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Research Article

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Comparing nitrification inhibitors to mitigate cadmium accumulation in pakchoi and elucidating the associated microbial mechanisms

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Abstract: The use of nitrification inhibitors has been suggested as a strategy to decrease cadmium (Cd) accumulation in crops. However, the most efficient nitrification inhibitor for mitigating crop Cd accumulation remains to be elucidated, and whether and how changes in soil microbial structure are involved in this process also remains unclear. To address these questions, this study applied three commercial nitrification inhibitors, namely, dicyandiamide (DCD), 3,4-dimethylpyrazole phosphate (DMPP), and nitrapyrin, to pakchoi. The results showed that both DCD and DMPP (but not nitrapyrin) could efficiently decrease Cd concentrations in pakchoi in urea- and ammonium-fertilized soils. In addition, among the three tested nitrification inhibitors, DMPP was the most efficient at decreasing the Cd concentration in pakchoi. The nitrification inhibitors decreased pakchoi Cd concentrations by suppressing acidification-induced Cd availability and reshaping the soil microbial structure; the most effective nitrification inhibitor was DMPP. Ammonia oxidation generates the most protons during nitrification and is inhibited by nitrification inhibitors. Changes in environmental factors and predatory bacterial abundance caused by the nitrification inhibitors changed the soil microbial structure and increased the potential participants in plant Cd accumulation. In summary, our study identified DMPP as the most efficient nitrification inhibitor for mitigating crop Cd contamination and observed that the soil microbial structural changes caused by the nitrification inhibitors contributed to decreasing Cd concentration in pakchoi.

Key words: Cadmium; Nitrification inhibitor; Soil microbial structure; Safe crop production

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1 Introduction

Cadmium (Cd) is an environmentally toxic substance that poses a significant risk to human health because of its notable biological toxicity (Souza-Arroyo et al., 2022; Li et al., 2021; Satarug et al., 2010; Clemens et al., 2013). Humans accumulate Cd through food intake, with Cd-contaminated food representing a worldwide problem (Schaefer et al., 2020). The consumption of crop foods produced in contaminated soil is a major route of Cd uptake by humans (Zulfiqar et al., 2022; Clemens et al., 2013; Ryan et al., 1982). Therefore, lowering Cd accumulation in soil and crops is crucial for protecting humans from Cd ingestion. However, consistent relationships have not been observed between soil and plant Cd concentrations because soil properties, soil Cd contents, plant genetics, and nutrient management all affect plant Cd concentrations (Schaefer et al., 2020; Nazar et al., 2012; Smolders, 2001). Many nutrients are associated with the availability of Cd in soil and the absorption capacity of Cd (Liu et al., 2022; Lux et al., 2011; Sarwar et al., 2010; Dheri et al., 2007), including

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nitrogen (N), which has the largest mineral nutrient demand among plants. Therefore, adjusting nutrient management is considered an effective, inexpensive, and time-saving strategy for minimizing Cd accumulation in plants (Zhu et al., 2023; Sarwar et al., 2010).

N management is one of the most common agronomic practices. In China, urea and ammonium account for over 95 % of the total N fertilizer applied in crop production (Zhang and Zhang, 2008). Under most conditions, urea in the soil is quickly converted to ammonia by urease, and ammonia is finally converted to nitrate (NO_3^-) by nitrification, which produces protons (H^+) (Rysa et al., 2019). This process leads to soil acidification, which increases the availability of Cd in the soil (Li et al., 2023; Yu HY et al., 2016; Kirkham, 2006). In addition, several lines of evidence have shown that the uptake of NO_3^- by root cells is accompanied by the promotion of Cd^{2+} uptake, which results from the induction of the Fe^{2+} transporter *IRT1* and the requirement for an anion-cation balance (Guan et al., 2015; Mao et al., 2014; Luo et al., 2012). Therefore, inhibiting nitrification may effectively lower Cd uptake by crops in the case of urea or ammonium fertilization.

Nitrification inhibitors are widely used to suppress nitrification, and a few types, such as dicyandiamide (DCD), have been shown to decrease plant Cd accumulation (You et al., 2020; Xie et al., 2009). Although several commercial nitrification inhibitors have been used for N management in agricultural production, there are significant differences among them in terms of their nitrification inhibition efficiency (Zhou et al., 2020; Wang et al., 2017). Moreover, little information is available on which inhibitor is the most effective chemical agent for mitigating Cd accumulation in crops. In addition, nitrification inhibitors have been shown to strongly affect the microbial community in soils (Kleineidam et al., 2011; Carneiro et al., 2010; O'Callaghan et al., 2010; Di and Cameron, 2005); however, whether and how nitrification inhibitor-mediated changes in the microbial community play a role in mitigating plant Cd accumulation remain unknown. Soil microorganisms have been shown to alter the availability and uptake of Cd by plants through various pathways (Shahid et al., 2019; Dong et al., 2008). Therefore, the most efficient nitrification inhibitors for mitigating Cd accumulation in crops must be screened from among the main commercial inhibitors, and how nitrification inhibitor-mediated changes in the microbial community affect Cd accumulation in crops must be determined.

Brassica rapa subsp. *chinensis* (pakchoi) is a vegetable widely cultivated and consumed in East and Southeast Asia. In this study, we conducted a pot experiment using pakchoi to compare the Cd-mitigation effectiveness of three commonly used commercial nitrification inhibitors (Zhou et al., 2020): dicyandiamide (DCD; CAS:461-58-5), 3,4-dimethylpyrazole phosphate (DMPP; CAS:202842-98-6), and nitrapyrin (NP; CAS:1929-82-4). 16S rRNA sequencing was performed to investigate the relationship between nitrification inhibitor-mediated changes in the microbial community and Cd accumulation in pakchoi.

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2 Materials and methods

2.1 Soil properties and plant cultivation

The experimental soil was collected from Jianggan District, Hangzhou, China (120° 12′ E, 30° 16′ N), and the following basic properties were determined according to the method described by Bao (2008): clay, 6.2 %; silt, 28.8 %; sand, 65 %; electrical conductivity (EC), 0.7 mS/cm; cation-exchange capacity (CEC), 7.9 cmol/kg; organic C, 11.5 g/kg; NH_4^+-N, 2.5 mg/kg; NO_3^--N, 13.4 mg/kg; total Cd, 0.4 mg/kg; and pH, 7.20. The air-dried soil was ground and passed through a 2-mm sieve, and part of it was mixed with $CdCl_2$ at 2.5 mg Cd/kg soil. The soil underwent an aging period of one year and was then used to fill pots at 450 g/pot. The experiments were conducted in a greenhouse. Pakchoi were germinated on TS-1 substrate (Klasmann-Deilmann, Germany). After 7 days, seedlings with uniform growth were selected and transplanted, one per pot. The soil in each pot was watered to 60 % of the field water-holding capacity (FWHC; 22 % water content, w/w), and water was subsequently added to compensate for evaporation and transpiration. The pots were divided into three groups based on the form of nitrogen fertilizer applied, i.e., $CO(NH_2)_2$, $(NH4)_2SO_4$, or $Ca(NO_3)_2$. They were then further divided into four subgroups (control, DCD, DMPP, and nitrapyrin) based on whether a nitrification

inhibitor was added and the type of nitrification inhibitor that was applied for 12 treatments. At the true leaf and vigorous growth stages (day 14 and day 28, respectively), 0.2 g N/kg nitrogen fertilizer with a pre-mixed nitrification inhibitor was applied to the appropriate pots. The recommended nitrification inhibitor use rate was taken from previous studies and suggestions provided by Kelliher et al. (2008), Zerulla et al. (2001), and Wolt (2000). DCD was added at 10 % of the nitrogen application rate, i.e., 0.02 g/kg, and DMPP and nitrapyrin were added at 2 % and 1 %, respectively. On day 35, the edible parts of the pakchoi were harvested and weighed, and soil samples were collected for further analysis.

2.2 Determination of plant cadmium concentration and soil properties

The edible parts of the pakchoi were dried at 65 °C for 48 h and were then digested (Zhu et al., 2020). The Cd concentration was measured using a 4200 MP-AES (Agilent Technologies, USA). Soil NH_4^+-N and DTPA-extractable Cd (DTPA-Cd) concentrations were determined according to standard procedures (Bao, 2008). NH_4^+-N was determined using the indophenol blue colorimetric method, and the absorbance was measured at 625 nm using a SpectraMax 13X (Melville Devices, USA). DTPA-Cd was measured using the 4200 MP-AES. Soil NO_3^--N was determined using dual-wavelength spectrophotometry at 220 nm and 275 nm according to the procedure of the National Standard of China (GB/T 32737-2016), and soil pH was measured using a pH electrode placed in a mixture of 1 g of fresh soil and 10 mL of deionized water.

Data analyses were performed using Excel 2019, IBM SPSS 21.0, and Origin 2018. Both one- and two-way ANOVAs were used to determine statistical significance (P<0.05), and Duncan's test was used for multiple comparisons.

2.3 Determination of plant cadmium concentration and soil properties

Total genomic DNA was extracted and purified from 0.5 g of each individual soil sample using a Mag-Bind Soil DNA Kit (Omega bio-tek, USA) following the manufacturer's protocol. DNA quality was checked using electrophoresis in 1 % (w/v) agarose gel (GUHE Info Technology Co., Ltd., Hangzhou, China).

The primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') were used to amplify the V4 region of bacterial 16S rRNA genes. PCR amplicons were purified, quantified, and sequenced on an Illumina NovaSeq 6000 platform. Barcodes were used to assign raw sequencing reads to the respective samples and were identified as valid sequences. Low-quality sequences were filtered (Chen and Jiang, 2014; Gill et al., 2006) and then operational taxonomic units (OTUs) were selected using Vsearch (v2.15.0), including dereplication, cluster, and chimera detection processes (Rognes et al., 2016). The OTU taxonomic classification was performed by searching the representative sequence set against the SILVA138 database using QIIME2 (v2020.6) (Bolyen et al., 2019; Quast et al., 2013). OTUs containing less than 0.001 % of the total sequences across all samples were discarded.

The OTU-level diversity indices, including the Shannon diversity index, Simpson index, Chao1 richness estimator, goods coverage, and rarefaction curves, were calculated using QIIME2 to compare the richness and evenness of the microbial communities in the different treatments. A β-diversity analysis was performed using Bray–Curtis distance matrices and was visualized via a constrained principal coordinates analysis (CPCoA) using the R (v.3.2.0) package "amplicon." A permutational multivariate analysis of variance (PERMANOVA) was performed using the R package "vegan" to investigate variations in microbial community structures across samples. A principal component analysis (PCA) was performed based on genus-level compositional profiles.

Linear discriminant analysis (LDA) effect size (LEfSe) was used to identify microbial taxa with different abundance levels (LDA>3; P<0.05) (Segata et al., 2011), and upset plots (R package UpSet) were used to visualize the results. A Pearson's correlation analysis (R package stats) was used to explore the relationships between the identified microbial taxa and soil properties, pakchoi yield, and Cd concentration. The ecological functions of different treatments were predicted using the Functional Annotation of Prokaryotic Taxa (FAPROTAX) database (Louca et al., 2016). Structural equation modeling (SEM; IBM SPSS Amos) was performed to evaluate the potential direct or indirect effects of the following factors: nitrification rate

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(proportion of NO_3^--N in the total NH_4^+-N and NO_3^--N) (Lin et al., 2004), soil pH, soil available Cd (DTPA-Cd concentration), soil microbial diversity (NMDS2; Bray–Curtis distance matrices, non-metric multidimensional scaling), and pakchoi Cd accumulation.

3 Results

3.1 Effects of different nitrification inhibitors on the yield and Cd concentration of edible parts

In uncontaminated soil, the application of DCD, DMPP, or nitrapyrin had little effect on the yield of edible pakchoi under all three nitrogen fertilizer treatments (Fig. 1 and Table S1). Cd contamination of the soil significantly inhibited the yield of the edible parts in the ammonium and nitrate treatments and had little effect on the urea treatment. Applying DCD and DMPP (but not nitrapyrin) completely abolished the decreased yield from Cd contamination during the ammonium treatment. However, none of the tested nitrification inhibitors affected the yield of the edible parts of pakchoi under the nitrate treatment. Compared with the effect on yield, the application of DCD or DMPP lowered the Cd concentration in the edible parts of pakchoi in the urea treatment (Fig. 1, Fig. S1, and Table S1). This effect was particularly significant for the application of DMPP, which decreased the Cd concentration by approximately 60 % compared to that of the control (without nitrification inhibitor application). In the ammonium treatment, DCD and DMPP applications similarly decreased the Cd concentration in the edible parts at a rate of approximately 40 %. Nitrapyrin application had little effect on the Cd concentration in the edible parts in both the urea and ammonium treatments. In addition, the tested nitrification inhibitors did not affect the Cd concentration in the edible parts of the three nitrogen fertilizer treatments. The above results suggest that the effects of nitrification inhibitors on the growth and Cd concentration of pakchoi depend on the type of nitrification inhibitor and the form of nitrogen fertilizer. Since urea is the most commonly used nitrogen fertilizer in agricultural production, DMPP could be considered the most efficient nitrification inhibitor for mitigating Cd accumulation in pakchoi.

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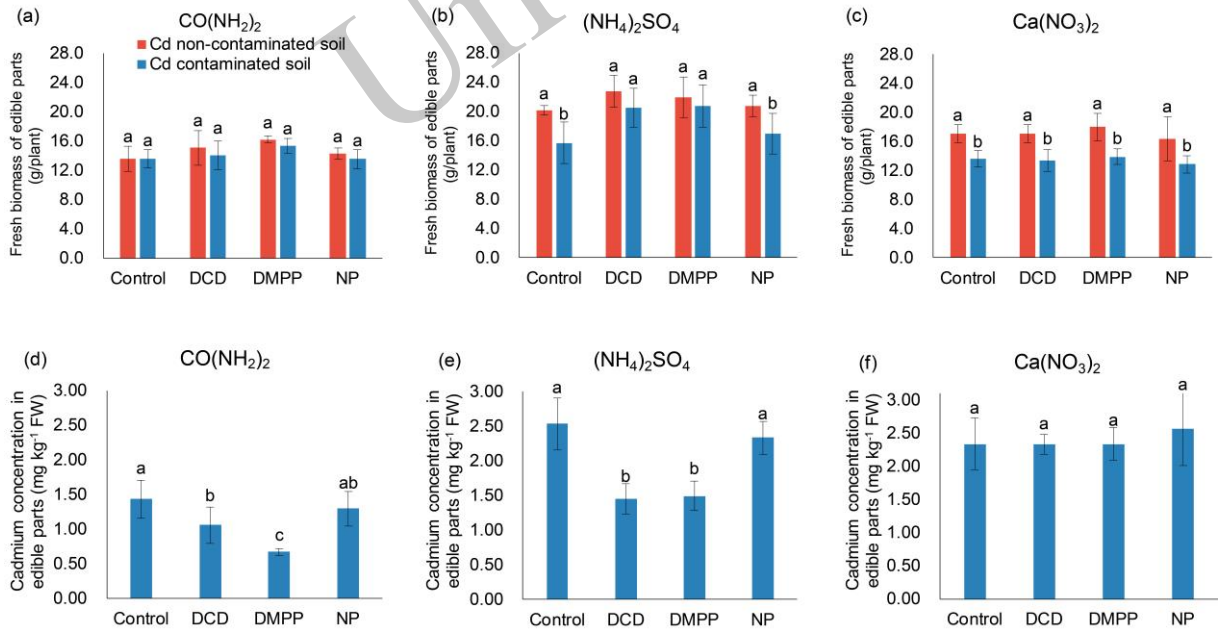

Fig. 1. Effects of the nitrification inhibitors on the yield and Cd concentration of the edible parts of pakchoi (fresh weight). $CO(NH_2)_2$, $(NH_4)_2SO_4$, and $Ca(NO_3)_2$ were used as the N fertilizers. Nitrification inhibitors were not applied to the control treatment. Data are expressed as the mean ± standard deviation (SD) (n = 5). Different letters represent significant differences (Duncan's test; P<0.05).

3.2 Effects of different nitrification inhibitors on the soil properties

In the soil fertilized with urea, applying the three tested nitrification inhibitors significantly increased the NH_4^+-N concentration in the soil at harvest compared with that of the control treatment (Fig. 2 and Table S1), with DMPP showing the most significant effect. Compared with the effect on NH_4^+-N concentration, the three tested nitrification inhibitors significantly decreased the NO_3^--N concentration in the soil, with DMPP showing the most significant effect. The effects of all three tested nitrification inhibitors on the NH_4^+-N and NO_3^--N concentrations in soil fertilized with ammonium were similar to those in soil fertilized with urea. None of the tested nitrification inhibitors affected the NH_4^+-N and NO_3^--N concentrations in soil fertilized with nitrate. Consequently, DMPP had the lowest nitrification rates (ratio of NO_3^--N to total mineral N), with urea and ammonium fertilizer at only 5.61 % and 4.04 %, respectively. The above results suggest that the effects of nitrification inhibitors depend on the type of nitrification inhibitor and the form of nitrogen fertilizer, and that DMPP could be the most efficient nitrification inhibitor when urea or ammonium are used as fertilizers.

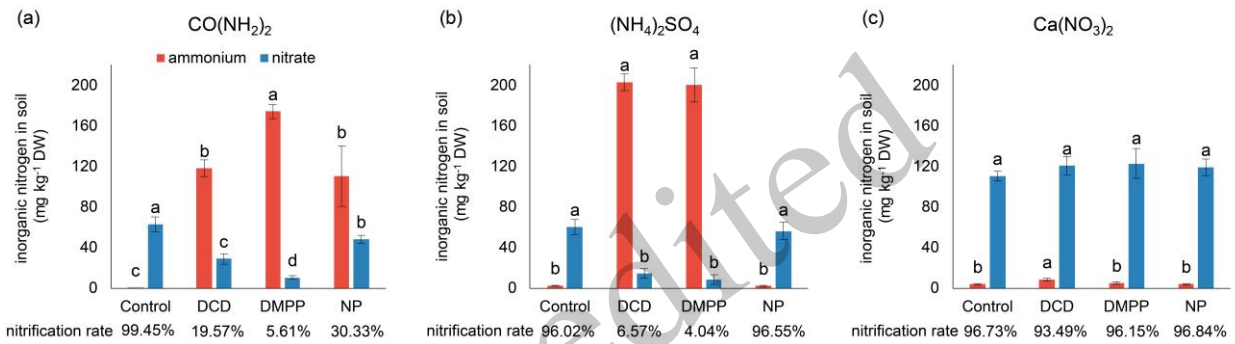

Fig. 2. Effects of the nitrification inhibitors on the concentrations of NH_4^+-N and NO_3^--N in the soil. $CO(NH_2)_2$, $(NH_4)_2SO_4$, and $Ca(NO_3)_2$ were used as the N fertilizers. Nitrification inhibitors were not applied to the control treatment. Data are expressed as the mean ± standard deviation (SD) (n = 5). Different letters represent significant differences (Duncan's test; $P<0.05$).

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DCD and DMPP significantly elevated the soil pH in the urea and ammonium fertilizer treatments (Fig. 3 and Table S1), with DMPP showing greater effectiveness. As expected, the three nitrification inhibitors had little effect on the pH of soil fertilized with nitrate. DCD and DMPP (but not nitrapyrin) decreased DTPA-Cd in soil fertilized with urea or ammonium compared to that of the control. The greatest decrease was observed with DMPP and urea at approximately 46 % compared to that in the control. However, the nitrification inhibitors did not affect DTPA-Cd in the soil fertilized with nitrate. These results suggest that DCD and DMPP can efficiently mitigate acidification and Cd availability in soil fertilized with urea or ammonium; DMPP had a greater effect on soil fertilized with urea.

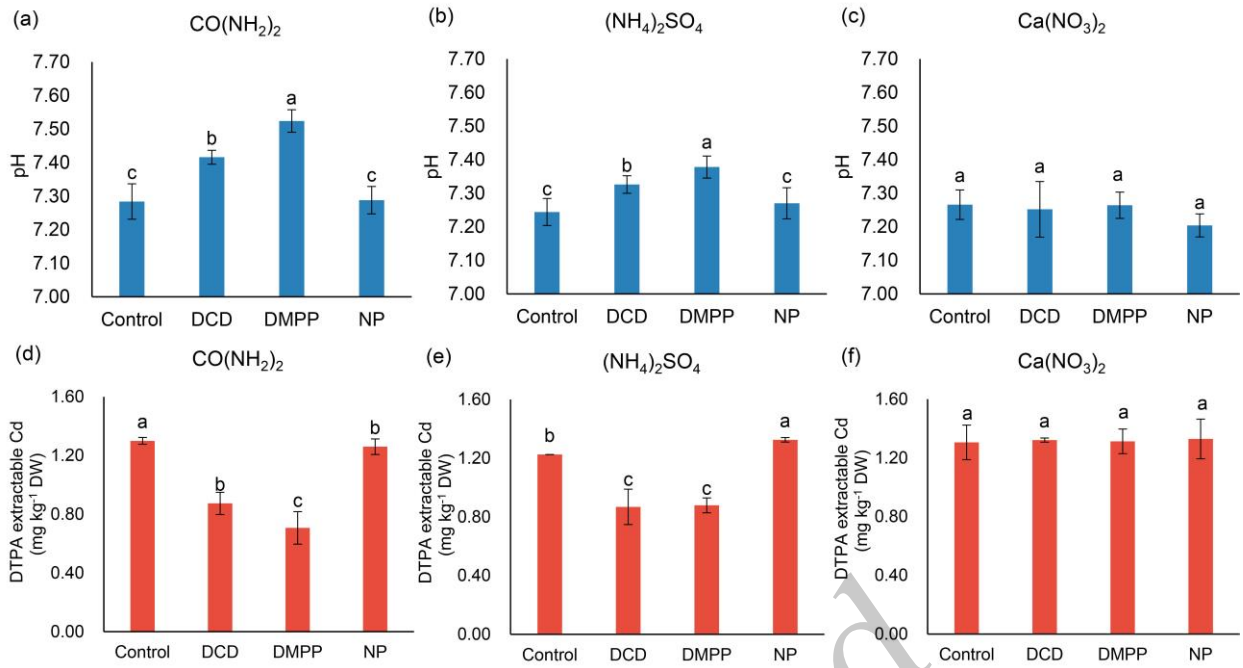

Fig. 3. Effects of the nitrification inhibitors on the pH and concentrations of available Cd in the soil. $CO(NH_2)_2$, $(NH_4)_2SO_4$, and $Ca(NO_3)_2$ were used as the N fertilizers. Nitrification inhibitors were not applied to the control treatment. Data are expressed as the mean ± standard deviation (SD) (n = 5). Different letters represent significant differences (Duncan's test; $P<0.05$).

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3.3 Effects of different nitrification inhibitors on the soil microbial structure

We first analyzed the effects of different nitrification inhibitors on the microbial structure in Cd-contaminated soils at the phylum level. The dominant phyla (average relative abundance greater than 1 %) in all treatments accounted for >95 % of the total abundance (Fig. 4). DCD and DMPP (but not nitrapyrin) significantly decreased the abundance of *Actinobacteriota* in soil fertilized with urea compared to that of the control. All three nitrification inhibitors decreased the abundance of *Patescibacteria* in soils fertilized with urea or ammonium, with the greatest decrease observed for DMPP in ammonium-fertilized soil. The abundance of *Proteobacteria*, the most abundant phylum (32.34 %), varied significantly between the different nitrification inhibitors, with DMPP presenting the lowest abundance in soils fertilized with urea or ammonium.

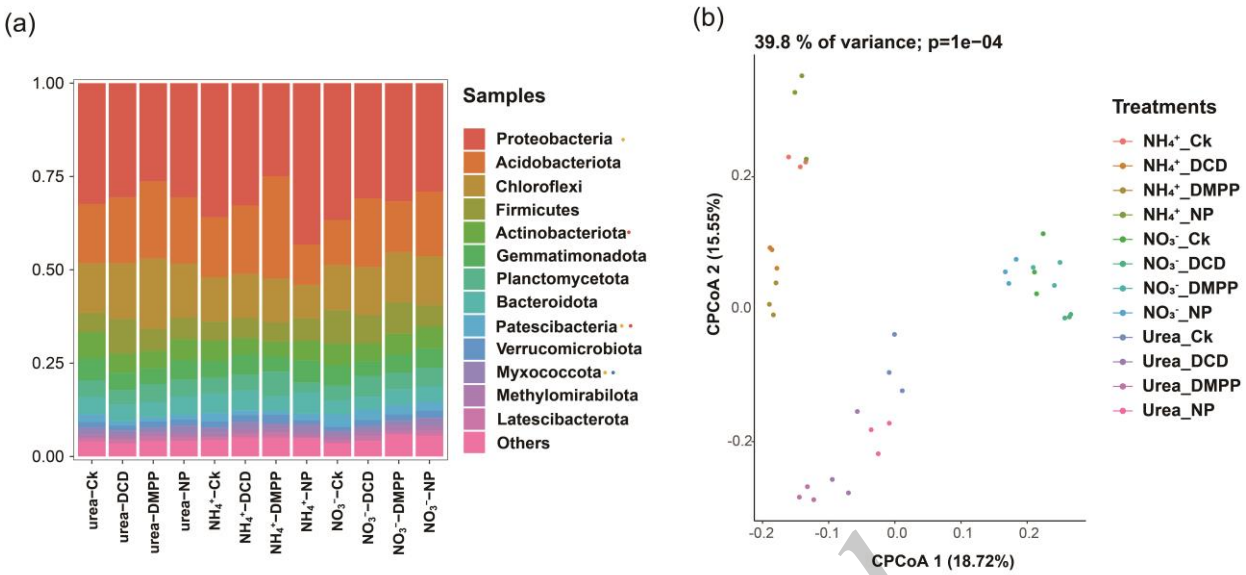

Fig. 4. (a) Phylum-level relative abundance in soil treated with different nitrification inhibitors and different fertilizers. Abundance represents the average (n = 3). Red, yellow, and blue dots represent significant differences (one-way ANOVA; Duncan's test; $P<0.05$) in soil fertilized with urea, ammonium, or nitrate, respectively. (b) A constrained principal coordinates analysis (CPCoA) plot was used to characterize the differences in soil β-diversity between different treatments (39.8 % of variance; $P = 0.0001$, n = 36; based on the Bray–Curtis distance).

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CPCoA based on Bray–Curtis distance matrices was performed to visualize the differences in soil microbial structures. The results revealed that approximately 39.8 % of the overall variance in soil microbial structure was attributed to the nitrification inhibitor type and nitrogen fertilizer form (Fig. 4). CPCoA2 explained 15.55 % of the variation in the microbial structure, with the nitrification inhibitor type representing a significant source of variation. Variance in microbial structures among the different nitrification inhibitors was observed in soils fertilized with urea or ammonium, but not with nitrate. This finding was further verified by PERMANOVA (Table S2). These results suggest that the effect of nitrification inhibitors on soil microbial structure is related to the type of nitrification inhibitor and the form of nitrogen fertilizer. Considering the above results and the effect of the nitrification inhibitors on the nitrification rate, a higher inhibitory effect on nitrification could lead to greater variation in microbial structure.

3.4 Significant microbial taxa and functional groups in the different nitrification inhibitor treatments

Since nitrification and microbial structure in the soil were significantly affected by nitrification inhibitors, we performed an LEfSe analysis to identify microbial taxa with significant differences in relative abundance between each nitrification inhibitor-treated soil sample and their corresponding controls (Fig. 5 and Fig. S3). A total of 135 microbial taxa with significant differences in relative abundance (70 % of the total relative abundance) were identified, and more taxa were significantly depleted in the nitrification inhibitor-treated soil than were significantly enriched. Additionally, soils with greater nitrification inhibition (urea-DCD, urea-DMPP, NH_4^+-DCD, and NH_4^+-DMPP) had more significant microbial taxa. Urea-DMPP and NH_4^+-DMPP led to the most significant microbial taxa (n = 59 and 57, respectively). Furthermore, significantly depleted taxa in the nitrification inhibitor-treated soil exhibited a greater overlap across different pairwise comparisons, particularly in the nitrification inhibitor-treated soil with significantly suppressed nitrification. In contrast, enriched taxa in the nitrification inhibitor-treated soil showed less overlap across the pairwise comparisons.

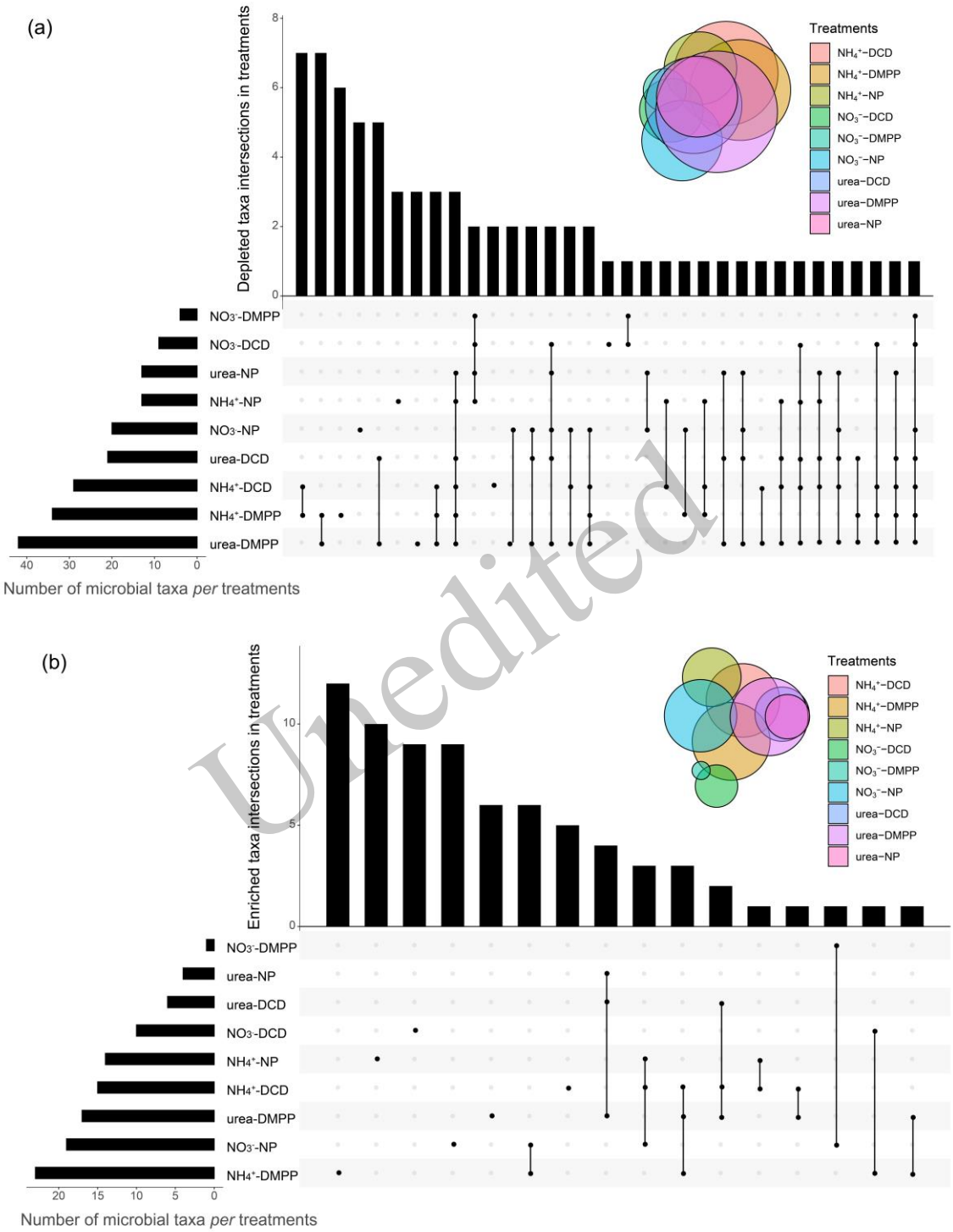

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Fig. 5. Upset plots visualizing the significantly depleted (a) and enriched (b) microbial taxa in different LEfSe pairwise comparisons of nitrification inhibitor-treated soil samples and their corresponding controls. The black bars in the left panel for each nitrification inhibitor treatment depict the number of taxa. Dark dots in the matrix indicate sets of treatments that intersect. The black bars in the upper panel describe the number of taxa at the

corresponding intersection.

The Pearson correlation analysis identified microbial taxa with significant differences in relative abundance whose relative abundance was significantly correlated with at least one of the soil properties: pH, DTPA-Cd, NH_4^+-N, NO_3^--N, pakchoi yield, and Cd concentration. Among the 99 screened microbial taxa, 57 were significantly correlated with Cd concentration in pakchoi (Fig. 6), suggesting that these microbial taxa may affect Cd uptake by pakchoi. At the phylum level, *Acidobacteriota* (17 % of the total relative abundance, including *Blastocatellia* 3 %) was significantly enriched in urea-DMPP and NH_4^+-DMPP soils compared to in their respective controls. Additionally, *Chloroflexi* (13 %, including *Anaerolineae* 7 % and *Chloroflexia* 3 %) was significantly enriched in the urea-DMPP soil. The application of nitrogen inhibitors (DCD, DMPP, and nitrapyrin) to soils fertilized with urea or ammonium resulted in the depletion of *Patescibacteria* (2 % relative abundance). Moreover, *Proteobacteria* (32 %) was significantly depleted in the urea-DMPP, NH_4^+-DCD, and NH_4^+-DMPP soils, and *Gammaproteobacteria* (17 %) was significantly depleted in the NH_4^+-DCD and NH_4^+-DMPP soils. *Proteobacteria* and *Chloroflexi* had a strong linear correlation with the Cd concentration in pakchoi (|rho|>0.5). *Acidobacteriota* and *Patescibacteria* showed significant correlations with soil pH, soil DTPA-Cd, soil NH_4^+-N, NO_3^--N, pakchoi yield, and Cd concentration.

At the genus level, *Devosia* and *Pedobacter* were significantly depleted in the NH_4^+-DCD and NH_4^+-DMPP soils compared with their corresponding controls. In contrast, *Lysobacter* was significantly depleted in all nitrification inhibitor treatments in soils with urea fertilization, in the DCD treatment in soils with ammonium fertilization, and in the nitrapyrin treatment in soils fertilized with nitrate. *Herpetosiphon* was significantly enriched in all nitrogen inhibitor treatments in soil fertilized with urea. *Pedobacter* and *Herpetosiphon* showed a strong linear correlation with the Cd concentration in pakchoi (|rho|>0.5). *Devosia* and *Lysobacter* were significantly correlated with soil pH, soil DTPA-Cd, soil NH_4^+-N, NO_3^--N, pakchoi yield, and Cd concentration.

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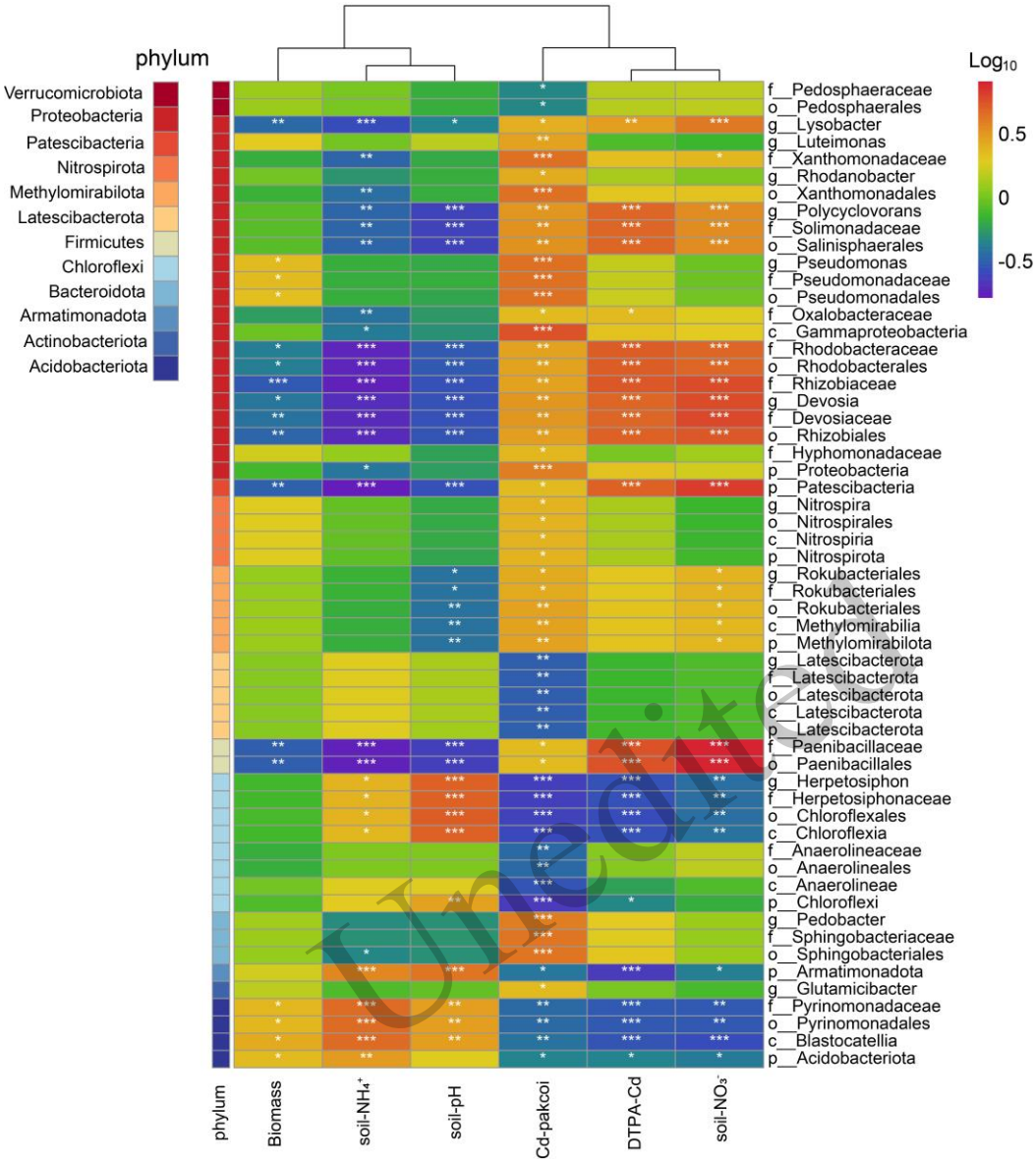

Fig. 6. Pearson correlation heatmap of significant microbial taxa abundance and soil properties, pakchoi yield, and pakchoi Cd concentration. The color scale indicates the strength and direction of the correlation, with warmer colors indicating a positive correlation and cooler colors indicating a negative correlation. The p-values for each correlation are indicated by asterisks ("*" $P<0.05$, "**" $P<0.01$, "***" $P<0.001$). Strong correlations had absolute correlation coefficient values greater than 0.5.

FAPROTAX was used to assess the functional diversity of the microbial communities under different treatments (Fig. 7). Of the 1498 analyzed OTUs, 305 (20.4 %) were assigned to 41 functional groups. PERMANOVA revealed that different nitrification inhibitors, nitrogen fertilization, and their interactions significantly influenced the functional microbial groups, reporting 61.95 % of the variation (Table S3). Functional groups with significant differences under different nitrification inhibitors were screened in soil fertilized with urea, ammonium, or nitrate. The proportion of microorganisms involved in photoautotrophy and chitinolysis after DMPP or DCD application was significantly lower than that in the control soil fertilized with urea. In soil with ammonium fertilization, low-abundance OTUs with cellulolysis function were detected in the DCD, DMPP, and nitrapyrin treatments compared to the control soil with ammonium fertilization. Meanwhile, DCD and DMPP decreased the abundance of OTUs associated with ureolysis and methylotrophy, and increased

dark hydrogen oxidation. The highest abundance of OTUs with aerobic ammonia oxidation and nitrification was detected in the DMPP treatment. In contrast, OTUs involved in chemoheterotrophy were not as abundant in the control, DCD, and nitrapyrin treatments. Additionally, DMPP significantly decreased the number of microorganisms, such as human pathogens, animal parasites, and symbionts. Nitrapyrin decreased chemoheterotrophy, phototrophy, and chitinolysis; however, it increased predatory or exoparasitic microorganisms in nitrate-fertilized soil.

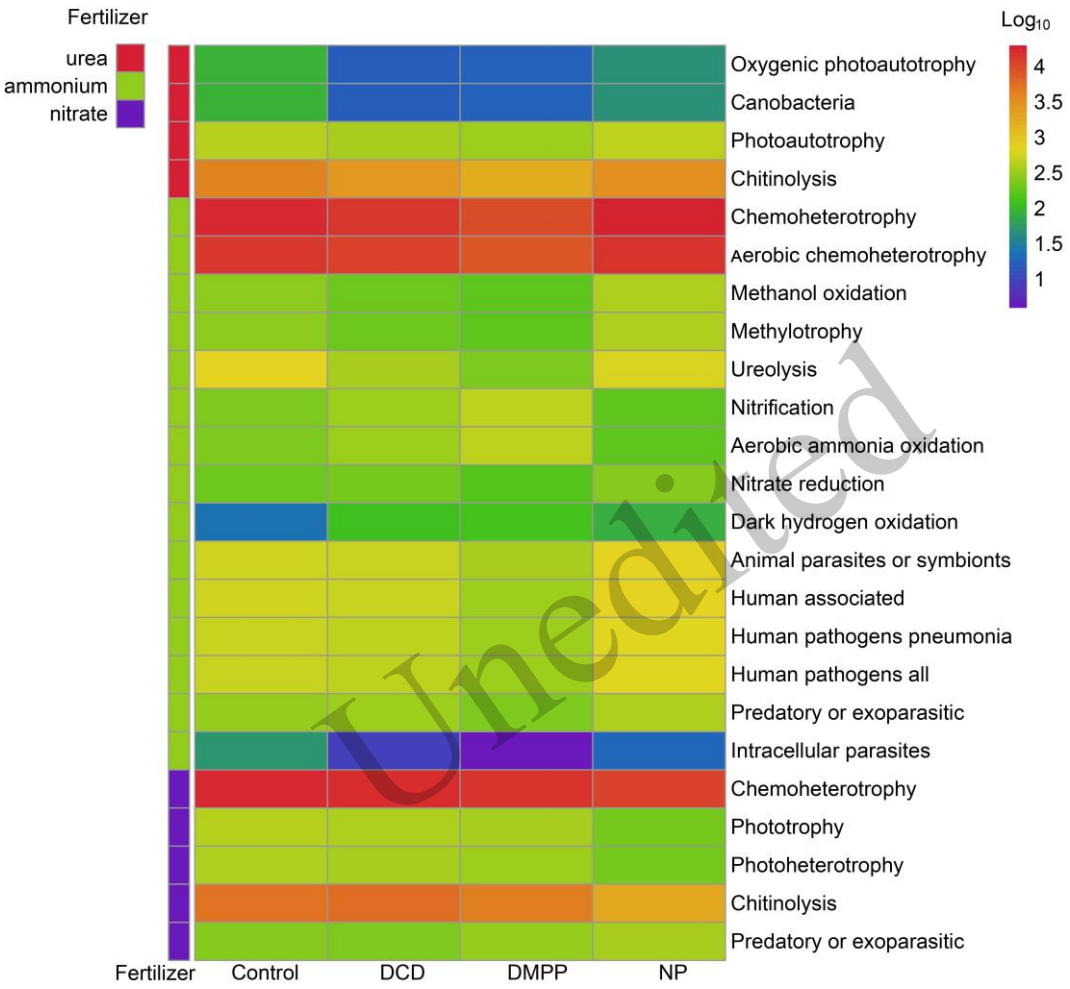

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Fig. 7. FAPROTAX heatmap showing significant functional microbial groups (one-way ANOVA; Duncan's test; $P<0.05$, n = 3) and their OTU number under different nitrification inhibitor treatments.

The LEfSe and FAPROTAX analyses showed that the abundance of ammonia-oxidizing microorganisms increased with the DMPP treatment in soil with ammonia fertilization. To further understand the variations in abundance, we summarized the genus-level relative abundances of ammonia-oxidizing archaea (AOA), ammonia-oxidizing bacteria (AOB), and nitrite-oxidizing bacteria (NOB) in the treatments that had significant inhibitory effects on nitrification (Fig. 8). The abundance of *Nitrosomonas* was significantly lower in the DCD and DMPP treatments than in the control soil with urea and ammonia fertilization. However, the DMPP treatment decreased the abundance of *Nitrosospira* in urea- and ammonia-fertilized soil. In contrast, *Nitrosococcus* was more abundant in the DCD and DMPP treatments than in the control soil fertilized with ammonia. In soil fertilized with ammonia, DMPP application significantly increased the abundance of *Nitrososphaeraceae*, *Candidatus_Nitrosotenuis*, and *Candidatus_Nitrososphaera*; *Nitrososphaeraceae*

increased in soil with DCD application. In addition, the relative abundance of *Nitrospira* (NOB) was significantly decreased in the ammonia-fertilized soil and DMPP compared to that of the control, consistent with the LEfSe analysis. Furthermore, the Pearson correlation analysis showed that the abundance of *Nitrospira* was significantly and positively correlated with the Cd concentration in pakchoi.

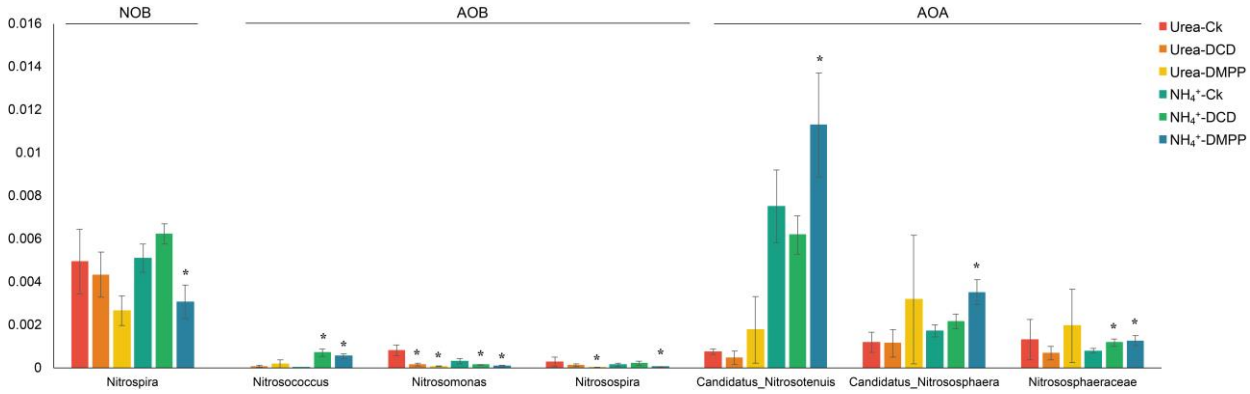

Fig. 8. Relative abundance of AOA, AOB, and NOB in the different treatments. Data are expressed as the mean ± standard deviation (SD) (n = 3). "*" Significant difference compared to the control (one-way ANOVA; Duncan's test; $P<0.05$).

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3.5 Factors contributing to the decrease of Cd in edible parts by the application of nitrification inhibitors

The question then arises of how nitrification inhibitors lower Cd concentration in the edible parts of pakchoi. SEM was used to analyze the relationship among nitrification rate, pH, DTPA-Cd, soil microbial diversity, and Cd concentration in the edible parts of pakchoi (Fig. 9). The results showed that both pH and microbial diversity were negatively correlated with the nitrification rate. In contrast, soil pH was negatively correlated with soil DTPA-Cd. Therefore, the decrease in available Cd due to DMPP or DCD application in the urea or ammonium treatments should be attributed to an increase in pH resulting from the inhibition of nitrification. As expected, the Cd concentration in the edible parts of pakchoi was positively correlated with that of DTPA-Cd. However, a negative correlation was observed between the β-diversity and Cd concentration of pakchoi. Consequently, the decrease in available Cd and the increase in microbial diversity because of the inhibited nitrification should contribute to the decreased Cd accumulation in the edible parts of pakchoi in the urea or ammonium treatments with DMPP or DCD application.

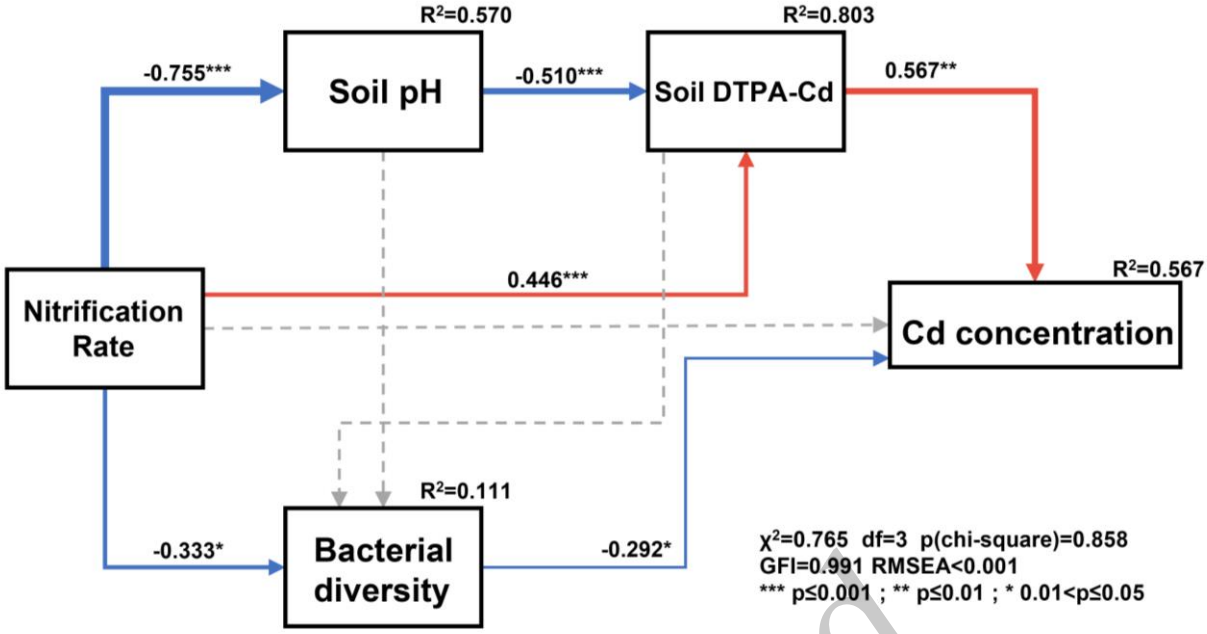

Fig. 9. SEM illustration of the direct or indirect effects of various factors, including the inhibitory effect of nitrification inhibitors (larger nitrification rates indicate a worse inhibitory effect), soil properties (soil pH and DTPA-Cd), microbial diversity, and Cd concentration of pakchoi. The red line represents a significant positive correlation, and the blue represents a significant negative correlation. A thicker line indicates a stronger correlation between factors. The dotted lines indicate no significant correlation.

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4 Discussion

4.1 DMPP is a highly efficient nitrification inhibitor for mitigating Cd accumulation in pakchoi

DCD, DMPP, and nitrapyrin are three commercial nitrification inhibitors that are commonly used in agricultural production. In this study, we observed that DCD and DMPP (but not nitrapyrin) could efficiently lower the Cd concentration in the edible parts when urea or ammonium was used as the N fertilizer. This was particularly true when applying DMPP with urea fertilization, which decreased the Cd concentration in the edible parts of pakchoi much more than the application of DCD did. Since urea is widely used as an N fertilizer in agricultural production (Singh et al., 2023; Witte, 2011), DMPP can be considered the most efficient nitrification inhibitor for mitigating Cd accumulation in pakchoi. Considering that DCD has a high nitrogen content (66.7 %) and requires a high input amount to realize an efficient inhibitory effect on nitrification, the nature of the decomposition of DCD to ammonia in the soil after its application may intensify soil acidification caused by nitrification (Vilsmeier, 1980), which is not favorable for the mitigation of Cd contamination in crops. Nitrapyrin had little effect on the mitigation of Cd concentration in the edible parts (Fig. 1 and Table S1), which may have been due to the unstable nature of nitrapyrin in soils (Papadopoulou et al., 2020; Lewis et al., 2016).

Nitrification inhibitors may negatively affect plant growth, thereby inhibiting Cd absorption by plant roots (Kelliher et al., 2008; Macadam et al., 2003). However, this should not be the case in this study because the three tested nitrification inhibitors had little effect on Cd concentration in the edible parts of pakchoi, provided nitrate was the N source. SEM (Fig. 9) revealed that the differences in pakchoi Cd concentration between treatments were mainly related to the reduced availability of Cd in the soil due to the inhibition of nitrification. This was expected because nitrification results in soil acidification, increasing Cd availability. A decrease in available soil Cd by increasing soil pH decreases Cd accumulation in crops. For example, Cd concentrations decreased in lettuce, cabbages (Jackson and Alloway, 1991), and rice (Zhu et al., 2016) as the soil pH increased. The SEM

results suggested an important role for soil microbial diversity in mitigating Cd concentrations, as discussed below.

Additionally, the nitrification inhibitory effect of nitrification inhibitors on soil was observed to be correlated with soil pH. As soil pH increases, the dominance of AOB with lower ammonia affinity increases relative to that of AOA with nitrification (Jung et al., 2022; Amoo and Babalola, 2017; Kits et al., 2017). These AOBs are the main targets of nitrification inhibitors (Papadopoulou et al., 2020; Shi et al., 2016). In addition, nitrification inhibitors exhibited more robust inhibitory effects on nitrification in alkaline soils compared to that in acidic or neutral soils (Cui et al., 2021). The above findings suggest that applying nitrification inhibition may be more effective in inhibiting soil acidification and decreasing Cd solubility in alkaline soils.

4.2 Possible mechanisms of nitrification inhibitor-altered soil microbial community effects on decreasing Cd content in pakchoi

Soil microorganisms play an important role in the biogeochemical cycles of soil ecosystems (Leibold and McPeek, 2006). The application of nitrification inhibitors resulted in significant changes in the soil microbial structure. This phenomenon was particularly evident in urea- and ammonium-fertilized soils treated with DMPP. Meanwhile, the SEM results showed that nitrification inhibitors suppressed the acidification-induced availability of Cd in soil by inhibiting nitrification and reshaping microbial communities to lower the Cd concentration in pakchoi. Specific microbial groups may be associated with the mitigating effects of nitrification inhibitors on Cd accumulation in pakchoi; alterations in nitrifying microorganisms may be the key factors. Among the three tested nitrification inhibitors, DMPP had the greatest inhibitory effect on the abundance of *Nitrosomonas* and *Nitrosospira* (Fig. 8), which are known to have higher maximum rates of ammonia oxidation than AOA (Jung et al., 2022; Kits et al., 2017; Martens-Habbena et al., 2009). This effect may be the main reason for the inhibition of nitrification in the tested soils. Additionally, although AOAs are more abundant than AOBs, they do not dominate the nitrification process (Schauss et al., 2009). Given that the ammonia oxidation process is the main step of nitrification that produces H^+, a decrease in the ammonia oxidation rate may decrease the available Cd in the soil. Additionally, DMPP has a slight inhibitory effect on NOB (Papadopoulou et al., 2020), which may be coupled with the inhibition of the upstream limiting step (ammonia oxidation), thus leading to a decrease in *Nitrospira* abundance in the NH_4^+-DMPP treatment. This decrease may further lower nitrite oxidation to decrease NO_3^--N production, which could decrease Cd^{2+} uptake by root cells. This is because the decrease in root cell uptake of NO_3^- lowers Cd^{2+} uptake by inducing the Fe^{2+} transporter *IRT1* and the requirement for anion-cation balance (Guan et al., 2015; Mao et al., 2014; Luo et al., 2012).

We focused on *Herpetosiphon* and *Lysobacter* in the LEfSe analysis because of their significant correlation with Cd concentration in pakchoi and their high abundance (>1 %) in soils (Fig. 6 and Fig. S2). Both are predatory bacteria that may enhance the effects of nitrification inhibitors on the soil microbial structure (Pérez et al., 2016; Pasternak et al., 2013) and ultimately affect plants' uptake of Cd by preying on other bacteria (Yang et al., 2020). In addition, due to changes in the soil chemistry and biological environment, significant changes occurred in the abundance of many microorganisms that have been reported to promote plant growth or decrease soil Cd availability. For example, *Pedosphaeraceae*, *Anaerolineaceae*, *Gemmata*, and *Terrimonas* increased with DMPP or DCD in the urea and NH_4^+ treatments. *Terrimonas* and *Pedosphaeraceae* exhibit Cd tolerance and plant growth-promoting capabilities (Yuan et al., 2022; She et al., 2021). Similarly, *Gemmata* abundance negatively correlates with heavy metal concentrations in plants, and a few of its species have heavy metal resistance genes (Singh et al., 2020; Aghnatios and Drancourt, 2016). *Anaerolineaceae* lowers Cd solubility by coexisting with methane-metabolizing microorganisms (Meng et al., 2019). In contrast, a few microorganisms decreased in the DMPP or DCD treatments. For example, *Devosia* had a high abundance (0.6 %) and was significantly positively correlated with the Cd concentration in pakchoi. Previous research has shown a significant positive correlation between *Devosia*, soil heavy metal availability, and plant Cd concentration, suggesting that it may play a role in shaping the soil microbial community (Wang et al., 2021;

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Chen et al., 2018).

Certain microbial groups may indirectly decrease soil Cd availability by influencing the soil environment. For example, in the NH_4^+-DMPP and NH_4^+-DCD treatments, the number of functional groups involved in dark hydrogen oxidation increased significantly (Fig. 7). Hydrogen-oxidizing microorganisms (HOM) play an important role in the soil–carbon cycle (Piché-Choquette and Constant, 2019) and CO_2 fixation, which may contribute to the formation of complexes between Cd and organic matter (Stein et al., 2005; Dong and Layzell, 2001). Moreover, HOM is believed to enhance the soil nutrient status and serve as a plant growth-promoting rhizobacteria (PGPR) (Dong et al., 2003), which may decrease the Cd content in pakchoi because of the dilution effect resulting from growth promotion. However, the effect of nitrification inhibitors on the microbial community structure is poorly understood, and the function of microorganisms involved in Cd accumulation remains unclear. Future studies on microbial functions via the isolation of individual bacterial strains and metagenomics may provide a better understanding of how nitrification inhibitors reshape the microbial community structure and subsequently influence crops' uptake of Cd.

5 Conclusion

DCD and DMPP (but not nitrapyrin) efficiently mitigated Cd concentration in pakchoi grown in soil fertilized with urea or ammonium. DMPP showed a greater effect than DCD. The difference in the rate of decrease in the pakchoi Cd concentrations between different nitrification inhibitor treatments was related to the inhibition of the acidification-induced availability of Cd in the soil. The changes in the soil microbial community caused by nitrification inhibitors played an important role in mitigating the Cd concentration in pakchoi. Many microorganisms are regulated by changes in soil chemistry and the biological environment and directly or indirectly participate in the mitigation of Cd accumulation in pakchoi in diverse ways.

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Data availability statement

All data generated or analyzed during this study are included in this published article. For further detailed information, please refer to us.

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Author contributions

Wenxin DU: Investigation, Formal analysis, Visualization, Writing-original draft. Qingyang ZHU: Methodology, Writing-review & editing. Xiangting JING: Methodology. Weijie HU: Methodology. Yao ZHUANG: Methodology. Yijie JIANG: Data Curation. Chongwei JIN: Conceptualization, Writing - original draft.

Compliance with ethics guidelines

Wenxin DU, Qingyang ZHU, Xiangting JING, Weijie HU, Yao ZHUANG, Yijie JIANG and Chongwei JIN declare that they have noconflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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Supplementary information:

Tables S1-S3; Figs. S1-S3