Applied Soil Ecology 154 (2020) 103665

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil

Nitrification and urease inhibitors improve rice nitrogen uptake and prevent denitrification in alkaline paddy soil

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ARTICLE INFO

Keywords:
Nitrification inhibitor
Nitrification
Denitrification
Ammonia oxidizers
Denitrifier

ABSTRACT

Increasing evidence indicates that nitrification is a vital factor in crop growth and nitrous oxide emission. Nitrification and urease inhibitors have been demonstrated to be effective in inhibiting the nitrification process and are widely used as fertilizer additives in agricultural soils. However, the effects of these inhibitors on rice N uptake and N_2O production through denitrification in paddy soils remain unclear. In the present work, we compared the influences of nitrification inhibitors dicyandiamide (DCD), nitrapyrin (2-chloro-6-(trichloromethyl) pyridine; NP) and N-(n-butyl) thiophosphoric triamide (NBPT) on rice growth, the fate of urea nitrogen (N), and the abundances and activities of ammonia oxidizers and denitrifiers. The fate of urea N was determined by the ^{15}N isotope labeling technique, and the abundances of ammonia oxidizers and denitrifiers were determined using qPCR. All three inhibitors improved rice growth mainly due to the increase in urea N use efficiency. Urea N uptake was negatively correlated with nitrification. The growth of ammonia-oxidizing bacteria (AOB), important in nitrification, was directly blocked by DCD. Additionally, NP and NBPT impeded the growth of ammonia-oxidizing archaea (AOA). In addition, NP significantly increased the microbial biomass to promote more residual urea N in soil and increased soil N transformation. NBPT significantly inhibited urea hydrolysis indirectly affecting nitrification. All three inhibitors decreased the potential denitrification rate (PDR) at the rice heading stage but had little effect on the denitrifier gene abundance except for nitrapyrin, which decreased the *nirK* gene abundance. DCD and NBPT may reduce the denitrification activity by decreasing the denitrification substrate (NO_3^-) concentration. These results suggest that DCD, NP and NBPT have a beneficial effect on improving rice N uptake and have the potential to reduce N_2O generation through denitrification.

1. Introduction

Nitrification is a biologically mediated process performed by nitrifying microbes whereby ammonia is converted into nitrate via nitrite (Yao et al., 2016). It is a vital step in the global nitrogen (N) cycle, plant nutrition and environmental pollution (Kuypers et al., 2018; Li et al., 2018), and nitrification has received worldwide attention in the past decades (Wang et al., 2015). Previous studies have found that a high nitrification rate may result in a low crop N use efficiency and more N loss to the atmosphere (e.g., as nitrogen oxides) or leaching into ground or surface waters (e.g., as NO_3^- or NO_2^-) (He et al., 2018; Herrera et al., 2016; Yang et al., 2017). The first and rate-limiting step in nitrification is that from NH_4^+ to NH_2OH (Amberger, 1989; Di et al., 2014). This process utilizes the critical enzyme ammonia monooxygenase (AMO), encoded by the *amoA* gene and conducted by ammonia-oxidizing archaea (AOA) or ammonia-oxidizing bacteria (AOB)

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https://doi.org/10.1016/j.apsoil.2020.103665
Received 25 June 2019; Received in revised form 2 May 2020; Accepted 16 May 2020
Available online 26 May 2020
0929-1393/

(Li et al., 2018). Although AOA and AOB use the same substrate in nitrification, many of their cellular and molecular features are notably different. In general, NH_4^+ addition promotes the growth and activity of AOB, while AOA are more competitive when ammonia availability is limited (Fisk et al., 2015; Q. Wang et al., 2017). The soil pH, by affecting the substrate concentration and ammonia-oxidizer activities, is a critical factor in nitrification. It has been previously reported that nitrification is stronger in alkaline soils, and in certain cases, AOB have been shown to contribute more to ammonia oxidation, even when AOA are the numerically dominant population (Meinhardt et al., 2018).

Nitrification and urease inhibitors have been demonstrated to be effective in inhibiting the nitrification process (Di et al., 2014; Guardia et al., 2018; Yang et al., 2017) and are widely used as fertilizer additives in agricultural soils (Xi et al., 2017) and grassland soils (Wu et al., 2017). Common nitrification and urease inhibitors include dicyandiamide (DCD), nitrapyrin (2-chloro-6-(trichloromethyl) pyridine; NP) and N-(n-butyl) thiophosphoric triamide (NBPT) (Modolo et al., 2018). The core mechanism of nitrification inhibitors is impeding ammonia oxidizer increase and activity by deactivating the ammonia monoxygenase enzyme through copper chelation (Di et al., 2014; Subbarao et al., 2006). It has been reported that nitrification inhibitors do not exclusively target AOA or AOB, the dominant ammonia oxidizer groups (Zhang et al., 2012). In general, DCD significantly inhibited AOB rather than AOA (Kawakami et al., 2012), while nitrapyrin had the opposite inhibition effects (Ruser and Schulz, 2015), and DCD greatly impeded AOA in strongly acidic soils (Zhang et al., 2012). However, alkaline paddy soil is a more complex system with a high pH and alternating flooding (reduction) and draining (oxidation) conditions, and most experiments have been conducted using only microcosms, and it remains unclear whether inhibitors affect either AOA or AOB during rice growth in the field.

Unlike nitrification inhibitors, NBPT, a urease inhibitor, effectively blocks three active sites of the urease enzyme, forming a tridentate bond, with two nickel atoms at the center and one oxygen atom from the carbamate bridge linking both metals, which reduces the probability of urea reaching nickel atoms, thus inhibiting hydrolysis and enhancing the N supply cycle (Cantarella et al., 2018). The advantages of NBPT are a quick effect (the reaction occurs in minutes or hours), long duration and suitable application in a wide variety of soils. However, the effect of NBPT on the nitrification process in addition to reducing the substrate supply remains unclear.

Current knowledge on how inhibitors improve rice N uptake states that inhibitors notably impact the fate of fertilizer N through decreased loss and increased uptake, while the effect of inhibitors on the transformation of fertilizer N in paddy soil is uncertain, and there are few studies that focus on the fate of fertilizer N (Liu et al., 2017). The ^{15}N isotope labeling technique is a useful means of determining the distribution of labeled ^{15}N in plant-soil N cycling (Y. Li et al., 2019), which provides precise information on the fate of fertilizer N, including N uptake and residual soil N (Cao and Yin, 2015). Moreover, the ^{15}N isotope labeling technique is a more precise method than the traditional method (using the differences between fertilized and control plots), which ignores any added N interaction (ANI, aka priming) (Asagi and Ueno, 2009). Therefore, we adopted ^{15}N isotope labeling technique to study the effect of inhibitors on fertilizer N transformation and postulated that these inhibitors may reduce residual fertilizer N in soil by hindering microbial growth.

Nitrous oxide (N_2O), a trace gas of great concern contributing to global warming and stratospheric ozone destruction, is a byproduct of both nitrification and denitrification (Long et al., 2017; Yu et al., 2018). Soil moisture content effects on the oxygen concentration also change the contribution to N_2O emissions through nitrification and denitrification (Di et al., 2014). Under aerobic conditions, AOA and AOB produce N_2O when NH_3 or NH_4^+ is oxidized to NO_2^- (Meinhardt et al., 2018), but under anaerobic conditions, denitrifiers produce N_2O as an intermediate product when NO_3^- is reduced to N_2 (Huang et al., 2019; Volpi et al., 2017). In addition to conventional heterotrophic denitrification, nitrifier denitrification has also been implicated as a source of N_2O in soils under oxygen stress (Meinhardt et al., 2018). Inhibitors have been developed that can reduce N_2O emissions by blocking the nitrification process. However, most studies on inhibitors in reducing N_2O emissions were conducted on non-paddy soils, which lack an anaerobic environment, and focused more on N_2O generated from nitrification and nitrifier denitrification (Di et al., 2014; Yu et al., 2016). Moreover, the effect of inhibitors on N_2O emissions reduction in previous studies is simply ascribed to nitrification inhibition and denitrification substrate reduction. However, N_2O generated through denitrification accounts for a considerable proportion and cannot be neglected in paddy soils. However, it is unknown whether inhibitors also affect the N_2O generation capacity through denitrification or the denitrifier community in paddy soils.

In the present work, we hypothesized that the studied nitrification and urease inhibitors impeded nitrification and reduced the microbial biomass to increase fertilizer N use efficiency, decrease residual fertilizer N in soil, and hinder denitrifier growth to reduce the produced N_2O by denitrification. The objectives of the study were to investigate the effects and mechanisms of the above inhibitors on rice N uptake and N_2O production through denitrification in alkaline paddy soils.

2. Material and methods

2.1. Site description and basic soil properties

The study was conducted in Ningbo (29°46′N, 121°52′E), Zhejiang Province, China, from 16 July 2017 to 29 October 2017. The deposited materials in the area originated from the nearby Yangtze and Qiantang Rivers, which were translocated to the south bank of Hangzhou Bay influenced by the warm Taiwan Strait current (Zou et al., 2015). The annual mean temperature was 18.3 °C, and the annual mean rainfall was 1480 mm in 2017. The sampling site had been planted with double-cropping rice during the seasons of March to July and July to November since 2010. In the soil tillage layer (0–20 cm), the soil organic matter, soil total nitrogen (TN), bulk density and pH were 28.0 g kg^{-1}, 1.2 g kg^{-1}, 1.28 g cm^{-3} and 7.91, respectively. Before transplanting, the soil nitrate nitrogen (NO_3^-) and soil ammonium nitrogen (NH_4^+) levels were 1.31 and 3.85 mg N kg^{-1}, respectively.

Twelve trial plots (1 m long and 1 m wide) were laid out in a completely randomized block pattern including four treatments with three replicates: a control treatment containing urea without inhibitors (CK); one group containing urea with dicyandiamide as nitrification inhibitor (DCD); one group containing urea with nitrapyrin as nitrification inhibitor (NP); and one group containing urea with N-(n-butyl) thiophosphoric triamide as urease inhibitor (NBPT). According to the fertilization practices of local farmers, 180 kg N ha^{-1} ^{15}N-labeled urea (5.10% ^{15}N atoms) was similarly applied across all treatments on July 15th. The nitrapyrin, NBPT and DCD dosages were 0.2%, 0.45% and 10% urea by weight, respectively, and were applied after mixing with urea. Phosphate and potassium fertilizers were applied as K_2HPO_4 at a rate of 300 kg ha^{-1} at the same time. In the rice growing season, a flood level higher than 2 cm is maintained until the mature stage, and the soil water content is then maintained near the water-holding capacity.

2.2. Sample collection and preparation

Soil samples (0–20 cm) from each treatment were collected at both the rice heading stage (August 24) and harvest stage (October 29), which were immediately placed in an ice box and transported to the laboratory. Each sample was divided into three aliquots. One aliquot was processed through a 2.0-mm sieve, thoroughly mixed and stored at 4 °C for subsequent analysis of the soil inorganic N content (NH_4^+ and NO_3^-), net nitrification rate, denitrification rate, microbial biomass

carbon (MBC) and microbial biomass nitrogen (MBN). One aliquot was air dried and processed through a 2.0-mm sieve for determining the soil pH and urease activity and then processed through a 0.125-mm sieve for obtaining TN and ^{15}N abundance. The last aliquot was freeze dried and stored at −80 °C for DNA extraction and real-time PCR analysis. Rice straw (stems, sheaths and leaves) and grains were separately collected at the mature stage (110 days after transplantation). Rice tissues were oven dried at 70 °C to a constant weight, weighed and then ground into powder. The total N and ^{15}N abundance in the samples were then analyzed.

2.3. Soil chemical analysis and calculation

The total N in the soil and plant samples was determined with a CNS elemental analyzer (Vario MAX, Elementar Analysensysteme GmbH, Germany), and the ^{15}N enrichment was measured with a stable isotope ratio mass spectrometer (Flash 2000 HT/Conflo IV/Delta V, Thermo Fisher Scientific, Germany).

The amounts of urea N uptake (total input of 18 g m^{-2}) by rice (N_U) and the residual N in soil (N_R) were calculated with Eq. (1), where a is the atom% of ^{15}N in the rice plant or soil, b is the atom% of ^{15}N in the added urea, M is the dry weight of rice or soil (0.2-m depth), and C_N is the rice or soil N concentration.

$$N_{U\text{ or }R} = M \times C_N \times \frac{a - 0.365}{b - 0.365} \quad (1)$$

The amount of urea N lost (N_L) (g m^{-2}) was calculated with Eq. (2).

$$N_L = 18 - N_U - N_R \quad (2)$$

The percentage of N uptake by rice derived from urea N ($\%N_{dfU}$) was calculated with Eq. (3), where N_U is the amount of urea N uptake by rice, M is the rice dry weight and C_N is the N concentration in rice.

$$\%N_{dfU} = \frac{N_U}{M \times C_N} \times 100\% \quad (3)$$

The soil NO_3^- and NH_4^+ contents were extracted from the fresh soil samples using 1 mol L^{-1} KCl (soil/KCl, 1:10) via shaking at 220 rpm for 1 h; the suspension was filtered through a 0.45-μm membrane and tested with a continuous flow injection analyzer (FLA star 5000 Analyzer, Foss, Denmark) (Yao et al., 2016).

The ^{15}N abundances of NO_3^- and NH_4^+ in the extracts were determined using the diffusion method described by Sebilo et al. (2004) with some changes. Briefly, 0.2 g light magnesium oxide (MgO) was added to convert the dissolved NH_4^+ into ammonia (NH_3), and the released NH_3 was trapped on filter paper (diameter 1 mm) by potassium persulfate ($KHSO_4$) (20 μL, 2.5 M) covered with Teflon (breathable yet waterproof). Seven days later, the filter paper was dried and placed in 5 × 8 mm universal soft tin containers (Thermo-Fisher Scientific, USA) and analyzed with a stable isotope ratio mass spectrometer. Thereafter, the cap was removed for one day to flush out any residual NH_3. Next, 0.4 g of Devarda's alloy (copper/aluminum/zinc, 50/45/5) was added to transform the NO_3^- in the extracts into NH_3; the absorption and determination methods were the same as those for NH_4^+. The recovery rate of inorganic N during the diffusion procedure was above 95%.

MBC and MBN were determined by the fumigation-extraction method (Liao et al., 2018; Zhang et al., 2019). Briefly, the soil samples were fumigated with $CHCl_3$ for 24 h at 25 °C in the dark. The fumigated samples and those without fumigation were extracted with 0.5 M K_2SO_4 for 30 min on a shaker and filtered. The filtrates were tested with an automated total organic carbon (TOC) analyzer (TOC-500, Japan). Extraction efficiency coefficients K_{EC} of 0.45 and 0.54 were used to measure MBC and MBN, respectively.

The atom% of ^{15}N in the extracts was measured by a stable isotope ratio mass spectrometer after freeze drying and grinding (Zhu et al., 2017). The ^{15}N incorporated into the microbial biomass (^{15}N-MBN) was calculated with Eq. (4), where f indicates the fumigated extracts, uf indicates the unfumigated soil extracts, and N_f and N_{uf} are the total N contents of the fumigated and unfumigated soil extracts, respectively.

$$\text{atom\%of}^{15}N - MBN = \frac{[(atom\%^{15}N)_f - 0.365] \times N_f - [(atom\%^{15}N)_{uf} - 0.365] \times N_{uf}}{100 \times 0.54} \quad (4)$$

The amounts of residual urea N (mg kg^{-1}) in the soil as NH_4^+, NO_3^- and MBN were calculated with Eq. (1), where a is the atom% of ^{15}N - NH_4^+, ^{15}N - NO_3^- or ^{15}N - MBN, b is the atom% of ^{15}N in the added urea, M is the dry soil weight (0.2-m depth), and C_N is the soil N concentration as NH_4^+, NO_3^- or MBN.

The urease activity (UA) was assayed using the indophenol blue method (Qin et al., 2010). Two grams of soil (2-mm sieved and air-dried soil) was preincubated with 1 mL toluene for 15 min and then incubated with 10 mL urea solution (100 g L^{-1}) and 20 mL citrate buffer (pH = 6.7) at 37 °C for 3 h. The accumulated NH_4^+ content was measured by spectrophotometry at 690 nm using salicylic acid colorimetry, and the urease activity was expressed as μg NH_4^+-N g^{-1} dry soil d^{-1}.

The potential nitrification rates (PNRs) were measured by the shaken-slurry method (Yao et al., 2011). Fifteen grams of fresh soil was mixed with 7.5 mL 0.2 M KH_2PO_4, 17.5 mL 0.2 M K_2HPO_4 and 75 mL 0.05 M $(NH_4)_2SO_4$ and incubated in the dark at 25 °C for 24 h on a 180-rpm shaker. Suspension aliquots of 10 mL were sampled at incubation times of 0.25, 8, 16 and 24 h and immediately analyzed on a continuous flow analyzer to determine their nitrate concentrations. The net nitrification rate was calculated from the rate of increase in NO_3^- concentration over time in the slurry using linear regression.

The potential denitrification rates (PDRs) were measured using the acetylene inhibition technique (Liu et al., 2014) with some changes. Briefly, 10 g fresh soil was added to 15 mL 10 mg L^{-1} NO_3^- solution to simulate field flooding in 120-mL serum bottles. The serum bottles were then sealed and purged three times by evacuating the ambient air and filling with helium (He), followed by equilibration under atmospheric pressure using a glass syringe. Part of the headspace in the serum bottle (10 mL) was removed and replaced with 10 mL acetylene (C_2H_2). The serum bottles were incubated at 25 °C, and N_2O was analyzed at the beginning and after 8 h by gas chromatography (Agilent 7890A, Agilent, Palo Alto, CA, USA). PDR was calculated by the change in N_2O concentration in the headspace during the incubation period with Eq. (5), where C_0 and C_t are the N_2O concentrations in the tube at the beginning and end of incubation, respectively (ng N L^{-1}); 0.1 is the headspace volume (L); W is the dry weight of the soil sample in the tube (g); and 8 is the incubation time (h).

$$\text{PDR (ng N } g^{-1}h^{-1}) = \frac{(C_t - C_0) \times 0.1}{W \times 8} \quad (5)$$

2.4. DNA extraction and molecular analysis

DNA was extracted from 500 mg frozen soil using the Fast DNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's instructions, immediately diluted ten times and stored at −20 °C for molecular analysis. The DNA concentration was measured on a NanoDrop ND-2000 UV–vis spectrophotometer (NanoDrop®, USA). Quantitative PCR was conducted on a Light Cycler 480 real-time PCR detection system (Roche480, USA). The *amoA* gene was studied for AOA and AOB, and the *nirK*, *nirS* and *nosZ* genes were studied for the denitrifiers. The specific primer combinations and thermal cycling conditions are listed in Table 1. Each PCR was performed in a 20-μl reaction mixture consisting of 0.5 μL of each primer, 10 μL SYBR® Premix, 1 μL tenfold labeling DNA template, and 0.5 μL bovine serum albumin (BSA, 20 mg mL^{-1}), and the residual volume was replenished with deionized water. For quantification, the amplification efficiencies were in the range from 93 to 106%, and the correlation coefficient (r^2) of the determination ranged from 0.95 to 0.99 for all of the standard curves.

Table 1
Primer pairs and PCR conditions used in real-time qPCR analysis.

Targer group	Primer	Sequence (5′-3′)	Length of amplicon (bp)	Thermal profile	Amplification efficiency (R2 > 0.99) (%)	References
AOA	CrenamoA23f	ATGGTCTGGCTWAGACG	635	95 °C for 2 min × 1 cycle; 95 °C for 20 s, 55 °C for 20 s, 72 °C for 30 s, 80 °C for 15 s × 40 cycles;	92–94	(Long et al., 2018a)
	CrenamoA616r	GCCATCCATCTGTATGTCCA				
AOB	amoA-1F	GGGGTTTCTACTGGTGGT	491	95 °C for 2 min × 1 cycle; 95 °C for 20 s, 57 °C for 30 s, 72 °C for 30 s, 85 °C for 15 s × 40 cycles;	96–98	(Rotthauwe et al., 1997)
	amoA-2R	CCCCTCKGSAAAGCCTTCTTC				
nirK	FlaCu	ATCATGGTSCTGCCGCG	474	95 °C for 2 min × 1 cycle; 95 °C for 20 s, 63 °C for 30 s, 72 °C for 30 s, 85 °C for 15 s × 40 cycles;	98–100	(Hallin and Lindgren, 1999)
	R3Cu	GCCTCGATCAGRTTGTGGTT				
nirS	cd3aF	GTSAACGTSAAGGARACSGG	410	95 °C for 2 min × 1 cycle; 95 °C for 45 s, 55 °C for 45 s, 72 °C for 20 s × 40 cycles;	93–95	(Michotey et al., 2000)
	R3cd	GASTTCGGRTGSGTCTTGA				
nosZ	nosZ-F	CGYTGTTCMTCGACAGCCAG	424	95 °C for 2 min × 1 cycle; 95 °C for 20 s, 58 °C for 30 s, 72 °C for 15 s × 40 cycles;	94–99	(Kloos et al., 2001)
	NosZ-1662R	CGSACCTTSTTGCCSTYGCG				

2.5. Statistical analysis

Analysis of variance (ANOVA) was conducted to test the effects of the treatments (CK, DCD, NP and NBPT) at each time point. The differences in the soil physicochemical and microbial properties were tested using the least significant difference (LSD) test at the 0.05 probability level, and the correlation coefficient was obtained using Pearson's correlation analysis. Redundancy analysis (RDA) was performed to visualize the differences in urea N fate between the treatments. All statistical analyses were conducted using the statistical software SPSS 20.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Rice N uptake and fate of urea N

3.1.1. Rice biomass and N uptake

The dry weight of rice grains and straws ranged from 834 ± 45 to 1091 ± 21 g m^{-2} and 653 ± 21 to 749 ± 26 g m^{-2}, respectively (Table 2). Compared with the traditional fertilization treatment (CK), nitrification inhibitor addition treatments (DCD and NP) significantly ($P < 0.05$) increased rice total dry weight by 21.1 and 22.6%, respectively; in particular, DCD and NP increased the dry weight of grains by 28.8 and 30.8% respectively, only the DCD treatment significantly ($P < 0.05$) raised dry weight of straws by about 14.7%. The urease inhibitor addition treatment (NBPT) had no strong effect on dry weight compared with other treatments; however, there was a significant ($P < 0.05$) increase in rice grains dry weight by 15.2% compared with the CK treatment.

The N concentration of rice grains and straws ranged from 1.26 ± 0.06 to 1.37 ± 0.02% and 0.69 ± 0.01 to 0.76 ± 0.07%, respectively (Table 2). N content in grains were significantly ($P < 0.05$) higher in DCD, NP and NBPT treatments than in the CK treatment. Accordingly, these applications significantly ($P < 0.05$) increased N uptake by rice by 17.6 to 31.4%, especially the percentage of rice N uptake derived from urea N by 25.4 to 35.2%. A significant relationship was identified between rice N uptake and rice N uptake derived from urea (Fig. 1).

3.1.2. Fate of the urea-derived N

The urea-derived N uptake by rice ranged from 10.8 to 19.2% of the total N input (Fig. 2). The amounts of urea-derived N uptake by rice in the DCD, NP and NBPT treatments (3.46 ± 0.26, 3.41 ± 0.03 and 2.87 ± 0.36 g m^{-2}, respectively) were significantly ($P < 0.05$) higher than that in the CK treatment (1.95 ± 0.31 g m^{-2}). The nitrification inhibitors (DCD and nitrapyrin) were significantly ($P < 0.05$) more effective than the urease inhibitor in promoting the urea-derived N uptake in this alkaline paddy soil.

Approximately 28.0 to 34.2% of urea N remained in the soil (Fig. 2). The amounts of residual urea-derived N in the soil in the DCD and NBPT treatments (5.40 ± 0.15 and 5.04 ± 0.54 g m^{-2}, respectively) were significantly ($P < 0.05$) lower than that in the CK treatment (6.17 ± 0.05 g m^{-2}). Although the NP treatment was not remarkably different compared with the CK treatment, the amount of residual urea-derived N in the soil as NH_4^+, NO_3^- and MBN in the NP treatment (12.9 ± 1.4 μg kg^{-1}, 332 ± 24 μg kg^{-1} and 1.9 ± 0.2 mg kg^{-1}, respectively) was higher than that in the other treatments, despite these accounting for only a small fraction of the residual urea-derived N in the soil (Table 3).

The amount of urea-derived N loss was calculated by the subtraction method. The nitrification inhibitor treatments (DCD and NP) significantly ($P < 0.05$) reduced the urea-derived N loss compared with the CK and NBPT treatments. In addition, the loss of urea-derived N in the NBPT treatment showed a negligible difference from that in the CK treatment.

Table 2
Rice dry weight and nitrogen uptake with different fertilizers.

Treatments	Dry weight (g m^{-2})			Nitrogen concentration (%)		Nitrogen uptake (g m^{-2})	N_{dfU} † %
	Grains	Straws	Total	Grains	Straws		
CK‡	834 ± 45 c§	653 ± 21 b	1486 ± 45 b	1.26 ± 0.06 b	0.74 ± 0.06 a	15.3 ± 0.5 b	12.77 ± 2.27 b
DCD	1074 ± 59 ab	749 ± 26 a	1823 ± 79 a	1.34 ± 0.01 a	0.75 ± 0.07 a	20.0 ± 1.4 a	17.26 ± 0.10 a
NP	1091 ± 21 a	708 ± 67 ab	1799 ± 88 a	1.35 ± 0.04 a	0.76 ± 0.07 a	20.1 ± 1.3 a	16.99 ± 0.99 a
NBPT	961 ± 106 b	694 ± 44 ab	1655 ± 142 ab	1.37 ± 0.02 a	0.69 ± 0.01 a	18.0 ± 1.7 a	16.01 ± 1.53 a

† Abbreviations: N_{dfU} means the percentage of N uptake by rice derived from urea N.
‡ Abbreviations: CK, control (urea at traditional fertilization rate); DCD, DCD applied with urea; NP. nitrapyrin applied with urea; NBPT, NBPT applied with urea.
§ Values are means ± standard deviation (n = 3). Values with different lowercase letters within a column are statistically significantly different at P < 0.05.

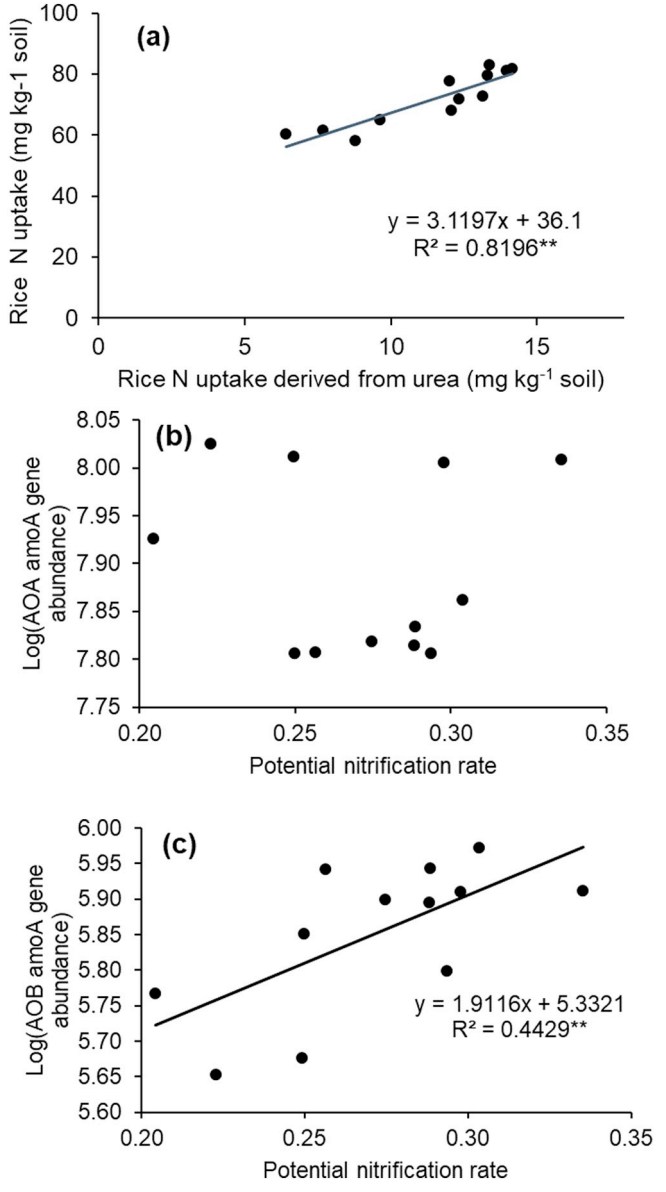

Fig. 1. Relationship analysis. Relationship between rice N uptake and rice N uptake derived from urea (a) and relationships between potential nitrification rate and soil AOB *amoA* gene copy numbers (b), and soil AOA *amoA* gene copy numbers (c) at rice heading stage. R denotes Pearson correlation coefficients, ** Significant at the 0.01 level.

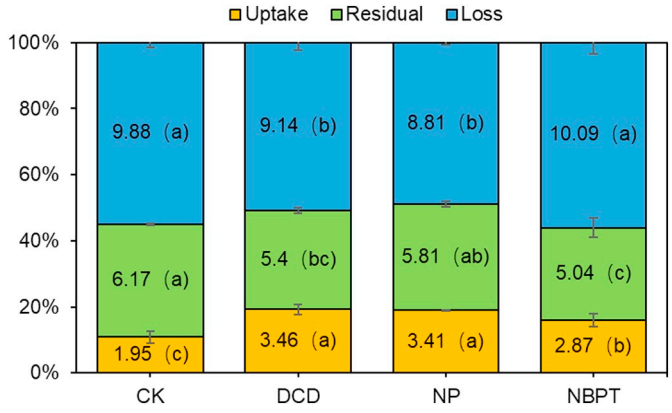

Fig. 2. Fate of urea N (uptake, residual and loss) as affected by nitrification inhibitors and urease inhibitor application. CK, control (urea at traditional fertilization rate), DCD, DCD applied with urea, NP, nitrapyrin applied with urea, NBPT, NBPT applied with urea. The figures in the column represent the average of fate of urea N (g m^{-2}), and different lowercase letters are statistically significantly different at P < 0.05.

Table 3
Residual urea N in soil as inorganic nitrogen and microbial biomass nitrogen.

Treatments	NH_{4dfU}^{+} †	NO_{3dfU}^{-}	MBN_{dfU}
	μg kg^{-1}	μg kg^{-1}	mg kg^{-1}
CK‡	3.4 ± 1.0 c§	273 ± 23 b	1.4 ± 0.1 b
DCD	5.3 ± 0.8 b	291 ± 8 b	1.3 ± 0.1 b
NP	12.9 ± 1.4 a	332 ± 24 a	1.9 ± 0.2 a
NBPT	6.4 ± 0.2 b	278 ± 13 b	1.4 ± 0.2 b

† Abbreviations: NH_{4dfU}^{+}, NO_{3dfU}^{-} and MBN_{dfU} means the amount of urea N residual in the soil as NH_4^+, NO_3^- and MBN.
‡ Abbreviations: CK, control (urea at traditional fertilization rate); DCD, DCD applied with urea; NP. nitrapyrin applied with urea; NBPT, NBPT applied with urea.
§ Values are means ± standard deviation (n = 3). Values with different lowercase letters within a column are statistically significantly different at P < 0.05.

3.2. Soil chemical properties and soil N transformation activities

3.2.1. Soil chemical properties

The chemical properties of soil samples at different stages are shown in Table 4. At the rice heading stage, the soil NH_4^+ and NO_3^- concentration ranged from 3.64 ± 0.70 to 7.29 ± 0.93 mg kg^{-1} and 2.96 ± 0.47 to 8.41 ± 1.12 mg kg^{-1}, respectively. The lowest NH_4^+ concentration was found in the NBPT treatment, which was half that of

Table 4
Chemical properties and soil N transformation activities affected by nitrification inhibitors and urease inhibitor application.

	Stage	CK[†]	DCD	NP	NBPT
NH_4^+ (mg kg^{-1} soil)	Heading	7.29 ± 0.93 a[§]	6.24 ± 1.02 a	5.82 ± 0.67 a	3.64 ± 0.7 b
	Mature	3.01 ± 0.19 b	3.39 ± 0.07 ab	3.54 ± 0.12 a	3.48 ± 0.34 a
NO_3^- (mg kg^{-1} soil)	Heading	4.33 ± 0.96 b	2.96 ± 0.47 b	8.41 ± 1.12 a	3.91 ± 0.29 b
	Mature	6.28 ± 0.06 ab	6.09 ± 0.01 b	6.50 ± 0.25 a	6.13 ± 0.05 b
MBC[‡] (mg kg^{-1} soil)	Heading	258.8 ± 25.4 b	209.5 ± 26.4 bc	322.1 ± 15.9 a	201.4 ± 41.2 c
	Mature	262.9 ± 4.2 a	218.9 ± 13.3 b	251.5 ± 5.9 a	231.4 ± 11.4 b
MBN (mg kg^{-1} soil)	Heading	37.0 ± 1.5 b	27.5 ± 3.1 c	50 ± 3.8 a	24.4 ± 2.0 c
	Mature	36.3 ± 0.8 b	32.7 ± 2.2 b	42.8 ± 3.2 a	32.8 ± 3.4 b
PNR (mg N kg^{-1} soil h^{-1})	Heading	0.31 ± 0.02 a	0.23 ± 0.02 c	0.27 ± 0.02 b	0.28 ± 0.02 ab
	Mature	0.29 ± 0.03 a	0.28 ± 0.03 a	0.26 ± 0.02 a	0.30 ± 0.01 a
PDR (μg N kg^{-1} soil h^{-1})	Heading	43.9 ± 4.0 a	33.8 ± 5.6 b	19.4 ± 4.8 c	19.6 ± 5.2 c
	Mature	87.9 ± 20.6 a	71.8 ± 27.4 a	60.7 ± 17.5 a	55.0 ± 19.5 a

[†] CK, control (urea at traditional fertilization rate), DCD, DCD applied with urea, NP. nitrapyrin applied with urea, NBPT, NBPT applied with urea.
[‡] Abbreviations: MBC, microbial biomass carbon content; MBN, microbial biomass nitrogen content; PNR, potential nitrification rate; PDR, potential denitrification rate.
[§] Values are means ± standard deviation (n = 3). Values with different lowercase letters within a column and same stage are statistically significantly different at $P < 0.05$.

the CK treatment and significantly ($P > 0.05$) lower than those of the DCD and NP treatments. The highest NO_3^- was found in the NP treatment, which was twice the other treatments. However, NH_4^+ and NO_3^- concentration have a negligible range of variation at the mature stage of rice, which ranged from 3.01 ± 0.19 to 3.54 ± 0.12 mg kg^{-1} and 6.09 ± 0.01 to 6.50 ± 0.25 mg kg^{-1}, respectively.

The soil MBC and MBN ranged from 201.4 ± 41.2 to 322.1 ± 15.9 mg kg^{-1} and 24.4 ± 2.0 to 50.0 ± 3.8 mg kg^{-1} at the heading stage and from 218.9 ± 13.3 to 262.0 ± 4.2 and 32.7 ± 2.2 to 36.3 ± 0.8 mg kg^{-1} at the mature stage, respectively. The DCD and NBPT treatments generally decreased MBC and MBN content at those two stages compared with the CK treatment. In contrast, the NP treatment significantly ($P < 0.05$) increased MBC content at the heading stage and increased MBN content in those two stages compared with the CK treatment.

3.2.2. Nitrification and denitrification activities

Potential nitrification, denitrification and urea hydrolysis were demonstrated in the present study, which were determined with enough substrate to reflect authentic activities (Table 4). At the rice heading stage, the PNR ranged from 0.23 ± 0.02 to 0.31 ± 0.02 mg N kg^{-1} soil h^{-1}. The lowest PNR was found in the DCD treatment, followed by the NP and NBPT treatments. The PDR ranged from 19.4 ± 4.8 to 43.9 ± 4.0 μg N kg^{-1} soil h^{-1} at the heading stage. The lowest PDRs were found in the NP and NBPT treatments, followed by the DCD treatment, which were significantly ($P < 0.05$) lower than the CK treatment. At the rice mature stage, nitrification inhibitors and urease inhibitor showed no effect on N transformation activities, and the PNR and PDR ranged from 0.26 ± 0.02 to 0.30 ± 0.01 mg N kg^{-1} soil h^{-1} and 55.0 ± 19.5 to 87.9 ± 20.6 μg N kg^{-1} soil h^{-1}, respectively.

In contrast to PNR and PDR, the present study measured UA at four stages (Fig. 3). The UA ranged from 57.1 ± 1.9 to 83.6 ± 1.2 μg N g^{-1} soil h^{-1} throughout. During the rice growth period (transplant, tiller and heading), the lowest UA was found in the NBPT treatment and was significantly lower than that in the DCD and NP treatments. At the transplant and tiller stages, the UA in the NBPT treatment was also significantly lower than that in the CK treatment.

3.2.3. Relationships between fate of the urea-derived N and soil chemical properties

In order to explore the relationship between the soil chemical properties at the rice heading stage, soil N transformation activities at the rice heading stage and urea-derived N fate at the mature stage, Pearson correlation analysis and Redundancy analysis were used in our study (Table 5 and Fig. 4). Significant relationships were identified

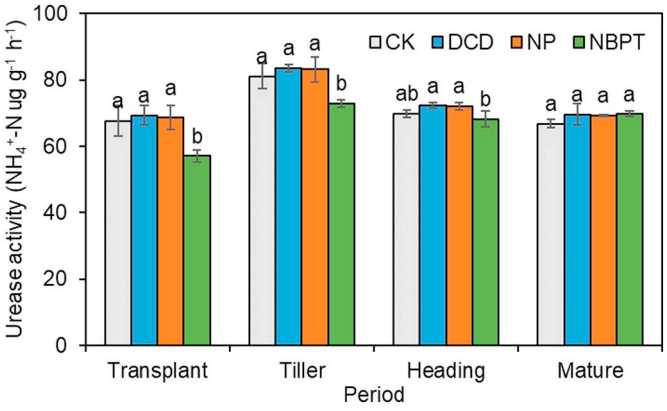

Fig. 3. Urease activities affected by nitrification and urease inhibitors application. CK, control (urea at traditional fertilization rate), DCD, DCD applied with urea, NP, nitrapyrin applied with urea, NBPT, NBPT applied with urea. The sampling time for rice transplant and tiller stage are July 18 and July 30, respectively. Values with different lowercase letters within a stage are statistically significantly different at $P < 0.05$.

between the soil chemical properties, soil N transformation activities and fate of urea-derived N. RDA showed that different inhibitor treatments changed the fate of urea-derived N; the first two axes of the RDA explained 90.3% of the fate of urea-derived N variation, PNR explained 37.5% of the variance with a P value of 0.01, MBC explained 21.1% of the variance with a P value of 0.08 and NH_4^+ explained 20.6% of the variance with a P value of 0.10. Pearson correlation analysis showed that urea-derived N uptake was significantly but negatively related to PNR; residual urea-derived N in the soil was significantly positively related to NH_4^+ and MBN content; as predicted, the urea-derived N loss was significantly negatively related to urea-derived N uptake.

3.3. The abundance of ammonia oxidizers and denitrifiers

The abundance of ammonia oxidizers and denitrifiers at different stages are shown in Figs. 5 and 6. At the heading stage, the AOA *amoA* and AOB *amoA* gene copy numbers varied from 6.5 × 10^7 ± 6.75 × 10^5 to 9.8 × 10^7 ± 1.17 × 10^7 g^{-1} dry soil and from 5.03 × 10^6 ± 7.18 × 10^5 to 8.6 × 10^6 ± 7.12 × 10^5 g^{-1} dry soil, respectively. Compared with the CK treatment, NP and NBPT treatments resulted in significantly lower AOA *amoA* gene abundances and the DCD treatment resulted in a significantly lower AOB *amoA* gene abundance. Significant relationships were identified between AOB

Table 5
Pearson correlation between urea N fate, soil chemical properties and soil N transformation activities.

	MBC[a]	MBN	NH_4^+	NO_3^-	PNR	PDR	UA	Uptake	Residual	Loss
Uptake	0.133	0.043	−0.295	0.260	−0.783**	−0.535	0.439	–	−0.432	−0.704*
Residual	0.572	0.617*	0.775**	0.300	0.326	0.570	0.064	−0.432	–	−0.337
Loss	−0.590*	−0.531	−0.303	−0.508	0.560	0.109	−0.508	−0.704*	−0.337	–

[a] Abbreviations: MBC, microbial biomass carbon content; MBN, microbial biomass nitrogen content; PNR, potential nitrification rate; PDR, potential denitrification rate; UA, urease activity; Uptake, urea N uptake by rice; Residual, urea N residual in soil; Loss, urea N loss.

* Indicates significant correlation at $P < 0.05$.

** Indicates significant correlation at $P < 0.01$.

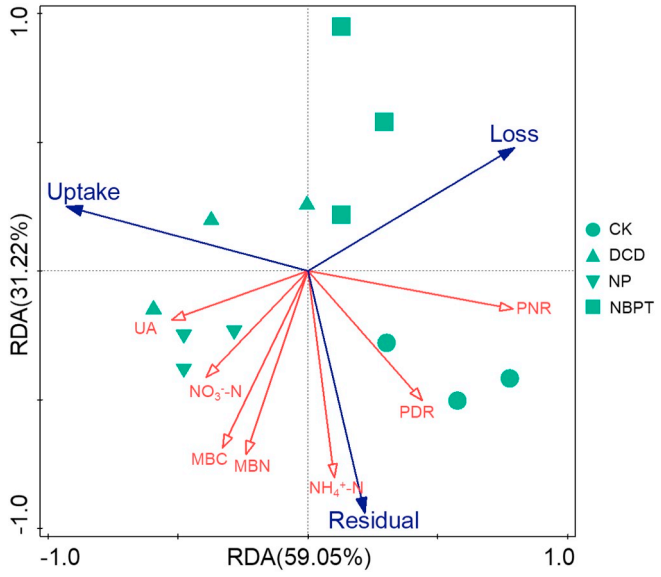

Fig. 4. Redundancy analysis (RDA) of urea N fate under different fertilizer treatments. Abbreviations: MBC, microbial biomass carbon content; MBN, microbial biomass nitrogen content; PNR, potential nitrification rate; PDR, potential denitrification rate; UA, urease activity; Uptake, urea N uptake by rice; Residual, urea N residual in soil; Loss, urea N loss.

amoA gene abundance and PNR, while no significant relationships were found between AOA *amoA* gene abundance and PNR (Fig. 1).

The *nirK*, *nirS* and *nosZ* gene copy numbers varied from $2.82 \times 10^6 \pm 3.28 \times 10^5$ to $4.40 \times 10^6 \pm 1.32 \times 10^5$, $5.73 \times 10^6 \pm 9.87 \times 10^5$ to $7.42 \times 10^6 \pm 7.06 \times 10^5$ and $1.35 \times 10^6 \pm 2.65 \times 10^5$ to $1.80 \times 10^6 \pm 1.88 \times 10^5$, respectively. Nitrification inhibitors and urease inhibitor showed no effect on denitrifier abundance except that the NP treatment significantly decreased the *nirK* gene copy number.

At the mature stage (Fig. 6), the AOA *amoA*, AOB *amoA*, *nirK*, *nirS* and *nosZ* gene copy numbers varied from $1.43 \times 10^8 \pm 6.90 \times 10^7$ to $2.04 \times 10^8 \pm 9.11 \times 10^7$, $2.26 \times 10^7 \pm 3.58 \times 10^6$ to $3.34 \times 10^7 \pm 6.34 \times 10^6$, $6.95 \times 10^6 \pm 2.44 \times 10^6$ to $1.85 \times 10^7 \pm 5.88 \times 10^6$, $1.16 \times 10^7 \pm 2.00 \times 10^6$ to $1.82 \times 10^7 \pm 8.99 \times 10^6$ and $1.38 \times 10^6 \pm 8.34 \times 10^4$ to $1.89 \times 10^6 \pm 5.55 \times 10^5$, respectively. Nitrification inhibitors and urease inhibitor showed no effect on ammonia oxidizer and denitrifier abundance except that the NP treatment again significantly decreased the *nirK* gene copy number.

4. Discussion

4.1. Fate of the urea-derived N

In practical production, inhibitors are generally used to increase crop yield and reduce fertilizer loss (Ding et al., 2018; Kim et al., 2012). As expected, the nitrification and urease inhibitors increased the grain dry weight and N content (Table 2) and improved the urea-derived N use efficiency (Fig. 2). These results reflected the effectiveness of these inhibitors for seasonal crops, consistent with previous studies who found that inhibitors could increase crop (e.g. rice) yield by > 6% (Abalos et al., 2014; Linquist et al., 2013; S. Wang et al., 2017). A meta-analysis found that the use of DCD and NBPT could increase N use efficiency by > 12%, and NBPT performed best in the alkaline soil (pH ≥ 8) (Abalos et al., 2014). Although the N use efficiency of rice was increased from 10.8% to 19.2% (Fig. 2), it was lower than that in China (30%–35%) (Zhu and Chen, 2002).This is mainly due to the differences in the calculation methods. The N use efficiency was normally calculated by the traditional method which was affected by the added N interaction (Asagi and Ueno, 2009). However, this method cannot be used in our study because of the lack of non-fertilization treatment. Instead, the amount of N uptake derived from soil could be calculated by subtraction, obtaining values for the CK, DCD, NP and NBPT treatments of 13.4 ± 0.7, 16.6 ± 1.1, 16.7 ± 1.3 and 15.1 ± 1.5 mg kg^{-1}, respectively. This clearly demonstrated that the inhibitors improved the rice N uptake derived from the soil (Ding et al., 2019). In addition, excessive fertilization, improper management under flooding conditions and a large amount of NH_3 volatilization in such alkaline environments may also cause lower N uptake efficiency (Dempsey et al., 2017; Long et al., 2018b).

Nitrification inhibitors (DCD and nitrapyrin) significantly decreased the loss of urea-derived N but urease inhibitor had no effect (Fig. 2). In the paddy field, the loss of fertilizer N is usually in the form of NH_3 volatilization, leaching and denitrification (Dempsey et al., 2017; Wang et al., 2016). The objectives of using inhibitors were to allow crop absorbing more fertilizer N, change the N conversion processes and thus extend the existence time of different forms of N. Nitrification inhibitors may reduce N loss through hindering nitrification and subsequent processes. It was reported that nitrification inhibitors could reduce N_2O emissions from the fertilizer in the range of 11% to 47% in the rice-wheat system (Lan et al., 2013). In contrast, NBPT reduced NH_3 volatilization > 50% and delayed the peak of NH_3 volatilization (Soares et al., 2012), but it may cause more leaching and denitrification loss due to the more ammonia involved in nitrification (Martins et al., 2017; Volpi et al., 2017). Martins et al. (2017) quantified the N_2O emissions from maize plants treated with a combination of urea and NBPT, and found that the application of NBPT resulted in an overall increase of 0.6–0.8 kg N_2O N ha^{-1} despite reducing NH_3 volatilization. These evidences suggested that nitrification inhibitors actually reduce the loss of fertilizer N, while urease inhibitors only change the forms of fertilizer N losses, but the total amount of N loss change insignificantly.

The residual urea N as inorganic N, MBN, and total N in soil were calculated using the ^{15}N isotope labeling technique, and the results revealed that the inorganic N and MBN accounted for 10% of the total urea N residual in the soil (Fig. 2). Accordingly, most of the total residual urea N in the soil was existed in the form of organic N. Quan et al. (2016), using a laboratory incubation experiment, found that urea N underwent a process from NH_4^+ to MBN and then to soil organic N which can be used by subsequent crops. In the present study, DCD and NBPT significantly decreased the residual urea-derived N in the soil, while nitrapyrin addition maintained the same residual amount of urea-

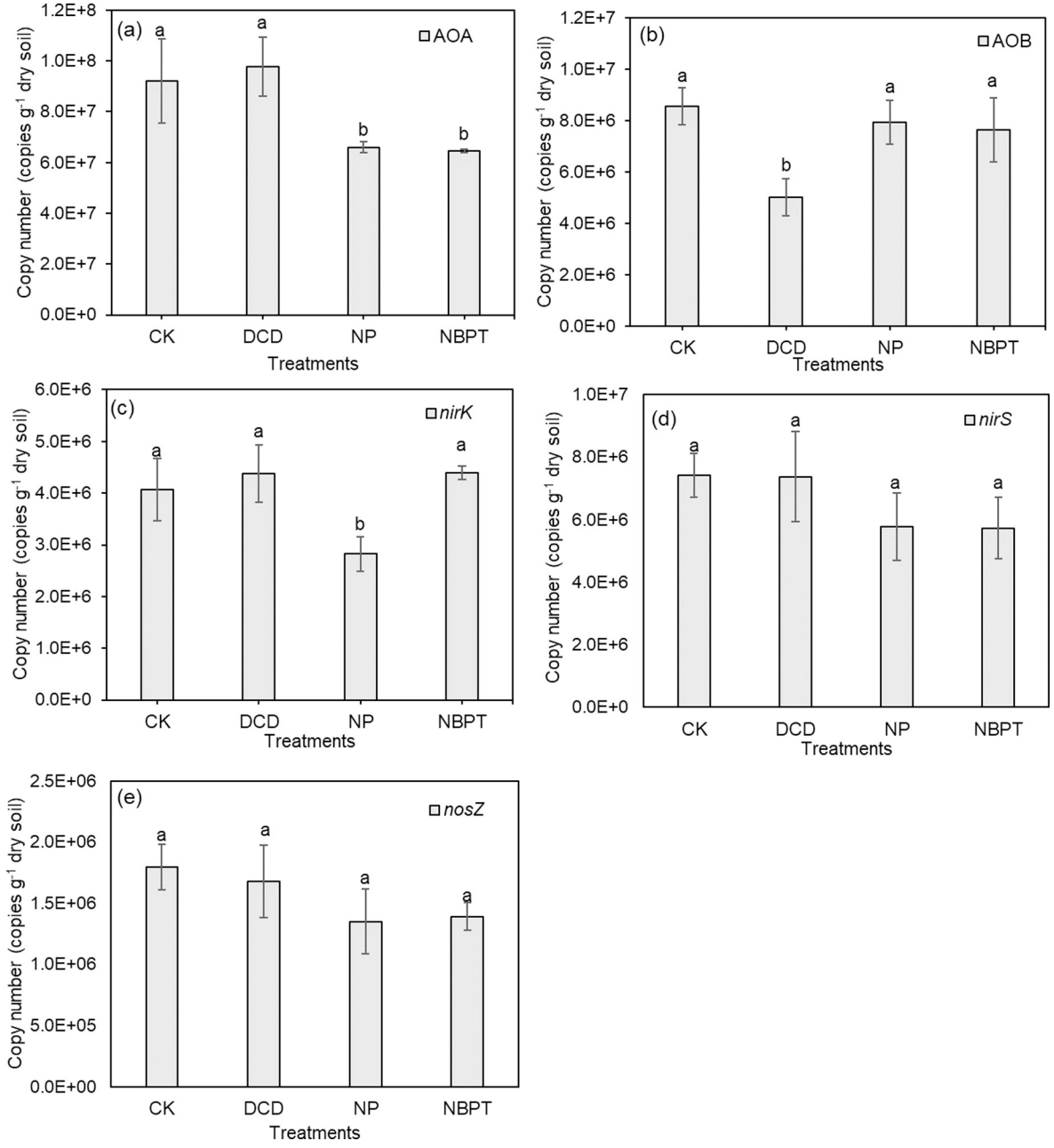

Fig. 5. Ammonia-oxidizer and denitrifier genes at the rice heading stage (a–e). Copy numbers of AOA *amoA* (a), AOB *amoA*(b), *nirK* (c), *nirS* (d) and *nosZ* (e) genes under different treatments. CK, control (urea at traditional fertilization rate), DCD, DCD applied with urea, NP, nitrapyrin applied with urea, NBPT, NBPT applied with urea. Different lowercase letters are statistically significantly different at $P < 0.05$.

derived N as with no inhibitor application (Fig. 2). This difference may be due to the effects of the different inhibitors on the microbial biomass, which contributed to N transformation including direct immobilization and indirect transformation through rhizosphere exudates. This conclusion is supported by the observation that the amount of residual urea-derived N in soil is significantly correlated with the microbial biomass content at the rice heading stage (Fig. 4). In addition, nitrapyrin tends to be adsorbed onto organic matter and experiences rapid photolysis and volatilization (Woodward et al., 2019), which provides a carbon source (from nitrapyrin and soil organic matter) for urea immobilization (Yu et al., 2019). The present study showed that the NP treatment significantly increased both the MBC and MBN contents at the rice heading and mature stages, similar to other studies (Yao et al., 2016). Moreover, the residual urea-derived N in the soil as NH_4^+, NO_3^- and MBN was significantly higher than that in the other treatments (Tables 3 and 4). These results may support our conclusion that NP increases the N transformation rate.

4.2. Nitrification and ammonia oxidizers

The PNR is an index that aims to determine the maximum capacity of nitrifiers in transforming ammonium (Li et al., 2018). Inhibitor addition significantly changed the PNR at the rice heading stage but not at the rice mature stage, suggesting that inhibitors only affect the current season's crop (Yao et al., 2016). All these inhibitors are susceptible to biodegradation (Lan et al., 2015); however, many field and incubation studies have shown that the effect of inhibitors on the ammonia-oxidizing microbial abundance and activity would last for 3 weeks or longer (Chen et al., 2015; J. Li et al., 2019; Wang et al., 2016). The PNR at the rice heading stage was significantly negatively correlated with

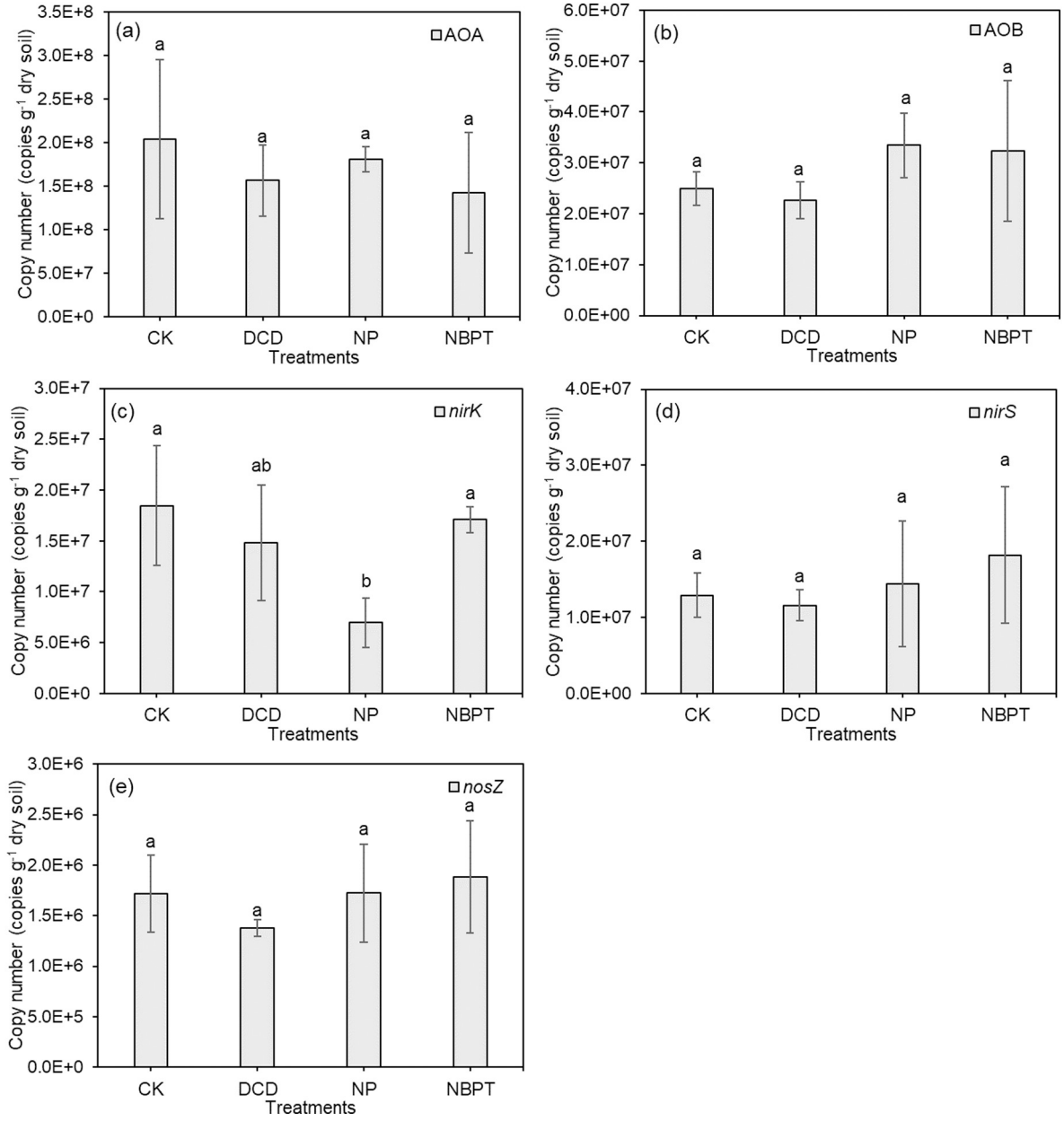

Fig. 6. Ammonia-oxidizer and denitrifier genes at the rice mature stage(a–e). Copy numbers of AOA *amoA* (a), AOB *amoA*(b), *nirK* (c), *nirS* (d) and *nosZ* (e) genes under different treatments. CK, control (urea at traditional fertilization rate), DCD, DCD applied with urea, NP, nitrapyrin applied with urea, NBPT, NBPT applied with urea. Different lowercase letters are statistically significantly different at $P < 0.05$.

the rice N uptake, indicating that nitrification plays an important role in rice growth (Yang et al., 2017) (Table 5). AOA and AOB are two important participants in nitrification. The present results showed that the PNR was linked with the gene abundance of AOB rather than with the abundance of AOA (Fig. 1), indicating that AOB play a critical role in nitrification in this alkaline paddy soil, consistent with the study of Jiang et al. (2015), who used a DNA-based stable isotope probing method and concluded that AOB dominate alkaline soils (pH = 8.2). In addition, the nitrification activity was stimulated by urea fertilization and accompanied by a significant increase in AOB in alkaline soils. However, the lack of correlation between the AOA and PNR may be due to the PNR method, in which we added excess NH_4^+. AOB often outcompete AOA for added inorganic ammonia (Hink et al., 2017) even to the extent of inhibiting the growth and functions of AOA. Moreover, certain species of AOA are inhibited at the ammonia concentrations typically used in potential assays (Martens-Habbena et al., 2009).

Although all three inhibitors decreased the PNR at the rice heading stage, the mechanisms were different. DCD directly blocked the growth of AOB and inhibited the AOB involved in the nitrification process (Fig. 5) and then fixed more NH_4^+ originating from the urea in the soil rather than allowing conversion (Di et al., 2014). This result was consistent with other studies (Akiyama et al., 2013; Ruser and Schulz, 2015). NP and NBPT only strongly inhibited the growth of AOA but not of AOB (Gu et al., 2019) and decreased the PNR to varying degrees. AOA prefer to use soil native N as a substrate rather than an exogenous N source from fertilizer (Fisk et al., 2015), and the soil organic matter level in the present study at 28.0 g kg^{-1} may have provided substrates for the AOA. The effect of NBPT on ammonia oxidizers was also encountered in other studies (Fan et al., 2018; Shi et al., 2017; Xi et al., 2017). Similar to our results, Xi et al. (2017) found that NBPT addition decreased the *amoA* gene abundance of AOA at pH = 7.04 but had no effect at lower pH values (3.97, 4.82, or 6.07) in vegetable soils,

suggesting that the inhibition of AOA by NBPT may only be effective at high pH values, and Shi et al. (2017) found that NBPT compensates for the decrease in soil pH to inhibit the growth of AOA. In contrast, Fan et al. (2018) suggested that NBPT decreased the *amoA* gene abundance of AOB rather than the *amoA* gene abundance of AOA in alkaline soils, albeit in an incubation experiment, and they also demonstrated the inhibitory effect of NBPT on ammonia oxidizers from another point of view. Future studies on the effects of NBPT on ammonia oxidizers should be performed. In general, the main mechanism of NBPT is to inhibit urea hydrolysis (Cantarella et al., 2018), and the present results suggest that NBPT decreased the urease activity at the rice heading stage (Fig. 3) by 15.7, 10.2 and 2.4% at the transplant, tiller and heading stages, respectively, compared with the CK treatment.

4.3. N_2O generation through denitrification

Under anaerobic conditions, N_2O is mainly produced by denitrification and anammox, and between these processes, > 90% of N_2O is produced by denitrification (Shan et al., 2018). The PDR is an index that aims to determine the maximum N_2O amount generated through denitrification. Nitrate addition provides adequate substrates, and acetylene inhibits nitrification, nitrifier denitrification and N_2O oxidation. Shan et al. (2018) used the ^{15}N tracer technique to confirm that when the NO_3^- concentration was higher than 4.5 mg L^{-1}, the PDR remained stable. In the present study, a 10 mg L^{-1} NO_3^- solution was added, and the NO_3^- concentration ranged from 10 to 20 mg L^{-1}. However, the PDRs in the inhibitor-added treatments were significantly lower than that in the CK treatment at the rice heading stage. These results suggested that the inhibitors control N_2O generation though denitrification (Table 4). Among these inhibitors, nitrapyrin significantly decreased the *nirK* gene abundance. It is generally believed that the *nirK* gene rather than the *nirS* gene in paddy soils is more readily affected by denitrification-inducing conditions (Yoshida et al., 2010). Q. Wang et al. (2017) found that DCD could significantly inhibit the *nirK* gene abundance in alluvial soils and that the total N_2O emissions were positively correlated with the *nirK* gene abundance. J. Li et al. (2019) reported that DMPP could significantly inhibit the *nirK* and *nirS* genes when incubated for 3 and 10 days, respectively. Both studies found that the *nirK* gene was positively correlated with the N_2O flux. While the abundances of denitrifiers in the DCD and NBPT treatments were not significantly different from that in the CK treatment (Fig. 5), on the one hand, the complex environment and buffering effects of the field experiment may mask any differences, while in comparison, incubation experiments may amplify any effect from DCD and NBPT (J. Li et al., 2019; Shi et al., 2017; Q. Wang et al., 2017; Zhou et al., 2018). On the other hand, it may be that DCD and NBPT maintain the soil NO_3^- content at a low level, and hence, denitrifiers exhibit a low level of activity. Many previous studies consider that NBPT has no effect on denitrification (Wang et al., 1991). In contrast, Sanz-Cobena et al. (2014) conducted an incubation experiment and observed that the applied NBPT with a urea treatment notably decreased N_2O compared with only the urea treatment when the water-filled pore space (WFPS) was 60% and 80%, but when the WFPS was 40%, no significant effect was observed between the NBPT applied with the urea treatment and the urea treatment alone. These results indicate that inhibitors have nonnegligible effects on N_2O generation through denitrification in some cases. Future studies on inhibitor effects on N_2O emission need to consider the contribution from denitrification.

5. Conclusions

In conclusion, our results highlighted the effects and mechanisms of two nitrification inhibitors (DCD and nitrapyrin) and one urea inhibitor (NBPT) on promoting rice growth, changing the fate of the urea-derived N and reducing the N_2O generation potential from denitrification. Briefly, the inhibitors improved rice growth mainly due to increasing the urea N use efficiency. The fate of the urea-derived N was correlated with the PNR and microbial biomass content. The inhibitors have various mechanisms: DCD directly blocked the growth of AOB and inhibited the AOB involved in the nitrification process, while NP and NBPT blocked the growth of AOA. In addition, NBPT significantly hindered urea hydrolysis to indirectly affect nitrification. For the N_2O generation potential through denitrification, all three inhibitors decreased the PDR at the rice heading stage. DCD and NBPT may reduce the denitrification activity by decreasing the denitrification substrate (NO_3^-) concentration, while nitrapyrin addition decreased the *nirK* gene abundance to reduce denitrification. Future studies on inhibitor addition effects should focus on the direction of the urea-derived N loss, and if N_2O emissions are considered, the contribution from denitrification should not be ignored.

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.

Acknowledgements

This work was supported by the National Natural Science Foundation of China [Grant Numbers 41525002, 4171101112, 41877051, 41701282], the National Key R & D Program of China [Grant Number 2017YFD0200102]. We thank Yongxiang Yu, Xien long, Xuechen Zhang, Haoxin Fan, Xing Li, Jiantao Xue, Yuzhen Luo and Yizu Zhu for technical assistance.

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