ*I-type denitrifiers adapt to various temperature conditions and are sensitive to high temperature, playing an important role in N_2O consumption (Cui et al., 2016; Xing et al., 2021). Thus, the niche separation of the two clades of *nosZ*-type denitrifiers in response to increased temperature remains unclear. Considering that these two clades dominate the only known biological sink of N_2O emissions, their responses to different temperatures need further investigation to better predict future N_2O emissions and develop mitigation strategies under global warming.

Dicyandiamide (DCD) is an effective nitrification inhibitor which is widely used to mitigate N_2O emissions (Di et al., 2014). The efficiency of DCD strongly depends on soil conditions, especially soil moisture contents and soil temperature (Di and Cameron, 2016; Wu et al., 2017; Guo et al., 2021). The application of DCD can effectively inhibit ammonia oxidization to decrease nitrate production, inhibit denitrification, and hence decrease N_2O emissions (Guo et al., 2014; Di and Cameron, 2016). Since DCD is susceptible to biodegradation, it is commonly used in autumn or winter when the temperature is relatively low (Kelliher et al., 2014). In the context of global warming, extreme heat climates will appear more frequently (IPCC, 2014; Zhang et al., 2020). However, the effectiveness of DCD at the temperature higher than 30°C has not yet been tested. Additionally, the efficacy of DCD can be modulated by different fertilizer types (Sha et al., 2020a, 2020b). The interactive effects of fertilizer types and increasing temperatures on the efficacy of DCD require further investigation to provide accurate fertilization tactics and mitigation strategies under the on-going climate warming conditions.

This study aimed to examine the interactive effects of temperature (20°C, 30°C, and 40°C) and fertilizer type (urea and manure) on N_2O-producers (including AOA, AOB), *nirS/K*-type bacterial denitrifiers and fungal *nirK*-type denitrifiers) and N_2O-reducers (including *nosZ*I and *nosZ*II clade) in a vegetable soil. It also examined the efficiency of DCD on reducing N_2O emissions under different temperature conditions. We hypothesized that 1) increasing temperature would decrease the inhibitory efficiency of DCD on N_2O emissions; 2) AOA and fungal *nirK*-type denitrifier would be more adapted than other N_2O-producers to high temperature with manure application; 3) different temperature conditions would lead to niche separation of the two clades of *nosZ*-type denitrifiers.

2 Materials and methods

2.1 Soil and manure used for the study

The soil used in this study was collected from a vegetable field in Quzhou, Zhejiang Province (28°45′ N, 118°20′ E) and is classified as Alfisol (yellow-brown soil). The sampling site was characterized as a subtropical monsoon climate, with a mean annual temperature of 17.3°C and precipitation of 1843 mm. The soil samples were collected from 0 to 20 cm surface soil at five random locations, and then mixed to make a composite sample. Fresh soil was passed through a 5-mm sieve and stored at 4°C until the commencement of the incubation experiment. About 500 g soil was air-dried to

determine the physical and chemical properties. The soil had the following properties: pH (1:2.5 H_2O) 7.2, organic C (carbon) 10.02 g kg^{-1}, total N (nitrogen) 1.21 g kg^{-1}, available P (phosphorus) 12.4 mg kg^{-1} and available K (potassium) 131.2 mg kg^{-1}.

Pig manure was obtained from a local pig farm and composted with sawdust. It had pH (H_2O) 7.3, the mean organic C, total N, total P and total K concentrations of 298.8, 15.5, 12.6 and 0.1 g kg^{-1} (dry matter), respectively, and the NH_4^+-N and NO_3^--N concentrations of 72.1 and 6.3 mg kg^{-1}, respectively.

2.2 Incubation experiment

2.2.1 Treatments

The experiment consisted of 5 fertilizer treatments, 3 temperatures and 3 replicates. The five treatments were: 1) Control (no fertilizer application); 2) Urea (400 kg N ha^{-1}); 3) Manure (400 kg N ha^{-1}); 4) Urea (400 kg N ha^{-1}) + DCD (20 kg ha^{-1}) and 5) Manure (400 kg N ha^{-1}) + DCD (20 kg ha^{-1}). The N amount was chosen to simulate the local management practice of the intensive vegetable production. The three selected temperatures were 20°C, 30°C, and 40°C. Two sets of parallel incubation experiments were set up, with one set for soil sampling and the other for gas sampling, according to Xu et al. (2017).

2.2.2 Experimental setup and sampling

For each treatment of the soil sampling experiment, 1 kg of sieved soil (dry weight basis) was placed into a 1-L plastic incubation container (12 cm diameter) with two 1-cm holes on the lid to maintain air exchange. Urea was dissolved in deionized water and added to soil evenly. Manure was passed through a 3-mm sieve and well mixed with soil. Soil sampling of each treatment was performed after 0, 5, 10, 19, 26, 41, 54, 68 and 90 days of incubation.

For the gas sampling set of the experiment, 500 g of sieved soil was placed into a 1-L glass jar (10.0 cm diameter). Three fifths volume of the jars was left empty for gas sampling. Gas sampling was carried out twice a week in the first month of incubation, and then weekly until the end of experiment. During the gas sampling, the jar was sealed by a lid with rubber stopper, which was linked with a three-way valve and syringe. Gas samples were taken at 0, 20 and 40 min and stored in 9-mL glass vials.

The soil water content was adjusted to field capacity (the water content was 23.4%) in all treatments. The weight of the containers was adjusted twice to three times per week to maintain the 100% field capacity of soil moisture content during the incubation period.

2.2.3 Analyses of mineral N and N_2O

Soil NH_4^+-N and NO_3^--N were extracted from 5 g soil sample with 50 mL of 1 M KCl, shaken at 250 rpm for 1 h. After filtration, NH_4^+-N and NO_3^--N in the filtrates were measured using a flow injection analyzer (SAN + + , Skalar, Netherlands). N_2O concentration of the gas samples was determined by gas chromatography (GC-2010 Plus SHIMADZU, Japan).

2.2.4 DNA extraction and quantification of key functional genes

Soil samples from days 0, 20, and 65 were used for DNA extraction and functional gene analyses. Soil DNA was extracted from 0.5 g fresh soil using a FastDNA spin kit for soil (MP Biomedicals, OH, USA), according to the manufacturer's protocol. The quality of DNA extracts was checked by gel electrophoresis and concentrations were determined using a Nanodrop® ND-2000 UV-vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Real-time fluorescent quantitative PCR analysis of the functional genes of ammonia oxidizers (bacterial and archaea *amoA* genes) and denitrifiers (*nirS*/*nirK*, fungi *nirK*, *nosZ*I and *nosZ*II) was performed by the LightCycler® 480II (Roche, German). The details of the primers and thermal profiles for each target gene were described in Liu et al. (2019). Each 20-μL reaction system included 1−10 ng of the template DNA, 0.16−0.3 μL of each primer, 10 μL SYBR® Premix Ex Taq™ II (TaKaRa, Japan) and milli-Q water to the final volume. Melting curve analysis was performed at the end of each run to confirm the PCR product specificity. Standard curve for qPCR were made as follows: each target gene was first PCR amplified and then purified by a PCR cleanup kit (Macherey-Nagel Inc, Germany). The purified PCR products were cloned into the pGEM-T Easy Vector (TransGen Biotech, China), and then transformed into *Escherichia coli* JM109 competent cells (TransGen Biotech, China). The plasmid was extracted by a MiniBEST Plasmid Purification Kit (TaKaRa, Japan). The concentration of the plasmid was measured by a Nanodrop® ND-2000 UV–vis, and 10-fold serial dilutions of plasmid with known gene copy number were used as standard curve. To avoid possible inhibitions of the qPCR reactions, a series of 10-fold dilutions of the DNA samples were included during the assessment. The amplification efficiencies reached 90%−101% after 10-fold dilution which was used for the final qPCR analysis. The reaction specificity was confirmed by the melting curve analysis at the end of each run.

2.2.5 MiSeq sequencing and phylogenetic analysis

The AOA and AOB communities were analyzed by MiSeq

sequencing of 16S rRNA genes in the V4-V5 regions with the universal primers 515F and 907R (Xu et al., 2020). A total of 2 361 339 16S rRNA gene high quality sequence reads were obtained. The raw data were processed using the Quantitative Insight Into Microbial Ecology (QIIME2). Only sequences with quality score > 20 and read length > 200 bp and without ambiguous calls were included for the subsequent analyses. These selected sequences were then binned into one operational taxonomic unit (OTUs) at a 97% identity threshold. Reads classified as AOB and *Thaumarchaeota* or unclassified archaea were screened out for phylogenetic trees construction by the Molecular Evolutionary Genetics Analysis (MEGA 4.0) with 1 000-fold bootstrap. The data set of gene sequences was deposited under BioProject accession number PRJCA005798 in National Genomics Data Center's Genome Sequence Archive (GSA).

2.3 Statistical analysis

Hourly N_2O emissions were calculated according to Hutchinson and Mosier (1981) by using the slope of glass jar headspace N_2O concentration change from the collected three samples. Cumulative emissions were calculated according to Di et al. (2007) by integrating the measured daily fluxes which were calculated using hourly N_2O fluxes.

One-way analysis of variance (ANOVA) followed by Duncan-test was performed to check the differences in total N_2O emissions and functional gene abundances (Wang et al., 2023). Two-way ANOVA was conducted to test the significance of the effects of fertilizer type and temperature and their interactions on the abundances of N_2O-producers and N_2O-reducers. One-way and two-way ANOVA were performed by SPSS version 20 (IBM Co., Armonk, NY, USA). $P < 0.05$ was regarded as statistically significant. The correlation analysis of inorganic N, functional gene abundance and N_2O emission was calculated and visualized in GGally, ggplot2 and wesanderson packages of R. Structure equation modeling (SEM) was used to evaluate the direct and indirect effects of temperature on mineral N concentrations, functional gene abundances and N_2O emissions using AMOS 24.

3 Results

3.1 Soil mineral N concentrations

The NH_4^+-N concentrations in the urea treatments increased rapidly first and then decreased with incubation time at 20, 30, and 40°C (Fig. 1A−C). In contrast, NH_4^+-N concentrations in the manure treatments decreased slowly over time (Fig. 1A−C). In the treatments of urea + DCD, NH_4^+-N reached to the highest concentration at day 10 and remained stable thereafter at 20°C, but decreased with incubation time at 30°C (Fig. 1 A−B). In the treatments of manure + DCD, soil NH_4^+-N concentration increased gradually at 20°C while decreased gradually at 30°C (Fig. 1 A−B). The addition of DCD showed no significant difference in NH_4^+-N concentration regardless of fertilizer type at 40°C ($P > 0.05$) (Fig. 1C).

Soil NO_3^--N concentrations changed conversely to those of NH_4^+-N. Specifically, in the urea and manure treatments, NO_3^--N concentrations increased over time. The NO_3^--N concentrations in the urea treatment was significantly higher than those in the manure treatment after 41 days of incubation at 20°C, but significantly lower than those in the manure

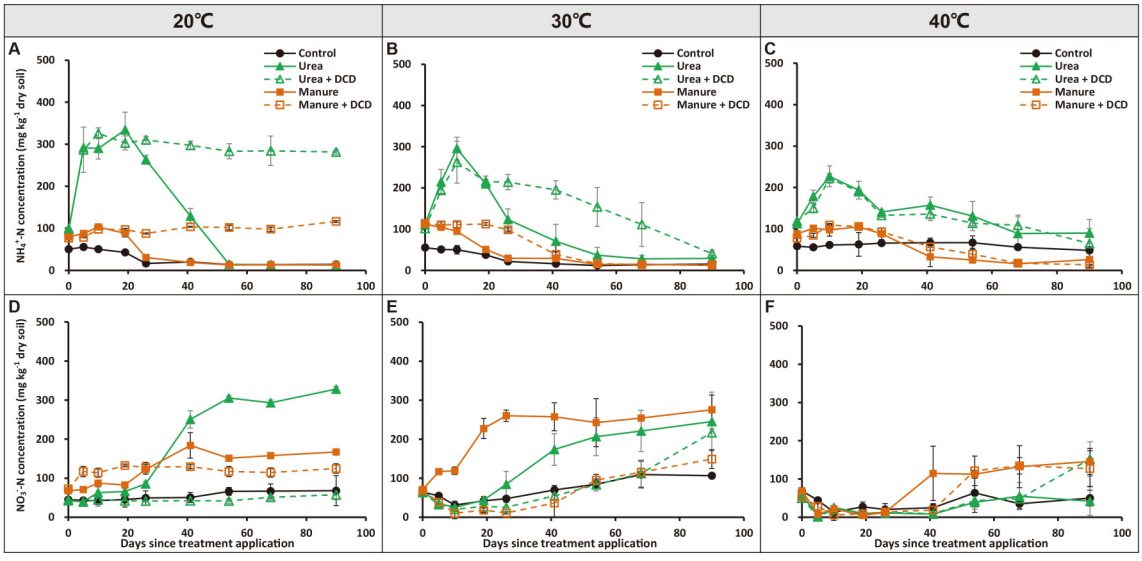


Fig. 1 The effects of fertilizer type (urea and manure) and addition of dicyandiamide (DCD) on the concentrations of NH_4^+-N (A−C) and NO_3^--N (D−F) at soil temperatures of 20, 30 and 40°C during 90 days of incubation. The vertical bars indicate the standard error of the means (S.E.M.).

treatment during 41 days of incubation at 30°C ($P < 0.05$) (Fig. 1D, E). The application of DCD reduced NO_3^--N concentrations at 20 and 30°C (Fig. 1D, E). However, the NO_3^--N concentration at 40°C was low and was not significantly affected by fertilizer treatment (Fig. 1F).

3.2 N_2O emissions and DCD inhibition efficiency

At 20°C, the application of manure significantly increased hourly N_2O emissions compared with the urea and control treatments ($P < 0.05$) with a significant N_2O peak of 0.64 mg N_2O-N m^{-2} h^{-1} at day 20 (Fig. 2A). The hourly N_2O emissions with urea were significantly higher than those in the control treatment between days 11 to 27 ($P < 0.05$) (Fig. 2A). At 30°C, significant N_2O peaks of 0.12 mg N_2O-N m^{-2} h^{-1} at day 20 and 0.25 mg N_2O-N m^{-2} h^{-1} at day 27 were observed in the urea and manure treatments, respectively (Fig. 2B). However, at 40°C, the application of manure hardly affected N_2O emissions with a small peak of 0.06 mg N_2O-N m^{-2} h^{-1} and 0.04 mg N_2O-N m^{-2} h^{-1} observed at days 33 and 56, respectively (Fig. 2C).

Total N_2O emissions in the manure treatment were significantly higher than those in the urea and control treatments at 20°C and 30°C ($P < 0.05$) (Fig. 2D). At all temperature conditions, the application of urea significantly increased total N_2O emissions compared with the control ($P < 0.05$) while DCD addition significantly decreased total N_2O emissions (Fig. 2D).

The inhibition efficiency of DCD at 20°C was higher than that at 30 and 40°C in the urea and manure treatments. At 40°C, DCD decreased N_2O emission by approximately 60% (Table 1).

3.3 N_2O related functional microbes

Fertilizer type and temperature and their interaction had significant effects on AOA and AOB abundance ($P < 0.01$) (Table 2). Specifically, the application of manure significantly increased the abundance of archaeal *amoA* gene by one to two orders of magnitude at 20°C and 40°C after 65 days of incubation (Figs. 3A and C) and at 30°C after 20 days ($P < 0.05$) (Fig. 3B). The application of urea significantly increased the bacterial *amoA* gene abundance at 20°C and 30°C by day 65 ($P < 0.05$) (Fig. 3D and E). Manure addition significantly increased bacterial *amoA* gene abundance at 30°C and 40°C compared with the control treatment after incubation for 65 days ($P < 0.05$) (Fig. 3E and F). The application of DCD significantly decreased the abundance of AOA and AOB in the urea and manure treatment at 20°C and 30°C after 65 days of incubation, but decreased the

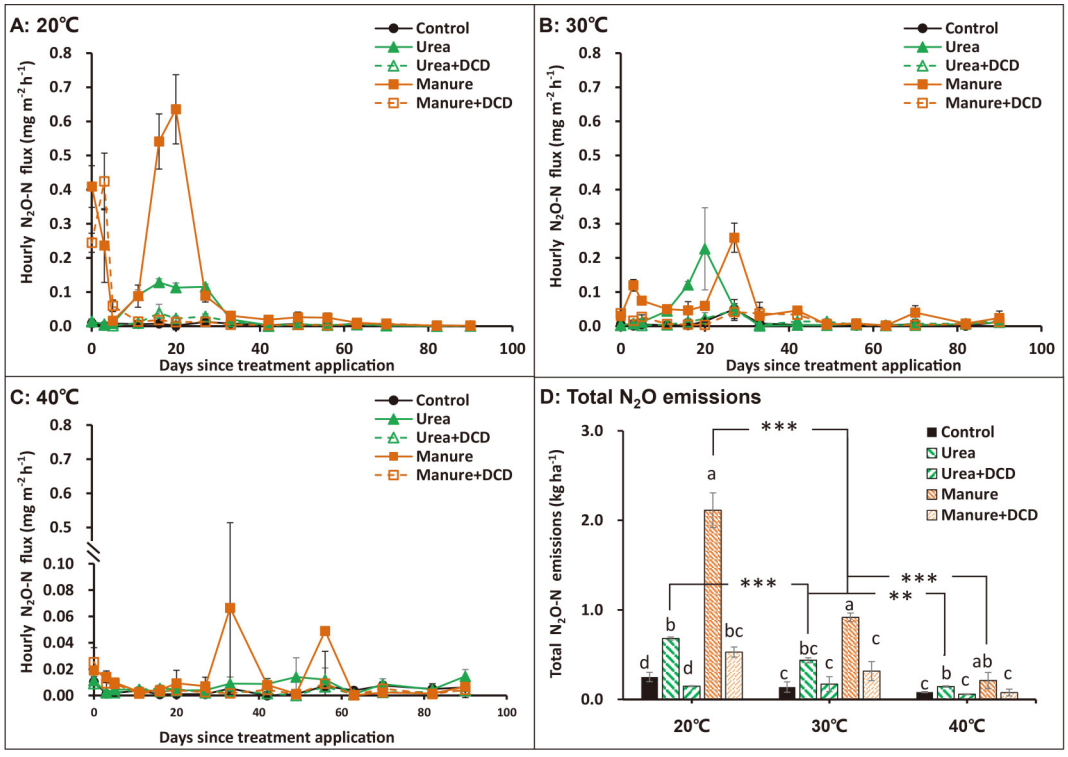


Fig. 2 The effects of fertilizer type (urea and manure) and addition of dicyandiamide (DCD) on hourly N_2O fluxes (A–C) and total N_2O emissions (D) at soil temperatures of 20, 30, and 40°C during 90 days of incubation. The vertical bars indicate the standard error of the means (S.E.M.). Different letters indicate significant differences ($P < 0.05$) in the N_2O emissions with different fertilizer types and DCD application, as indicated by the analysis of variance (LSD) method. Asterisks ** and *** indicate a significant difference between different temperature conditions at $P < 0.01$ and $P < 0.001$, respectively.

abundance of AOA and AOB only in the manure treatment at 40°C. Phylogenetic analysis of 16S rRNA genes showed that 98.8% of the AOA-like sequences affiliated to group 1.1b (Fig. S1A). Specifically, half of the total archaeal 16S rRNA gene sequences were affiliated with *Nitrosocosmicus* cluster with manure application at 30°C. In addition, at 40°C, 15.9%, and 15.2% of the total archaeal 16S rRNA gene sequences were classified into *Nitrososphaera* cluster in the manure and control treatments, respectively (Fig. S1). As for AOB community, most of the AOB 16S rRNA gene sequences were affiliated with *Nitrosospira* cluster 3 (Fig. S1B). At 20°C, the sequences affiliated with *Nitrosospira* cluster 3 occupied 7.7%, 29.2% and 5.6% of the total AOB 16S rRNA gene sequences in the control,

Table 1 The inhibition efficiency of dicyandiamide (DCD) to N_2O emissions in urea and manure treatments at different temperatures.

DCD efficiency (%)	Urea	Manure
20°C	78.1±0.8a	74.9±1.3a
30°C	62.9±1.7b	64.8±1.5b
40°C	58.2±1.4c	60.1±1.0c

Table 2 Two-way ANOVA revealing the effects of fertilizer type (Fer), temperature (Tem) and their interaction (Fer×Tem) on the abundances of functional genes at days 20 and 65.

Gene		Day 20			Day 65		
		Fer	Tem	Fer×Tem	Fer	Tem	Fer×Tem
AOA	*F*	13.252	8.563	4.746	13.252	8.563	4.746
	p-value	**< 0.001**	**0.002**	**0.009**	**< 0.001**	**0.002**	**0.009**
AOB	*F*	1.101	8.933	3.666	36.903	21.075	12.008
	p-value	0.354	**0.002**	**0.024**	**< 0.001**	**< 0.001**	**< 0.001**
nirS	*F*	12.595	21.912	14.513	23.113	20.232	23.950
	p-value	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
nirK	*F*	12.239	9.171	18.096	1.187	16.835	3.730
	p-value	**< 0.001**	**< 0.001**	**< 0.001**	0.328	**< 0.001**	**0.022**
Fungal *nirK*	*F*	2.595	9.676	4.846	22.521	23.420	13.315
	p-value	0.102	**0.001**	**0.008**	**< 0.001**	**< 0.001**	**< 0.001**
*nosZ*I	*F*	11.128	11.239	0.788	12.748	1.431	2.199
	p-value	**0.001**	**0.001**	0.426	**< 0.001**	0.265	0.110
*nosZ*II	*F*	12.122	7.934	0.728	11.328	11.419	5.117
	p-value	**0.001**	**0.003**	0.136	**< 0.001**	**< 0.001**	0.116

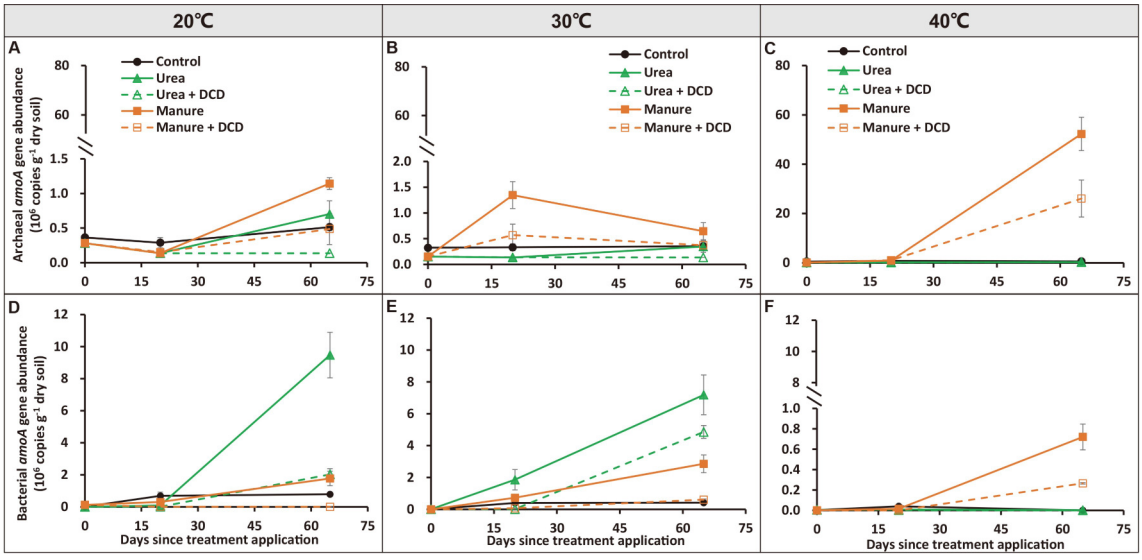


Fig. 3 The effects of fertilizer type (urea and manure) and addition of dicyandiamide (DCD) on the abundances of archaeal *amoA* gene (A−C) and bacterial *amoA* gene (D−F) at soil temperatures of 20, 30, and 40°C at 0, 20, and 65 days of incubation. The vertical bars indicate the standard error of the means (S.E.M.).

urea and manure treatments, respectively. At 30°C, 6.5%, 35.3% and 5.3% of the total AOB 16S rRNA gene sequences were classified into *Nitrosospira* cluster 3 in the control, urea and manure treatments, respectively (Fig. S1B).

Fertilizer type, temperature and their interaction had significant effects on *nirS* gene abundance (Table 2). There were significant increases of the *nirS* gene abundance in all treatments over time after incubation for 65 days at 20 and 30°C ($P < 0.05$) (Fig. 4A and B). At 40°C, only manure application significantly increased the *nirS* gene abundance which was higher than that in 20°C and 30°C (Fig. 4C). The application of DCD significantly increased the abundance of *nirS* gene in the urea treatments after 65 days of incubation at 20°C and 30°C. Manure application significantly increased the *nirK* abundance at 20°C at day 65 ($P < 0.05$) (Fig. 4D), but did not affect it at 30°C and 40°C (Fig. 4E and F). The addition of urea and manure significantly increased the *nirK* abundance after incubation for 20 and 65 days at 20°C and 20 days at 30°C in the urea and manure treatments (Fig. 4D and E). The application of DCD significantly decreased the abundance of the *nirK* gene abundance in manure treatment after incubation for 20 and 65 days at 20°C. Fertilizer type had no significant effect on the fungal *nirK* gene abundance at day 20 ($P > 0.05$) (Table 2). The fungal *nirK* gene abundances at 40°C were higher than those at 20°C and 30°C (Fig. 4G−H). Moreover, the fungal *nirK* gene abundances significantly increased from 5.43×10^5 at day 0 to 2.53×10^6 and 2.86×10^6 at days 20 and 65 with urea application at 40°C ($P < 0.05$) (Fig. 4I).

Significant increases of *nosZ*I gene abundance were observed in the control, urea and manure treatments at all three temperatures after incubation for 20 and 65 days ($P < 0.05$) (Fig. 5A−C). Fertilizer type and temperature had significant effects on *nosZ* gene abundance at day 20 ($P < 0.01$), but only fertilizer type affected *nosZ* gene abundance at day 65 ($P < 0.01$) (Table 2). Comparably, the abundance of *nosZ*II gene was significantly affected by both fertilizer types and temperature (Table 1). After 20 and 65 days of incubation, the application of urea and manure significantly decreased *nosZ*II abundance at 20°C, while the application of urea significantly increased its abundance at 30°C. The application of urea and manure had no significant effect on the abundance of *nosZ*II gene at day 20, but the application of urea and manure, together with the DCD addition, significantly increased their abundance at day 65 (Fig. 5D−F).

3.4 Relationship between mineral N concentrations, N-cycling functional genes and N_2O emissions

Linear and polynomial regression analysis showed that N_2O emissions had a positive correlation with the abundances of AOA, AOB, *nirK* gene, *nirS* gene and *nosZ*II at 20°C, with the abundance of *nirK* gene at 30°C and with *nirS* gene, fungal *nirK* and *nosZ*I gene at 40°C ($P < 0.05$) (Fig. 6).

The impact of temperature on mineral N concentrations, related microorganisms and N_2O emissions varied, depended on fertilizer type (Fig. 7). In the control treatment, temperature had negative direct influence on NO_3^--N concentration ($P < 0.001$), AOB abundance ($P < 0. 01$) and

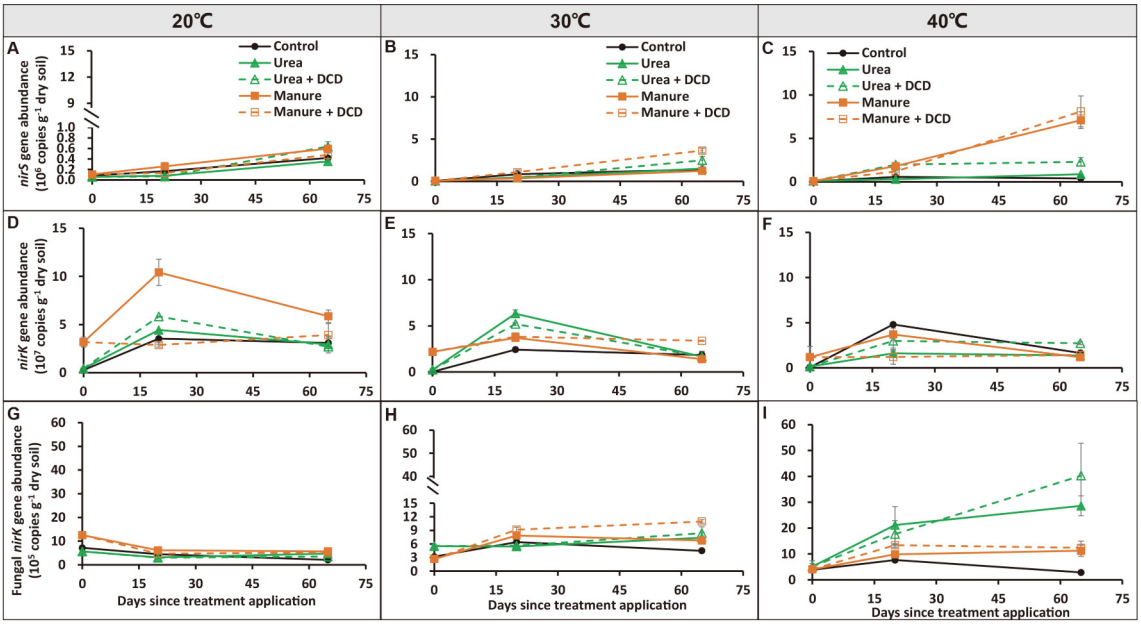


Fig. 4 The effects of fertilizer type (urea and manure) and addition of dicyandiamide (DCD) on the abundances of *nirS* gene (A−C), *nirK* gene (D−F) and fungal *nirK* gene (G−I) at soil temperatures of 20, 30, and 40°C at 0, 20, and 65 days of incubation. The vertical bars indicate the standard error of the means (S.E.M.).

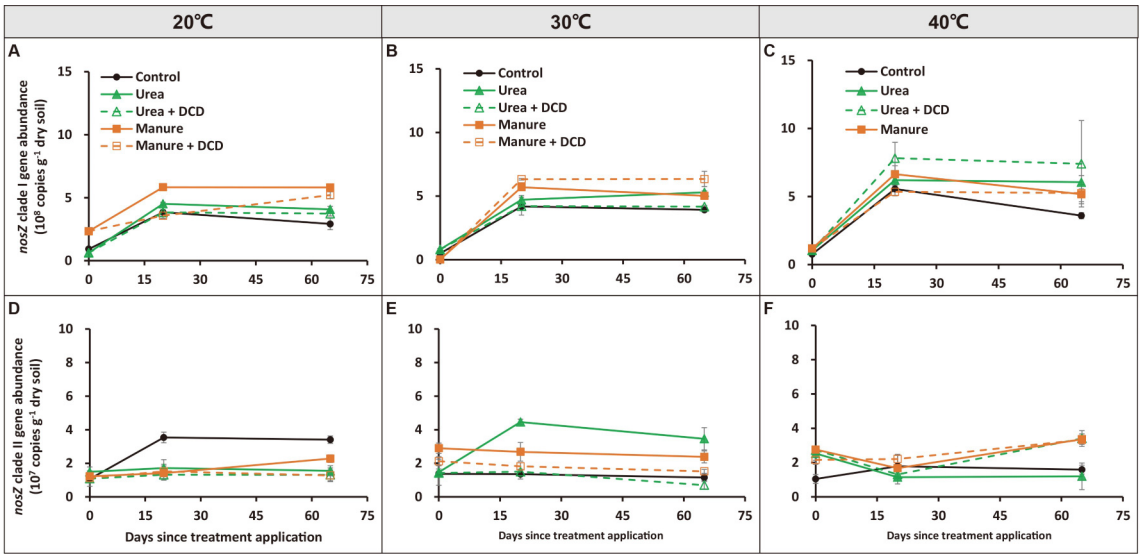


Fig. 5 The effects of fertilizer type (urea and manure) and addition of dicyandiamide (DCD) on the abundance of *nosZ* clade I gene (A−C) and *nosZ* clade II gene (D−F) at soil temperatures of 20, 30, and 40°C during at 0, 20, and 65 days of incubation. The vertical bars indicate the standard error of the means (S.E.M.).

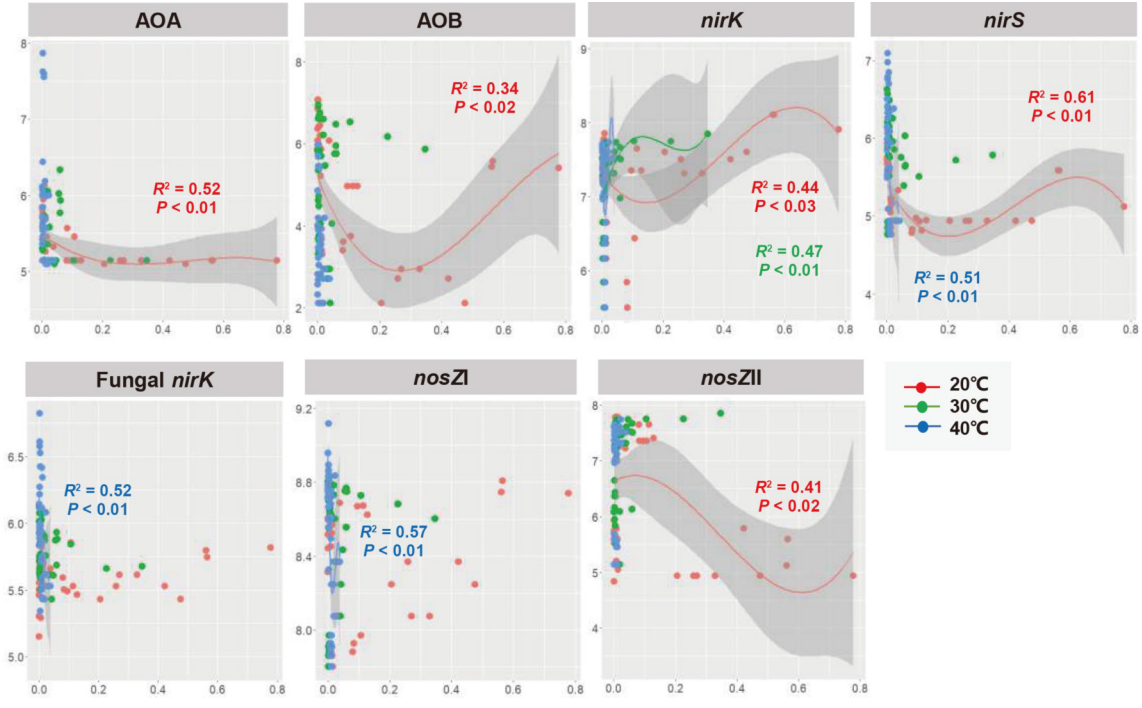


Fig. 6 Linear and polynomial regression relationships of N_2O-producers (including AOA, AOB, *nirK*-type, *nirS*-type and fungal *nirK*-type denitrier) with *nosZ* clade (I and II) and N_2O emission at temperatures of 20, 30, and 40°C. Only correlations that are significant at $P < 0.05$ are given.

N_2O emissions ($P < 0.001$). The concentration of NO_3^--N had a negative effect on the abundances of *nosZ*I ($P < 0.05$) and fungal *nirK* genes ($P < 0.001$). The abundance of AOB and fungal *nirK* respectively had direct negative and positive effects on N_2O emissions (Fig. 7A). In the urea treatment, temperature also had direct negative effect on NO_3^--N concentration ($P < 0.01$). The concentration of NO_3^--N positively influenced the abundances of AOB ($P < 0.001$), AOA ($P < 0.001$) and *nirS* gene ($P < 0.05$), but negatively influenced the abundance fungal *nirK* gene ($P < 0.05$). AOA and *nirS*-type denitrifier were negative and positive direct contributors to N_2O emissions, respectively (Fig. 7B). In the manure treatment, temperature had direct negative effects on the abundances of *nirK* ($P < 0.001$) and *nirS* ($P < 0.001$) genes and N_2O emissions ($P < 0.05$), but had a direct positive effect on AOA abundance ($P < 0.05$). The abundance of

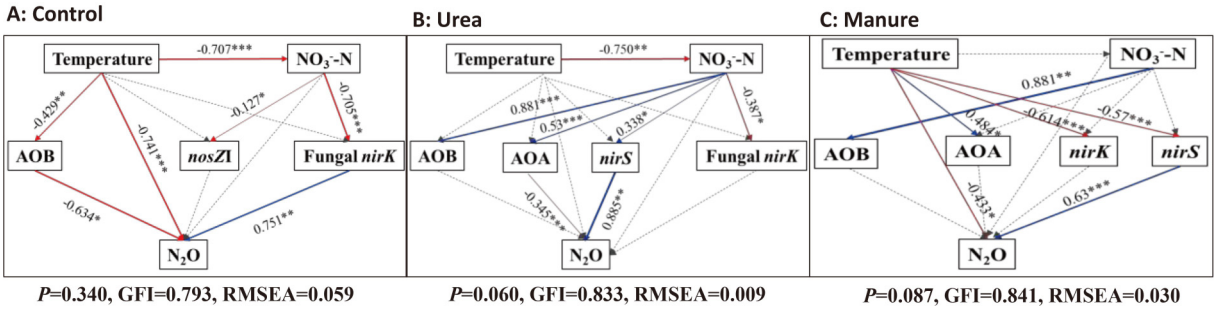


Fig. 7 Structural equation models based on the effects of temperature on mineral N concentrations, N_2O emissions and related microorganism abundance in the control (A), urea (B) and manure (C) treatments. Blue and red arrows indicate significant positive and negative relationships, respectively. Gray dotted arrows indicate non-significant relationships. Numbers at arrows are standardized path coefficients. The width of arrows indicates the strength of the relationships. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

nirS gene was positively related with N_2O emissions ($P < 0.001$) (Fig. 7C).

4 Discussion

4.1 The effect of temperature on N_2O emissions and the DCD efficacy

Our results showed that the N_2O emissions were significantly decreased with the increased temperature, and were the lowest at 40°C (Fig. 2). The decreased N_2O emissions had mainly resulted from lower nitrification rate as indicated by the concentrations of ammonium and nitrate at 40°C (Fig. 1). In addition, increasing temperatures had a significant negative effect on N_2O emissions in the control and manure treated soils but not in the urea-treated soils (Fig. 7). This might be because the application of different fertilizer types modulated the adaptability of the functional guilds under different temperatures and thus consequently affected the response of N_2O emissions (Duan et al., 2019b; Jansson and Hofmockel, 2020; Stein, 2020). Our results showed that N_2O emissions were linked to the abundances of AOA, AOB, *nirK*- and *nirS*-type denitrifiers, and *nosZ*II group at 20°C, but only linked to the abundance of *nirK*-type denitrifiers at 30°C, and to the abundances of *nirS*-type denitrifiers, fungal *nirK*-type denitrifiers and *nosZ*I clade at 40°C (Fig. 6). These findings indicate that ammonia oxidizers and bacterial denitrifiers were the dominant microbes at 20°C and 30°C, while bacterial denitrifiers and fungal denitrifiers were the dominant microbes at 40°C, showing a significant niche differentiation under different temperatures.

The application of DCD significantly inhibited the N_2O emissions at all temperature conditions, but the efficiency of DCD decreased with increased temperature, supporting our first hypothesis. The results are also consistent with a previous report that DCD was easy to biodegrade under high temperatures (Di and Cameron, 2016). It has been suggested that the DCD is better to be applied at a temperature under 10°C to achieve the maximum effectiveness and longevity (Kelliher et al., 2014). Nevertheless, under the high temperature of 40°C, DCD still had a significant effect to decrease N_2O emission by approximately 60%.

The inhibitory effect of DCD was linked to both of AOA and AOB in the urea- and manure- treated soils (Fig. 3). The targeted group of DCD could reflect the active ammonia oxidizers during nitrification (Fan et al., 2019; Prosser et al., 2020), and our results showed that both of AOA and AOB played significant roles during nitrification and N_2O emissions under different temperatures in this slightly alkaline vegetable soil. It was previously reported that the abundance of both AOA and AOB was significantly inhibited at 40°C in an acidic forest soil (Xu et al., 2017). Comparably, the abundance of AOA and AOB was significantly increased at 40°C and was inhibited by the application of DCD. This could cause a cascading effect on denitrification and lead to a significant reduction of N_2O emissions at 40°C.

4.2 Fertilizer modulated the responses of the N_2O-producers to high temperatures

In this study, the abundance of AOA and AOB was strongly affected by the fertilizer types and temperature. The application of manure rather than urea significantly increased AOA growth at the three selected temperatures (Fig. 3). The SEM result further suggested that temperature had a direct positive effect on AOA abundance in the manure treatment. Our results were consistent with previous studies showing that organic N rather than chemical fertilizer stimulated the abundance of AOA (Zhou et al., 2015; Gao et al., 2022). The AOA preferred N-poor environments compared with AOB, and high level of urea-N input (as high as 400 kg N ha^{-1}) would inhibit their growth (Di et al., 2009; Stein, 2019).

This study also showed that AOA abundance in the manure treatment at 40°C was an order of magnitude higher than that at 20°C and 30°C (Fig. 3). Previous studies found

some AOA were obligately thermophilic (e.g., *Ca. Nitrosocaldus yellowstonii* and *Nitrosocaldus islandicus*) which can grow well at extreme high temperature habitats (de la Torre et al., 2008; Daebeler et al., 2018) or even some moderately thermophilic AOA (e.g., Ca. *Nitrososphaera gargensis*) can grow at 46°C (Roland Hatzenpichler, 2008). However, phylogenetic analysis showed AOA in the manure treatment not only affiliated with *Nitrososphaera* cluster but also with *Nitrosocosmicus* cluster at 40°C (Fig. S1). Pure cultures demonstrated *Nitrosocosmicus franklandus* grew at 30−45°C and the optimal temperature was 40°C (Lehtovirta-Morley et al., 2016), which concurred with our results. Thus, the *Nitrososphaera* and *Nitrosocosmicus* clusters might be the dominant ammonia oxidizer archaea which were well adapted to high temperatures with manure application.

The significant growth of AOB at 20°C and 30°C with urea application demonstrated that AOB are favored with high-N environments (Xu et al., 2021). However, at 40°C, no significant increase of the AOB abundance with urea was observed, indicating that high temperature had inhibited AOB growth (Cui et al., 2016; Duan et al., 2018). Previous studies showed that the optimum temperature for AOB ranged between 20 and 31°C, and 12°C lower than that for soil AOA (Ouyang et al., 2017; Taylor et al., 2017). The community of AOB was mainly affiliated with *Nitrospira* cluster 3, and the maximum ammonia-oxidation activity for the isolated strains of *Nitrospira* was between 10°C and 33°C (Avrahami et al., 2003; Zhao et al., 2020). Interestingly, manure application significantly stimulated the abundance of AOB at 40°C (Fig. 3). This could be because the soil microenvironment in manured soil matrix had lower temperature than ambient temperature, thus more suited for AOB growth (Stein, 2019; Li et al., 2020; Xu et al., 2020). However, to date, no literature reported the activity of AOB at a temperature higher than 40°C.

Significant increases of *nirS*-type denitrifiers in all treatments and *nirK*-type denitrifiers in the manure treatment indicated that fertilizer type had a significant effect on the abundance of *nirS* and *nirK* genes. The high content of organic C in the manure could be an energy source for denitrifiers (Kuypers et al., 2018; Stein, 2020). Furthermore, the significant amount of C in the manure could accelerate microbial activity, and facilitated soil anaerobic conditions (Zhou et al., 2017; Xu et al., 2020), creating a more suitable environment for denitrifiers. Compared with *nirK*-type denitrifiers, *nirS*-type denitrifiers could be well adapted to high temperature as evidenced by their higher abundance. Our findings are consistent with previous studies showing that the abundance of *nirS* rather than *nirK* gene increased with increasing temperature with organic fertilizer in alkaline soils (Cui et al., 2016; Xu et al., 2020).

The application of manure also significantly increased fungal *nirK* gene abundance at 20°C and 30°C while urea addition enhanced it at 40°C (Fig. 4G−I), displaying an interactive effect of fertilizer type and temperature (Table 2). Furthermore, the fungal *nirK* gene abundances at 40°C especially with urea were one order of magnitude higher than those at 20°C and 30°C, suggesting some fungal *nirK*-denitrifiers had better tolerance to high temperature (Xu et al., 2017). However, the effect of fertilizer type on thermotolerant fungal *nirK* needs further investigation because the species of fungi denitrifiers have been reported to be thermotolerant with organic fertilizers (i.e., manure) rather than inorganic fertilizers (i.e., urea) (Chen et al., 2015; Xu et al., 2017; Xu et al., 2020).

Fertilization affected the responses of N_2O-producers to high temperature condition, thus modulating their dominant role in N_2O emissions (Figs. 6 and 7). Our results indicated that the fungal denitrifiers played a significant role in N_2O emissions under high temperature conditions in non-fertilized soil at 40°C, while the bacterial denitrifiers played a significant role in N_2O emissions with sufficient substrates (urea- and manure-treated soils), with the *nirS*-type denitrifiers being specifically important under high temperatures (40°C). This was consistent with a previous observation (Cui et al., 2016), and thus *nirS*-type denitrifiers might be targeted microbes to decrease N_2O emissions under the warming climate, especially in intensive agroecosystems with heavy use of fertilizers.

4.3 Niche separation of N_2O-reducers modulated by temperature

The responses of *nosZ*I and *nosZ*II clade microbes to different fertilizer types and temperatures differed largely (Fig. 5). The *nosZ*I group was well adapted to all the temperature conditions regardless of fertilizer treatment, and its abundance in both control and fertilized soils significantly increased with incubation time. Additionally, the growth of *nosZ*I group was not affected by temperature after 65 days of incubation, indicating that this clade *nosZ* microbes had a strong adaptability to various temperatures (Xu et al., 2020; Xing et al., 2021). Comparably, the abundance of *nosZ*II group microbes was significantly affected by both fertilizer type and temperature, showing a niche differentiation between these two different clades. Increasing temperature significantly decreased the abundance of *nosZ*II in the non-fertilized soil (control), but increased it when urea or manure was applied, indicating that the *nosZ*II clade microbes could only adapt to higher temperatures when substates were sufficient. This was consistent with a field study showing that the *nosZ*II clade microbes were adapted to a warmer and drier condition with the application of urea and manure (Xu et al., 2020). The regression analysis showed that the *nosZ*II group microbes were dominant at lower temperature (20°C), while the *nosZ*I clade microbes were dominant at higher

temperature (40°C) (Fig. 6). Similar results were observed in an acidic soil where the *nosZ*I group exhibited a significant variation at 35°C, and played a significant role in N_2O consumption (Xing et al., 2021). Comparably, the diversity of *nosZ*II rather than *nosZ*I explained more of the variation of *in situ* N_2O emissions at relatively lower ambient temperature conditions (Domeignoz-Horta et al., 2018). Taken together, these results indicate that different temperatures could lead to a significant niche separation of the two clades of *nosZ*-type denitrifiers as we hypothesized. We highlighted the important role of *nosZ*II at lower temperature (< 30°C), and of *nosZ*I at higher temperature (> 35°C) in N_2O consumption. As these two clades represented the only known biological sink of N_2O emissions, we suggest that this should be further studied in various agroecosystems in order to develop efficient mitigation strategies.

5 Conclusions

Our results showed that temperature and fertilizer types had interactive effects on both N_2O-producers and N_2O-reducers. AOA, AOB and *nirS*-type but not *nirK*-type denitrifiers were well adapted to high temperature as high as 40°C with manure application. Comparably, the fungal *nirK*-denitrifiers had better tolerance to high temperature with the application of urea. The *nosZ*I microbes had a strong adaptability to various temperatures while the *nosZ*II clade microbes were only adapted to higher temperatures with fertilization, showing a niche differentiation between these two different clades. Temperature and fertilizer types also caused a cascading effect on N_2O emissions and the dominant functional microbes. Increasing temperature and the application of DCD significantly decreased N_2O emissions. Generally, fungal denitrifiers played a significant role in N_2O emissions under high temperature conditions in non-fertilized soils, while bacterial denitrifiers were more important in soils fertilized with urea or manure, with the *nirS*-type denitrifiers being specifically important under 40°C. Our results indicate that different functional groups should be targeted to mitigate N_2O emissions under various temperature conditions. Specifically, the important role of *nirS*-type and *nosZ*I denitrifiers in N_2O emissions under high temperature conditions should be further studied in various ecosystems to better predict future N_2O emissions and develop effective mitigation strategies under global warming.

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Declarations

The authors declare no competing interests.

Electronic supplementary material

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