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Urease Inhibitors Weaken the Efficiency of Nitrification Inhibitors in Mitigating N_2O Emissions from Soils Irrigated with Alternative Water Resources

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Abstract It is generally accepted that inhibitors are effective in reducing agricultural nitrous oxide (N_2O) emissions from soils irrigated by groundwater. However, it was unclear whether these inhibitors effectively regulate N_2O emissions from soils irrigated with alternative waters, like reclaimed water and livestock wastewater. To clarify this, nitrapyrin, a nitrification inhibitor, and N-(N-butyl) thiophosphoric triamide, a urease inhibitor, were added separately or jointly to the soils irrigated by groundwater, reclaimed water and livestock wastewater through two consecutive cycles of pot experiment. Both the single and combined addition of inhibitors lowered N_2O emissions from soils irrigated with alternative water, while the reduction effect of the combined application decreased relative to that of the single application. The using of combined inhibitors did reduce the enrichment level of nitrification genes and slow down the nitrification process, but the associated relatively high *nirS*/*nosZ* ratio potentially discounted its ability to prevent N_2O emissions. Whereas under groundwater irrigation, treatment with combined inhibitors only decreased N_2O emissions in the first cycle but not in the second cycle. Inhibitor application affected the composition of soil bacterial communities, and in particular, urease inhibitor application increased community differences across the two cycles. Moreover, using inhibitors led to a general reduction in the enrichment level of the denitrification genes *narG* and *nosZ*, and we speculate that inhibitors could also indirectly manipulate N_2O release by involving the denitrification process. Structural equation model results further displayed that the relative abundance of the *nxrA* and *narG* genes and NH_4^+-N concentration played a vital role in the regulation of N_2O release from the alternative water-irrigated soils applied with inhibitors.

Keywords Reclaimed water · Livestock wastewater · Nitrous oxide · Soil · Inhibitor

1 Introduction

Nitrous oxide (N_2O) is a long-lived greenhouse gas that has 265 times the global warming potential

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of CO_2 over a 100-year time frame (IPCC, 2014). According to statistics, it accounts for about 6.24% of the global radiative forcing (Davidson, 2009), which greatly impacts global warming. In addition, N_2O is one of the principal ozone-depleting substances, which causes the ozone layer hole. Cropland is recognized as a main source of N_2O (Liu et al., 2019b), of which 60% are ascribed to nitrogen (N) fertilizer applications in agricultural production (Van Kessel et al., 2013). The application of N fertilizers, especially urea, can increase soil ammonium (NH_4^+) and nitrate (NO_3^-) levels and accelerate the nitrification and denitrification reactions, thus facilitating N_2O production. The amount of N fertilizers also increases as a result of the increasing global population, which may lead to a continued increase in N_2O emissions (Reay et al., 2012).

Apart from fertilization, irrigation is also an important part supporting agricultural production, and adequate water resources are a prerequisite. However, pollution and waste of water resources are progressively serious, making freshwater resources increasingly scarce (He et al., 2021; Hochstrat et al., 2006). Irrigation with alternative water, like reclaimed water (RW) and livestock wastewater (LW), has become an effective measure to alleviate water shortages (Mello Leite Moretti, 2017; Poustie et al., 2020). Numerous studies on alternative water irrigation have been carried out in recent years, which focused more on soil and crop safety (Li et al., 2024; Lu et al., 2016; Xiang et al., 2024), while less on N_2O emissions from soils when reclaimed water and livestock wastewater are used. These alternative waters contain some nutrients, hence irrigation with them may impact the content of soil nitrogen (Li et al., 2024; Lu et al., 2016) and dissolved organic carbon (Liu et al., 2023; Pereira et al., 2011), water filled pore space (WFPS) (Shang et al., 2016), pH (Solís et al., 2005), and thereby soil N cycle and N_2O emissions (Chi et al., 2020; Duan et al., 2019; Hu et al., 2022).

Given that alternative water use and urea application potentially affect the release of N_2O and the environment, it is necessary to take steps. Nitrification inhibitors (NIs) and urease inhibitors (UIs) addition is widely considered to be an effective strategy to suppress N_2O releases (Bohara et al., 2018; Cui et al., 2011). NIs can inhibit soil ammonia oxidation, improve N use efficiency, and thus decrease N_2O releases (Meng et al., 2020; Tao et al., 2024). Nitrapyrin (NP), dicyandiamide (DCD), and 3,4-dimethylpyrazole-phosphate (DMPP) are the main NIs widely used for agricultural emission abatement. Among them, NP is the only option that can effectively inhibit both ammonia-oxidizing archaea and ammonia-oxidizing bacteria, and also has outstanding effects on nitrite-oxidizing bacteria (Papadopoulou et al., 2020), so it was chosen as the NI for this experiment. NP works by oxidizing 6-chloropyrimidine carboxylic acid to chelate Cu at the active site of ammonia monooxygenase (Vannelli & Hooper Alan, 1992). N-(n-butyl) phosphorothioate triamide (NB), a major UI applied in agriculture, restrains urea hydrolysis and inhibits N_2O emissions by blocking the enzyme site of urease (Manunza et al., 1999; Recio Huetos et al., 2020), while it cannot prevent nitrifying bacteria and archaea from oxidizing NH_4^+ to NO_3^-. Moreover, NI application lengthens the retention time of soil NH_4^+ (Lam et al., 2017), leading to an increase in NH_3 release (Pan et al., 2016). Therefore, some studies have attempted to apply NIs and UIs in combination to further reduce N loss and inhibit N_2O emissions while circumventing NH_3 emissions (Ni et al., 2017; Wang et al., 2023). However, Zhao et al. (2017) found that inhibitors applied together are less effective than those applied alone in regulating N_2O emissions. It is also found that whether the combined application of inhibitors improves the regulation effect on N_2O emissions relative to the single application depends on cropping years (Sanz-Cobena et al., 2012).

These controversial findings stop us from deciding whether co-application of inhibitors is suitable for weakening soil N_2O emissions, especially under alternative water resource irrigation. Hence, this study aimed to answer this question. By monitoring N_2O emission and soil basic properties dynamics, soil bacterial community composition and the nitrogen cycle functional genes throughout the experiment, we try to: 1) clarify the response of soil N_2O emission to single application and joint application of inhibitors under different alternative water resources irrigation conditions; 2) evaluate the efficiency of NP and NB in controlling the abundance of N cycle function genes and N level in soil irrigated with different alternative water resources; 3) determine if the combined use of inhibitors modulates the release of N_2O more potently than the use of a single inhibitor.

2 Materials and Methods

2.1 Experimental Site and Materials

The experiment was operated at one of our institute's research stations (35.27°N, 113.93°E), which is located in Xinxiang City. The topsoil of 20 cm in a field 22 km from the station was gathered, air-dried, and mixed thoroughly. The soil properties are as follows: bulk density 1.50 $g{\cdot}cm^{-3}$, pH 8.27, total phosphorus 1.74 $g{\cdot}kg^{-1}$, total nitrogen 1.11 $g{\cdot}kg^{-1}$, ammonium-N 4.61 $mg{\cdot}kg^{-1}$, nitrate–N 3.23 $mg{\cdot}kg^{-1}$, and organic matter 19.22 $g{\cdot}kg^{-1}$.

The reclaimed water from a wastewater treatment plant and the livestock wastewater sourced from the biogas slurry in an intensive pig farm were used as the alternative waters, and both water intake sites were located in Xinxiang City. Groundwater (GW) was garnered from the experimental site. With reference to irrigation water quality standards, LW was diluted at a ratio of 1:10 before irrigation (GB 5084–2021) (2021). The water properties before use can be seen in Table 1.

2.2 Experiment Design

The three water resources mentioned above were used in this experiment. There were four inhibitor treatments: no substance (NS); nitrification inhibitor, nitrapyrin (NP); urease inhibitor, N-(n-butyl) thiophosphoric triamide (NB); both NP and NB (NPB). Thus, a total of 12 treatments were set, each of which had three replicates. The amount of each inhibitor added per pot was 1% of pure N in the fertilizer.

To closely simulate the field vegetable cultivation in the greenhouse, we adopted production management practices in this pot experiment akin to those used in the field, including sowing, fertilizing, and watering. Referring to the actual fertilization amount in the field, $CO(NH_2)_2$, KH_2PO_4, and KCl were applied at 0.326 (N 0.15 g kg^{-1}), 0.290 and 0.090 g kg^{-1} (P_2O_5 0.15 g kg^{-1} and K_2O 0.15 g kg^{-1}), along with inhibitors (except NS), respectively. The fertilizers combined with 6 kg of dry soil were then packed into per pot with a top diameter, bottom diameter, and height of 23.0 cm, 18.0 cm, and 21.5 cm, respectively. Next, 1200 mL of the corresponding water was immediately added to each pot to approach the field holding capacity (21%) (referred to day 0). Three days later, 15 Chinese cabbage (*Brassica chinensis* L.) seeds were sown in each pot; and then 300–400 mL of water were irrigated per pot every 2–4 days to meet the water needs. During the three-leaf stage, the seedlings were thinned to five evenly spaced in per pot. During the first cycle of the experiment, which spanned from 14 April to 19 May 2022, the vegetables were carefully tended to until they were ready for harvest. Then, the soil was thoroughly mixed in preparation for the second cycle. The second cycle lasted from 23 May to 27 June 2022 in the same location within the greenhouse, during which the sowing, vegetable variety, fertilization, water resources, and others were consistent with the previous cycle. However, since the second cycle started later with the higher temperature, it received two more irrigations than the first cycle.

2.3 Gas Flux Sampling

Gas samples were grasped by the static chamber method. The procedures used for gas sampling and the sampling times were identical for both cycles. The details of the chamber construction, gas collection method, and gas emission flux formula are described in supplementary materials.

Table 1 Properties of the studied water resources

Water type	COD ($mg{\cdot}L^{-1}$)	TN ($mg{\cdot}L^{-1}$)	TP ($mg{\cdot}L^{-1}$)	pH	NH_4^+-N ($mg{\cdot}L^{-1}$)	NO_3^--N ($mg{\cdot}L^{-1}$)	EC ($\mu S{\cdot}cm^{-1}$)
Groundwater	-	2.8	0.9	8.4	1.2	1.3	551
Reclaimed water	-	19.1	1	8.4	9.1	6.9	988
Livestock wastewater	943.1	166.9	64.5	8.3	80.8	65.2	1007

COD – chemical oxygen demand; TN – total N; TP – total P; NH_4^+-N – ammonium-N; NO_3^--N – nitrate–N; EC – electrical conductivity. Note: "-" refers to below the detection limit

2.4 Soil Sampling and Edaphic Properties Measurement

In both cycles, a total of 20 g of soil samples (0–10 cm) approximately 5 cm from the seedlings after each gas sampling were collected by a soil auger, the length and internal diameter of which are 20 cm and 1 cm, respectively. For the soil samples, one part was stored at − 80 ℃ immediately for nucleic acid extraction, one section to determine soil inorganic N was stored at 4 °C, one was oven-dried for mass water content determination, and the remains were naturally air-dried for the determination of pH and EC (5:1 water-soil ratio) using the potentiometric method. Ammonium nitrogen was characterized using indophenol blue colorimetry, and nitrate nitrogen was measured by ultraviolet spectrophotometry.

2.5 DNA Extraction, HT-qPCR and Analyses of 16S rRNA Gene

Soils collected from day 7, 21, and 35 of each cycle were selected for testing the functional genes and bacterial community composition. Using a FastDNA® Spin Kit for soil, DNA was extracted from 0.2 g of soil samples (MP Biomedical, Santa Ana, California, USA) depending on the manufacturer's instructions. High-throughput quantitative PCR (HT-qPCR) were conducted using the WaferGen SmartChip qPCR System by Hefei Yuan-zai Biotechnology Co., Ltd. (Hefei, China). Detailed HT-qPCR procedures with the primers used in this study as well as Illumina sequencing and analyses of 16S rRNA gene are described in the supplementary materials. The primer sequences of the targeted genes are shown in Table S1.

2.6 Data Analysis

To assess the average differences among treatments, a one-way analysis of variance (ANOVA) was conducted using SPSS (26.0). LSD test was utilized for comparing the averages and the difference is deemed significant when the p value is less than 0.05 (SPSS 26.0). Two-factor permutation multivariate ANOVA (PERMANOVA) was employed to compare the divergence of environmental data sets among different treatments throughout the experiment, and to compare the effects of inhibitors and water on the differences in the abundance of soil bacterial communities at the genus level by PAST 4.01. Network analyses were performed in the R 4.4.1 using igraph and RMThreshold packages. The Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) was used to predict potential pathways associated with the predicted amplicon sequence variants (ASVs) using the free online platform Personalbio GenesCloud (https://www.genescloud.cn). Automatic linear model was performed at the confidence level of 95% in SPSS. A structural equation model was established to identify the main predictors of soil N_2O fluxes from the soil environmental factors (AMOS 24.0).

3 Results

3.1 Edaphic Characters

At the beginning of the first cycle, the rapid hydrolysis of urea produced a large amount of NH_4^+-N. Subsequently, as nitrification proceeded, the concentration of NH_4^+-N decreased and NO_3^--N concentration steadily increased (Fig. 1). In both cycles, the NH_4^+-N concentration in NB-added soils at a low level during the first 7 days implied that the application of NB showed inhibition of urea hydrolysis. The greater soil NH_4^+ concentration and lower NO_3^- concentration in NP and NPB treatments relative to NS suggested that the nitrification inhibitor NP played a role in inhibiting nitrification, and the inhibitory effect of NPB was better than that of NP in most cases. Inhibitors and water resources affected NH_4^+-N concentrations significantly in both cycles, while for NO_3^--N concentration, this effect was only present in the first cycle (Table S2).

WFPS increased immediately after each irrigation, with little difference between different inhibitor treatments (Fig. S1). The change in soil pH was significantly affected not only by inhibitors but also by irrigation water resources (Table S2). In the second cycle, for most cases, the pH of the NPB treatments was highest (Fig. S2).

3.2 Yield

Compared with NS, inhibitor treatments increased the yield of Chinese cabbage (Fig. S3), the inhibitors

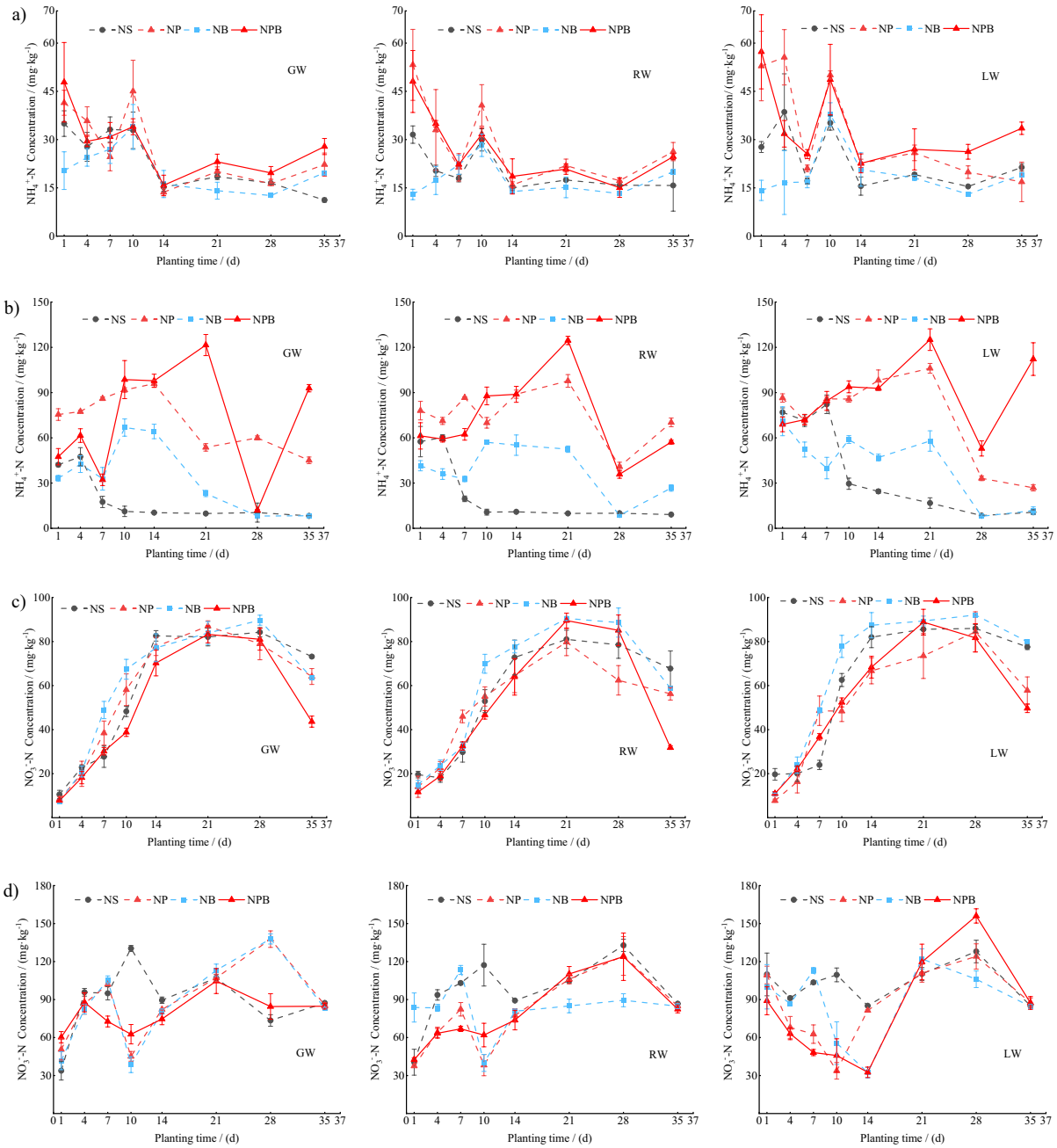

Fig. 1 Dynamics of soil NH_4^+-N concentration in (**a**) the first cycle and (**b**) the second cycle and NO_3^--N concentration in (**c**) the first cycle and (**d**) the second cycle. NS, no substance; NP, Nitrapyrin; NB, N-(N-butyl) thiophosphoric triamide; NPB, NP + NB. GW, groundwater; RW, reclaimed water; LW, livestock wastewater. Error bars indicate the standard deviations (n = 3)

can regulate soil N transformation and reduce nitrogen gaseous losses, thereby probably promoting N uptake by the crop. Compared with GW irrigation, RW and LW irrigation increased the yield. Inhibitors

and water resources significantly affected the yield (Table S2).

3.3 Emission Fluxes of N_2O

Inhibitor application significantly altered N_2O emission fluxes in both cycles, and the significant effect of the water resources on N_2O was present only in the second cycle (Table S2). N_2O emissions varied significantly between treatments and showed a significant increase between day 4 and day 10 in the first cycle, with the reduced emissions due to the addition of inhibitors (Fig. 2a and b). Taking the two cycles together, the application of NP and NB alone inhibited cumulative N_2O emissions regardless of the water resource (Fig. 2c and d). Compared with NS, in the first and second cycle, NB inhibited N_2O emission most obviously under GW irrigation by 38.3% and 29%, respectively; NP inhibited N_2O emission most efficiently by 29.3% and 41.4% in RW-irrigated soils and by 42.8% and 45.1% in LW-irrigated soils. The study showed that the impact of NPB on cumulative N_2O emission varied among different water resources (Fig. 2c and d). Under GW irrigation, compared with NS, NPB only reduced N_2O emission by 40.9% in the first cycle while promoting N_2O emission in the second cycle. On the contrary, in RW and LW-irrigated soils, NPB reduced N_2O emissions in both cycles

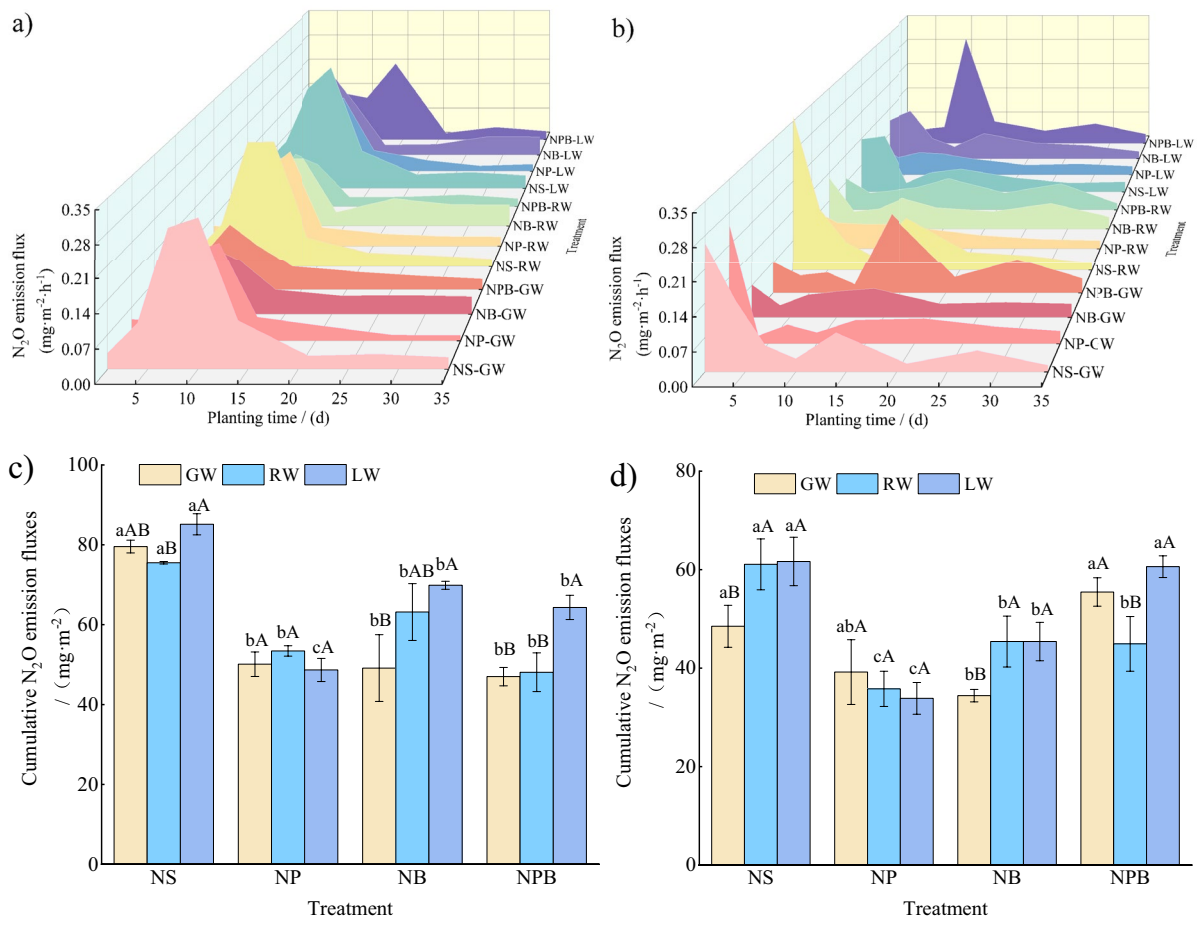

Fig. 2 Effects of alternative water and inhibitors on N_2O emission fluxes in (**a**) cycle 1 and (**b**) cycle 2 as well as N_2O cumulative emission fluxes in (**c**) cycle 1 and (**d**) cycle 2. NS, no substance; NP, Nitrapyrin; NB, N-(N-butyl) thiophosphoric triamide; NPB, NP + NB. GW, groundwater; RW, reclaimed water; LW, livestock wastewater. Different lowercase letters indicate significant differences between inhibitor treatments ($p < 0.05$), and different uppercase letters indicate significant differences between different water resources ($p < 0.05$). Error bars indicate the standard deviations (n = 3)

compared with NS. However, the regulatory effect of NPB was not significantly advanced than that of NP or NB applied alone. Overall, water resources played a part in the regulation of N_2O emission by inhibitors, NPB did not show a greater advantage in regulating N_2O emissions than NP and NB alone.

3.4 Abundance of Functional Genes and Bacterial Community Composition

Figure 3 display the enrichment level of nine functional genes related to the N cycle in the soil, including *ureC* associated with urease coding, *nifH* related to nitrogen fixation, four genes involved in nitrification (*amoA*, *amoB*, *hao*, and *nxrA*), and three genes engaged in denitrification (*narG*, *nirS*, and *nosZ*). The use of the urease inhibitor NB significantly reduced the enrichment level of the urease-coding gene *ureC* on day 7 of the experiment as expected (Fig. 3). Intriguingly, the nitrification inhibitor NP showed a similar performance with NB, which contradicted with that the NH_4^+-N concentration in NP soil was greater than NS soil. This was probably the combined results of the affected urea hydrolysis and nitrification. On the 7th and 21st day of the experiment, compared to NS, NP effectively suppressed the enrichment level of *amoB* and *hao*, meanwhile, NB increased the relative abundance of *nxrA* in RW- and LW-irrigated soils (Fig. 3). Both NP and NB mostly inhibited the relative abundance of the denitrification genes (*narG*, *nirS*, and *nosZ*) compared to NS under GW irrigation during the first cycle; however, they only decreased the enrichment level of *nirS* in the second cycle (Fig. 3). In addition, during the second cycle, the application of NPB under GW irrigation resulted in a higher relative abundance of the N-fixing gene *nifH* compared to NS (Fig. 3). As can be seen in Figure S4, the values of *nirS*/*nosZ* ratio were generally lower in the second cycle than in the first cycle, with the NPB treatment having a higher *nirS*/*nosZ* ratio in most cases.

The top 10 genus in each treatment comprised 23.6%—45.3% of the total community (Fig. 4a and b). Notably, *Bacillus* and *Pontibacter* exhibited relatively high proportions in both cycles. Overall, the percentage of *Massilia* decreased over time, and was generally lower in the second cycle compared to the first cycle across treatments. The soil 16S rRNA gene copy number was generally higher during the first cycle compared to the second cycle, particularly on day 7, when urea hydrolysis and nitrification reactions occurred at a relatively fast rate (Fig. S5a). Similarly, the Chao 1 index was also found to be higher in the first cycle than in the second cycle on day 7 (Fig. S5b). Additionally, the application of inhibitors resulted in an increase in soil 16S rRNA gene copy number compared to NS (Fig. S5a). Two-factor PERMANOVA demonstrated that inhibitor application

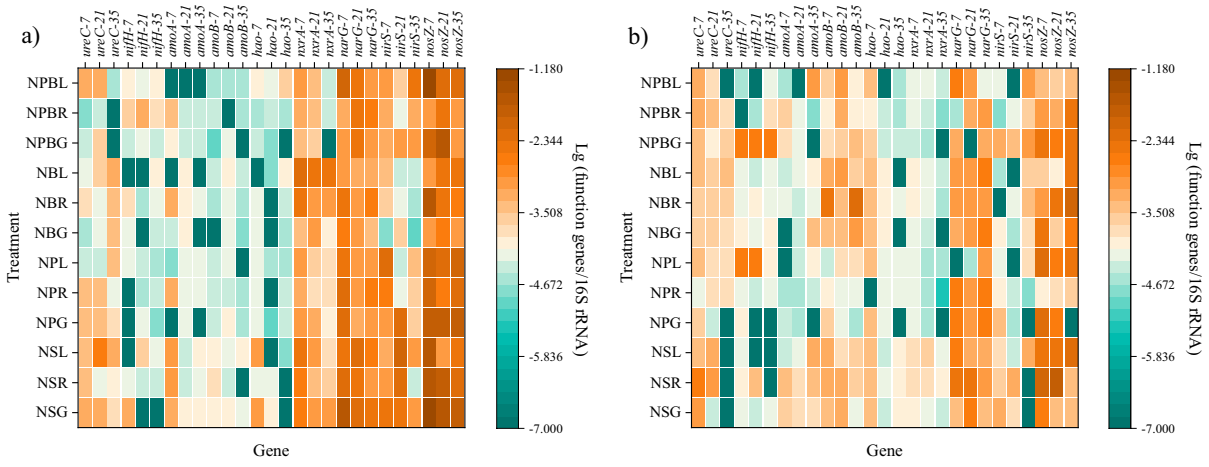

Fig. 3 The logarithmic relative abundance of selected function genes in soil from (**a**) the first cycle and (**b**) the second cycle. NS, no substance; NP, Nitrapyrin; NB, N-(N-butyl) thiophosphoric triamide; NPB, NP + NB. G, groundwater; R, reclaimed water; L, livestock wastewater. "7", "21", and "35" after the genes in the horizontal axis represent day 7, day 21, and day 35 respectively

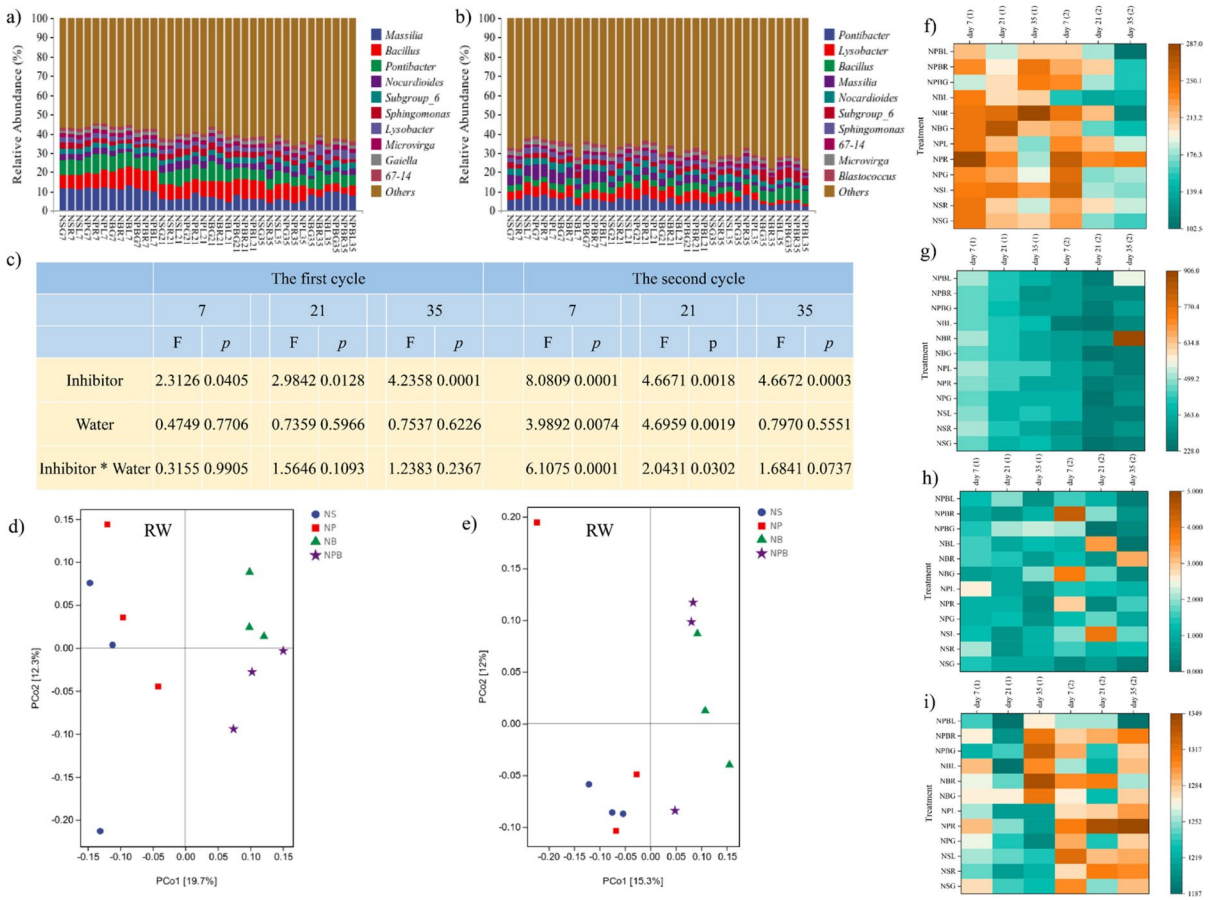

Fig. 4 The relative abundance of bacterial communities at the genus level for each treatment in (**a**) the first cycle and (**b**) the second cycle; (**c**) Two-way PERMANOVA dispalying the effects of inhibitors and waters on the differences in the abundance of soil bacterial communities at the genus level; the PCoA result based on the Bray–Curtis distance matrix (day 7) showing the distribution patterns of soil bacterial communities at the genus level under reclaimed water (RW) irrigation in (**d**) the first cycle and (**e**) the second cycle; and the relative abundance of the potential pathways for (**f**) nitrate reduction I (denitrification), (**g**) urea cycle, (**h**) nitrifier denitrification, and (**i**) nitrate reduction VI (assimilatory). NS, no substance; NP, Nitrapyrin; NB, N-(N-butyl) thiophosphoric triamide; NPB, NP + NB. G, groundwater; R, reclaimed water; L, livestock wastewater. "7", "21", and "35" in subgraphs (c) and in the horizontal axis in subfigures a) and b) represent day 7, 21 and 35 of each cycle, respectively. "1" and "2" in the brackets in subfigures f)-i) represent the first and second cycles, respectively

significantly affected bacterial communities at the genus level, but water resources did not cause a prominent effect (Fig. 4c). In both cycles, under RW irrigation, the application of NB and NPB was shown to cause community differences on day 7 (Fig. 4d and e). As time progressed, the bacteria communities in the NB and NPB treatment were generally observed to be different from those in the NS and NP treatments regardless of water resources (Figs. S6 and S7). Furthermore, there was more aggregation and similarity in the community among the samples from different treatments in cycle 2 compared to cycle 1.

As predicted by PICRUSt2, four potential pathways were linked to the nitrogen cycle (Fig. 4f-i). The nitrate reduction I (denitrification) and the nitrifier denitrification pathway influence N_2O emissions by engaging in denitrification and nitrification, respectively. The urea cycle pathway affects N_2O emissions indirectly by regulating urea hydrolysis, whereas in the nitrate reduction VI (assimilatory) pathway N_2O is an intermediate product. Among them, in the nitrate reduction I (denitrification) pathway and the urea cycle pathway, there were differences in the functional potentials across the two cycles, with the

first cycle being generally larger than the second. Conversely, the results were reversed in the nitrate reduction VI (assimilatory) pathway.

3.5 Relationships between Environmental Factors, N_2O Emissions, Functional Genes, and Microbial Taxa

The network analysis results revealed no significant co-occurrence or co-exclusion between the selected N-functional genes and microbial taxa at the genus level (Fig. 5). In the first cycle, a noteworthy pattern of co-occurrence between *Massilia* and N_2O emission fluxes was observed, but this pattern was not evident in the second cycle. Furthermore, the relationships between microbial taxa differed between the two cycles. For instance, in cycle 1, there was a co-exclusion between *Subgroup-6* and *Nocardioides*, while in cycle 2, *Subgroup-6* was found to be co-exclusion with *Lysobacter*. Structural equation model analyses indicated that inhibitors indirectly affected N_2O emissions by influencing soil properties and the relative abundance of N-cycle functional genes (Fig. 6). Notably, NB directly reduced N_2O emissions under RW and LW irrigation. The mechanisms through which inhibitors regulated N_2O emissions exhibit both similarities and differences across water sources. In all water resources, soil pH and NO_3^- concentration led to a decrease and an increase in N_2O emissions, respectively. Additionally, the indirect regulation of N_2O emissions by NP was mainly achieved by controlling soil NH_4^+-N concentration. Microbial

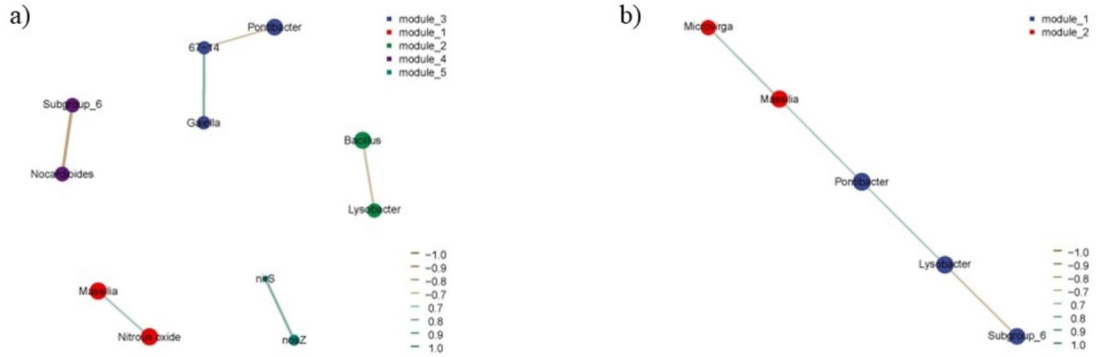

Fig. 5 Network analysis of associations between bacterial community (genus level), functional genes and N_2O emission fluxes in soil during (**a**) the first cycle and (**b**) the second cycle

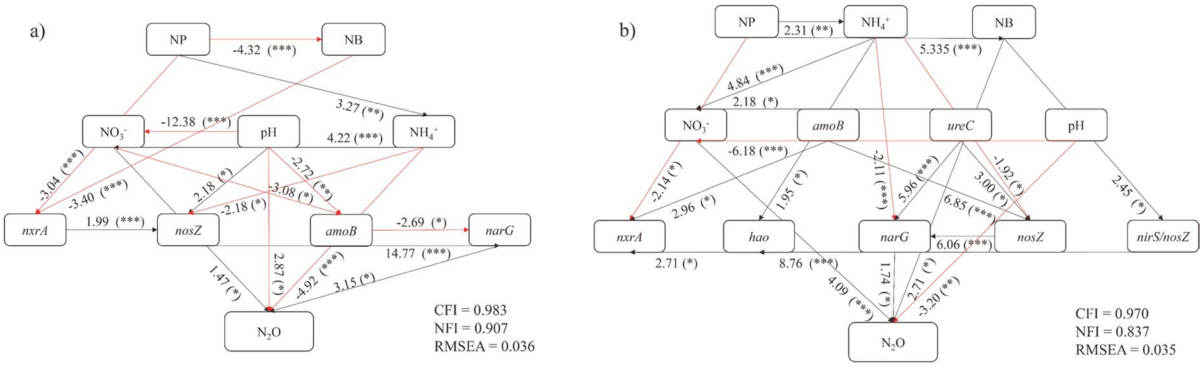

Fig. 6 Structural equation model depicts the causal relationships between soil N_2O emission fluxes and soil pH, water filled pore space (WFPS), concentrations of NH_4^+ and NO_3^-, and the abundance of N functional genes, based on data from (**a**) GW and (**b**) RW and LW in two cycles of experiments. Numbers adjacent to the lines are correlation coefficients. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

taxa were less important than functional genes and soil properties in contributing N_2O emissions, and a potential effect of *Massilia* on N_2O emissions was observed only in the first cycle (Fig. S8). Combining the results of all analysis methods, we inferred that soil NH_4^+-N concentration was the primary soil chemical property affecting N_2O emissions, and *nxrA* and *narG* were the main N cycle functional genes influencing N_2O emissions, with bacterial communities exerting a comparatively weaker influence on N_2O emissions in comparison to soil properties and functional genes (Figs. 5, 6 and S8).

4 Discussion

Two cycles of experiments showed that inhibitors had a positive reducing effect on the greenhouse effect of alternative water irrigation. Still, the effect of combined inhibitors on N_2O emission was not significantly improved relative to that of a single inhibitor application. Considering the total N_2O emission from the two cycles, it can be seen that the inhibition ability of N_2O emission by applying NP alone was sounder under alternative water irrigations, while this ability of NB was more prominent under groundwater irrigation.

4.1 Influence of Different Water Resources on N_2O Emissions

N_2O is mainly produced in agricultural soils through the processes of nitrification and denitrification driven by microbiomes. Alternative water usually contains more nutrients, promoting microorganisms' growth and activity (Ibekwe et al., 2018), and providing more reaction substrates for nitrification and denitrification. Taken together, the total N_2O emissions of the two cycles from RW- and LW-irrigated soils were higher than those from GW-irrigated soils when no inhibitors were added, consistent with previous findings (Shang et al., 2016; Zou et al., 2009). The relatively higher NH_4^+ concentration in RW- and LW-irrigated soils can provide more reaction substrate for the nitrification reaction. The increased production of NO_3^- from the nitrification also supplied the substrate for denitrification reactions, therefore both reactions may result in elevated emissions of N_2O. Besides, wastewater irrigation might reduce soil porosity, resulting in a slower diffusion rate of O_2, which in turn affects denitrification rates and ultimately raises N_2O emissions (Hernandez-Ramirez et al., 2021; Hilton et al., 1994; Leuther et al., 2019). We observed that RW irrigation reduced N_2O emissions in NS soils compared to GW irrigation in the first cycle, likely due to the higher pH in these soils under RW irrigation. According to the results of Wang et al. (2018), soil with a pH range of 8.0—9.0 (i.e., the range of pH variation in this experiment) showed a gradual decrease in N_2O emissions with increasing pH. The significance of soil pH in determining the outcome of denitrification might be the reason behind this, as pH increases could influence the overall denitrification activity, ultimately resulting in a decline of the $N_2O/(N_2O+N_2)$ ratio (Laughlin et al., 2010). Furthermore, N_2O emissions from NS-treated soils were lower in the second cycle compared to the first (Fig. 2). We conjecture that this may be linked to the generally lower abundance of nitrate reduction I (denitrification) pathway in the second cycle. The observed trend of higher NO_3^- concentrations in the second cycle than in the first cycle may side-step this hypothesis (Fig. 1). On the other hand, the number of 16S rRNA genes copies in the NS-treated soils from the second cycle was markedly lower than that observed in the first cycle at both day 7 and day 35 (Fig. S5). This decline in microbial populations during the second cycle may influence both the number and activity of microorganisms involved in the production of N_2O.

4.2 Regulation of N_2O Emission from Soil Irrigated with Different Water Resources by Inhibitors Applied Alone

Inhibitors influence the dynamics of N_2O emissions from the soil by modulating functional genes that contribute to nitrification and denitrification (Fan et al., 2018; Meng et al., 2020; Qu, 2022). This study evidenced that NP and NB alone were able to decrease N_2O emissions compared to NS, coincident with earlier findings (Borzouei et al., 2022; Dawar et al., 2011; Zhang et al., 2015). As for the regulation process of N_2O emission by NP, it was uncovered that the application of NP had the potential to affect the abundance of *amoB* and *nxrA* genes, which are the main drivers of the transformation from NH_4^+ to hydroxylamine (NH_2OH) and NO_2^- to NO_3^- in the

nitrification process, respectively. This may be one of the ways that NP inhibited N_2O emission in this study. Likewise, in close agreement with us, Cui et al. (2013) also documented that NP could decrease soil N_2O emission by lowering the abundance of nitrifying function genes. In addition, consistent with a previous study (Meng et al., 2020), we also found that NP not only affected the transformation of NH_4^+ to NH_2OH, but also reduced the relative abundance of the denitrification function genes, which may be another approach for NP to regulate N_2O emissions (Figs. 3 and 6). Usually, NB diminishes N_2O emission by postponing the hydrolysis of urea (Cantarella et al., 2018; Meng et al., 2020). Our results were in line with the findings of Fan et al. (2018) that NB application could reduce the enrichment level of *ureC* gene in the early days of the test, and lead to a lower soil NH_4^+ concentration and N_2O emission. The UreC is a part of the prokaryotic urease group that is usually used to sense urea hydrolysis in ureicidal prokaryotes and in soil (Oshiki et al., 2018). Moreover, NB reduced gene enrichment not only in *ureC* but also in *amoB* and *hao* in this study, suggesting that the application of NB may affect the subsequent nitrification reaction. Fan et al. (2018) pointed out that the application of NB may reduce the substrate availability for ammonia-oxidizing bacteria, and thus the abundance of ammonia-oxidizing bacteria, the primary microbes responsible for nitrification in alkaline soils (Shen et al., 2014).

Overall, the application of inhibitors under RW and LW irrigation suppressed soil N_2O emissions, with NP possessing the maximum effectiveness. As shown in Fig. 3, the relative abundance of the *nxrA* gene in the soil was lower in NP treatments than in the other three inhibitor treatments under RW and LW irrigation. According to the results of the structural equation model and automatic linear model, *nxrA* is one of the key factors in modulating N_2O emissions in this study (Figs. 6 and S8). In addition, the relative abundance of soil *ureC* gene under RW and LW irrigation was greater than GW irrigation in NB treatment (Fig. 3). As mentioned above, the negative effect of RW and LW on soil porosity promoted an increase in anaerobic sites in the soil, while NB is more suitable for the aerobic environment, and its role in delaying urea hydrolysis decreases under anaerobic conditions (Wang et al., 1991). This may indirectly hinder NB regulation of soil NH_4^+-N concentrations and subsequent N_2O production. Therefore, we argued that NP application under RW and LW irrigation would optimally reduce N_2O emissions by suppressing the relative abundance of functional nitrification genes, particularly *nxrA*.

4.3 Regulation of N_2O Emissions from Soil Irrigated with Different Water Resources by Combined Inhibitors

Our research confirmed the observations of Wang et al. (2023) that there was little difference in the effectiveness of NI and UI applied alone or in combination to reduce N_2O emissions. For the most part, NPB acted to reduce the enrichment level of nitrifying genes and denitrifying genes, but the difference was not large compared with NP or NB applied alone (Fig. 2). It is possible that the small discrepancies in soil WFPS and temperature between the three treatments of NP, NB, and NPB (Figs. S1 and S9) indirectly narrowed the differences in nitrification and denitrification reaction rates. In addition, although the difference in N_2O emissions between combined and mono application of inhibitors was not significant, it could be observed that N_2O emissions from NPB-applied soils were generally greater than those from NP or NB. This could be attributed to the accelerated rate of nitrification in the soils received both inhibitors compared to those received individual inhibitors, and that the co-existing nitrification inhibitors decreased the effectiveness of NB in inhibiting urea hydrolysis (Lasisi et al., 2020; Zhou et al., 2017). Further research is needed to fully comprehend this observation. In the second cycle, the effect of NPB on N_2O emission however changed from inhibition to promotion compared with NS in GW-irrigated soils, where the *nirS*/*nosZ* ratio of NPB treatment was significantly higher than that of NS at days 21 and 35, which implied that possibly the slower rate of conversion of N_2O to N_2 during this period resulted in relatively higher N_2O emissions from NPB than from NS. Additionally, in the second round, under GW irrigation, the relative abundance of *narG* and *nirS* was greater in NPB-added soils than in NS soils. The *narG* gene mediates the conversion of NO_3^- to NO_2^-, the beginning of the denitrification process, and the structural equation model indicated that its enrichment may contribute to N_2O emissions (Fig. 6). It was also observed in the second cycle that the ASVs

involving the nitrate reduction I (denitrification) pathway were more abundant for the NPB treatment than for the NS treatment. In addition to bacteria, microorganisms such as fungi and blue algae also affect the production and emission of N_2O in the soil (Liu et al., 2019a; Pan et al., 2022; Wei et al., 2015), and more attention needs to be paid to these microorganisms in future research.

5 Conclusions

We investigated the influences of NP and/or NB application on soil basic properties, bacterial community, N cycle function genes, and N_2O emissions under alternative water irrigations and the potential mechanisms through pot experiments. The findings indicated that irrigation with RW and LW would increase N_2O emissions by affecting soil NH_4^+-N and NO_3^--N concentrations and the abundance of functional genes. Fortunately, the addition of NP could reduce the enrichment level of nitrification genes, delay the transformation of NH_4^+-N to NO_3^--N, and thus reduce N_2O emission, regardless of the water resources. NB also played a delaying role in nitrification, but its inhibiting effect on the hydrolysis of urea was the more important reason for its regulation of N_2O emission. Moreover, NB caused more differences in soil bacterial communities than NP, both NP and NB appeared to decrease the relative abundance of *narG* related to the denitrification process. In addition, combining the results of the two cycles, we found that applying inhibitors together did not significantly precede applying inhibitors alone in regulating N_2O emissions, regardless of the type of water resource. Under GW irrigation, NB application was found to be more effective in controlling N_2O emissions; whereas under RW and LW irrigation, NP application emerged as the more applicable option for reducing N_2O emissions. This indicates that applying NP alone under RW and LW irrigation could be a cost-effective approach to enhance soil nutrients and mitigate N gas losses in practical production, while being cheaper than simultaneous application of both inhibitors. However, the ability of inhibitors to regulate N_2O emissions under irrigation with alternative water sources in the long run needs to be explored in the future.

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Data Availability All data are included in this article, and the data will be made available on request.

Declaration

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

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