COMPARISON OF DIFFERENT AGRONOMIC ACTIVITIES ON PHYSICOCHEMICAL PROPERTIES AND N-CYCLING GENE ABUNDANCES IN FARMLAND SOIL NEAR COPPER TAILINGS AREA

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(Received 15th Nov 2024; accepted 5th Feb 2025)

Abstract. To evaluate the impact of different agronomic activities on soil physicochemical properties and N-cycling gene abundances in farmland soil near copper tailings area. The pot experiments were performed from September 11 to December 5 in 2023, soil physiochemical properties, abundance of *nif*H, AOA-*amo*A, AOB-*amo*A, *nir*S, *nir*K and *nos*Z, and variation of N-cycling bacterial population were investigated. Results showed that combined application of Chinese milk vetch and N fertilizer significantly decreased soil pH while increasing the levels of total nitrogen, organic matter, available nitrogen, nitrate nitrogen, urease activity, alkaline proteinase activity, and abundances of *nifH*, AOA-*amo*A, and AOB-*amo*A. Results of multiple regression analysis suggested that the abundance of *nif*H, AOA-*amo*A, AOB-*amo*A, *nir*S and *nos*Z negatively correlated with pH ($p < 0.05$), and positively correlated with available N, nitrate nitrogen and alkaline proteinase activity ($p < 0.01$), and the abundance of *nos*Z correlated positively with available nitrogen and nitrate nitrogen. The main driving factors for N fixation, nitrification and denitrification, were *Hyphomicrobium*, *Nitrospira* and *Nitrosospira*, respectively. Combined agronomic practices had a better role in improving the cadmium-containing soil properties than the unitary Chinese milk vetch planting or N fertilizer alone. Therefore, pH, available nitrogen and nitrate nitrogen were crucial environmental factors for the abundances of nitrogen cycle genes.
Keywords: *Chinese milk vetch, nitrogen addition, soil properties, nitrogen cycle, gene abundance*

Introduction

Many studies have focused on the negative impacts of environmental pollutants on plant growth (Riyazuddin et al., 2021; Seneviratne et al., 2019; Sperdouli, 2022) and human health (Munene et al., 2023; Oyebamiji et al., 2024). It was previously reported that heavy metal pollution partly resulted from mining (Wan et al., 2024; Xie et al., 2024), moreover, the mining industry has altered soil enzyme activities, microbial functional diversity and nitrogen cycle (Aponte et al., 2021; Li et al., 2024).

Nitrogen (N) is an essential element for life (LeBauer and Treseder, 2008), and a vital component of biomacromolecules (Einsle, 2023). N deficiency in soil frequently inhibits the growth of microorganisms and plants and restricts the restoration of the ecosystem. The application of N fertilizer improves soil health mainly by altering soil organic matter (SOM) content, microbial life, and acidity (Bijay-Singh, 2018). At present, a severe eco-environmental problem has resulted from the excessive N addition, low utilization efficiency, and N leaching during agricultural practice. Farmland soil has been the primary sources of N_2O due to nitrogen application (Yang et al., 2023). The utiliztion of N by soil microorganisms is influenced by agricultural management practices. Some N-fixing genes such as *amo*A, *amo*B, *amo*C, *nir*S, *nos*Z and *nir*K all are invovled in N transformation, and have been regarded as crucial indicators for ecosystem function (Liu et al., 2023).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2303_43514367
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Leguminous plant has been applied in bioremediation of cadmium (Cd) contaminated soil (Gómez-Sagasti and Marino, 2015; Ike et al., 2007). Chinese milk vetch (*Astragalus sinicus* L.), a traditional leguminous green manure, is used to keep paddy soil fertile (Zhu et al., 2012), which can increase soil N content, thereby reducing agricultural costs (Chen et al., 2020; Ma et al., 2020). Limited research elucidated the impacts of Chinese milk vetch combined with nitrogen fertilizer on N utilization, abundances of nitrogen cycle genes in mining area soils.

Therefore, this paper focused on copper mining area soils to investigate the effects of different treatments on soil properties, nitrogen cycling bacteria and gene abundance, and to clarify the correlation between soil environmental factors and soil nitrogen cycling bacterial communities under different treatments, Our data may contribute to clarifying the change rule of soil nitrogen cycling in mining area, and provide theoretical support for the safe utilization of cultivated land.

Material and methods

Collection of soil samples

Soil samples were collected from a depth of 0–20 cm in the farmland in Tianmen Town, Tongling, Anhui, China (30°90'N 117°88'E). The samples were air dried and then passed through a 40-mesh screen. The resulting soil was used for test.

Experimental design

All treatments were divided into four groups: the untreated soil was used as a control group (CK), urea fertilizer (N group), Chinese milk vetch (CMV group), and Chinese milk vetch growth plus urea fertilizer (CMVN group). Each group has 3 parallel replicates. The pot size was as follows: 100 cm (length) × 33 cm (wide) × 23 cm (height). Then, Filling each pot with 25 kilograms of soil classified as Leptic Luvisol based on Harmoized world Soil Database (https://www.fao.org/home/en, GAEZ v4 Data Portal). The initial soil pH value was 7.91; The soil organic matter (SOM), total N, P, K and Cd were 21.26, 1.16, 0.50, 13.44 and 1.34 mg/kg, respectively, and the initial available content of N, P, K and Cd were 94.90, l6.76, 296.88 and 0.41 mg/kg, respectively. In addition, urea (CNSG Anhui Hong Sifang Co., China) was mixed well with the soil in CMVN before transplanting seedlings, and the final N concentration was 225 kg/hm^2.

The milk vetch seeds (Qingyijiang seed company, Wuhu, China) were disinfected in 1% NaClO solution for 10 min, and the rinsed at least 3 times by using deionized water. After germinating for 72 h, 30 seedlings were subsequently transferred to each pot sheltered from rain. The management and maintenance of all plants, including regular watering and weeding, was consistently carried out. The irrigation schedule involved watering twice a week with 500 mL of distilled water each time.

Sample collection

three soil samples underlying 0–2.5 cm (diameter 5 cm, 200 g) upper surface were randomly collected from each pot on December 5 for CK and N groups, on the other hand, Furthermore, with respect to CMV and CMVN groups, following the removal of soil tightly adhered to the root surface, three rhizosphere soil samples were collected from each pot, three soil samples from the same pot subsequently were combined to

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form a single composite sample for analysis. Finally, all composite samples were stored at –70°C.

Soil physiochemical property test

Dried soil samples were homogenized and the passed a 40-mesh screen. Soil pH was obtained in 1:2.5 (W/V) soil-water mixture by pH meter (PHS-3C, Shanghai Youke Instrument Factory, Shanghai, China). The contents of total nitrogen (TN), soil organic matter (SOM), available nitrogen (AN), NH_4^+-N and NO_3^--N in soil were determined by Kjeldahl determination, oxidation with potassium dichromate, alkaline diffusion method, Nessler's reagent colorimetric, Griess-Ilosvay method, respectively. Total soil Cd concentration was determined with the inductively coupled plasma mass spectrometry (ICP-MS NexION 5000, PerkinElmer Management (Shanghai) Co., Ltd., Shanghai, China), available Cd level was measured according to the method of Guan et al. (2018). The activities of alkaline protease (ALP) and urease were determined by soil alkaline protease (S-ALP) activity detection kits and phenol sodium hypochlorite colorimetric method, respectively.

DNA extraction

The total soil DNA was extracted by using the protcol of TIANamp soil DNA DP336 (Tiangen Biochemical Technology Co., Ltd., Beijing, China). Then extracted DNA was detected for quality and concentration by NanoDrop (SIM-100, Hangzhou Simgen Biotechnology Co., Ltd., Hangzhou, China) and 2% agarose gel electrophoresis.

Gene amplification and quantification

Gene abundance was quantified on an LightCycler 96 (Roche Inc., Switzerland). The reaction system was 25 μL containing 12.5 μL 2×Phanta Max Master Mix (Vazyme Biotech Co., Ltd., Nanjing, China), 0.25 μL each of forward and reverse primers (25 μmol/L), X μL of DNA solution corresponding to 12.5 ng of total DNA, (12-X) μL PCR grade water, and the primer sequences and PCR conditions for different genes were listed in the *Table 1*.

Purified PCR products were integrated into the pMD18-T vector (Takara, Dalian, China), then recombined plasmid constructs were individually transformed into the competent *E. coli* TOP 10 cells using standard protocols. Positive clones were confirmed by gene specific PCR amplification, Plasmid Miniprep Kit (Biomiga, Santiago, USA) was used for plasmid extraction. The plasmid DNA was detected by agarose gel electrophoresis and purified to determine the plasmid concentration, which was converted into plasmid copy number. The standard curve was amplified with the plasmid DNA diluted 10 times in sterile deionized water as a template, and the amplification curve and dissolution curve were drawn and generated. Absolute quantitative methods were utilized to quantify gene abundance, a reaction volume of 20 μl including primer concentration of 200 nmol/L, and DNA template dosage of 1 μL.

The standard curve R^2 value exceeded 0.99, the slope value falled within the range of –3.2 to –3.8, and the estimated amplification efficiency was 95%~105.35%. All standards, samples, and non-template controls underwent duplicate run with the sample being diluted by a factor of 10 prior to addition to the master mix. Gene copy calculations in soil were based on dry weight (105°C).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Table 1. *Sequences of primers and thermal profiles for gene quantification*

Gene name	Primer	Sequence	Product length (bp)	PCR profiles	Reference
*nif*H	*nif*H-F	AAAGGYGGWATCGGYAARTCCACCAC	458	95℃ for 1 min followed by 40 cycles of 95℃ for 10 s and 55℃ for 60 s	Rosch et al. (2002)
	*nif*H-R	TTGTTSGCSGCRTACATSGCCATCAT			
AOA-amoA	*amo*AF	STAATGGTCTGGCTTAGACG	635	95℃ for 1 min followed by 40 cycles of 95℃ for 10 s and 61℃ for 60 s	Francis et al. (2005)
	amoAR	GCGGCCATCCATCTGTATGT			
AOB-amoA	*amo*A-F	GGGGTTTCTACTGGTGGT	491	95℃ for 1 min followed by 40 cycles of 95℃ for 10 s and 60℃ for 60 s	Rotthauwe et al. (1997)
	*amo*A-R	CCCCTCKGSAAAGCCTTCTTC			
nirS	cd3AF	GTSAACGTSAAGGARACSGG	425	95℃ for 1 min followed by 40 cycles of 95℃ for 10 s and 58℃ for 60 s	Throback et al. (2004)
	R3cd	GASTTCGGRTGSGTCTTGA			
nirK	*nir*K-F	CGGCGAGCAGGACTTTTAT	222	95℃ for 1 min followed by 40 cycles of 95℃ for 10 s and 60℃ for 60 s	Liu et al. (2022)
	*nir*K-R	GTATCTCTGTTGGCTTGCGAAT			
nosZ	*nos*Z-F	CAACCACGACAAGGTGGAGG	149	95℃ for 1 min followed by 40 cycles of 95℃ for 10 s and 60℃ for 60 s	Liu et al. (2022)
	*nos*Z-R	GGCAGAAGTGCAGGCAGTAAC			

High throughput sequencing

The soil bacteria high-throughput sequencing was performed by Kaitai (Kaitai Biotechnology Co., Hangzhou, China). The V3-V4 region in 16S rRNA gene was amplified using the primer combination of B341F (CCTACGGGGCGWGCAG) and B785R (5GACTACHVGGGTATCTAATCC). The reaction system was 25 μL containing 12.5 μL 2×Phanta Max Master Mix (Vazyme Biotech Co., Ltd., Nanjing, China), 0.25 μL each of forward and reverse primers (25 μmol/L), X μL of DNA solution corresponding to 12.5 ng of total DNA, (12-X) μL PCR grade water, PCR condition was: 95°C for 3 min, 25 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s, and a final step at 72°C for 5 min. A library was constructed based on 16S rDNA sequence for purification, quantification, homogenization of PCR products, and the quality inspection was performed on Qsep100. Qualified libraries were subjected to the Illumina Novaseq 6000 platform with PE250 mode. Then, cutadapt was applied for primer removal, quality filtering, denoising, and chimerism removal on the original sequencing sequence, followed by 100% similarity clustering and bacterial species annotation based on the Silva database.

Data analysis

Each treatment was prepared in triplicated at all experimental times. The data were analyzed statistically with SPSS 19.0 (USA). The difference among treatments were analzyed by using the ANOVA followed by a LSD test. Figures were prepared using the Origin 2023 software (Origin Lab Corporation, USA).

Multiple regression analysis was carried to analyze the correlation among level of N cycle functional genes, soil physicochemical properties, and the density of nitrogen cycling-related bacteria genus. Additionally, Mantel tests were used to determine the associations between bactrerial community structure variation and environmental factors.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2303_43514367
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Results

Variations of physiochemical properties and enzyme activity in soil

Results showed that all of the three treatments had different degrees of soil remediation when compared to the CK group (*Table 2*). In detail, the soil pH in N, CMV and CMVN groups significantly decreased, while OM level in N, CMV and CMVN groups was increased by 20.04%, 14.35% and19.08%, respectively; the TN content of CMV group slightly increased but not significantly, and that of N and CMVN groups was significantly increased; the AN content of N, CMV and CMVN groups was significantly increased by 150.14%, 11.93% and 148.30%, respectively; the nitrate nitrogen content of N, CMV and CMVN groups was significantly increased by 736.64%, 28.48% and 730.03%, respectively; the content of ammonium nitrogen in N and CMVN groups was increased by 153.06% and 15.45%, respectively; and the urease activity in N and CMVN groups was increased by33.33% and 50%, respectively. and the alkaline proteinase activity in N and CMVN groups was increased by 30% and 46.67%. However, the effects of fertilization, CMV and CMVN on total cadmium and available cadmium were not significant. All these results implied that N fertilization had a stronger impact on pH and the levels of TN, available N, NO_3^--N, NH_4^+-N, urease and alkaline proteinase than Chinese milk vetch application.

Table 2. *Analysis of soil physicochemical properties for each treatment*

Soil physicochemical properties	Groups			
	CK	N	CMV	CMVN
pH	8.19 ± 0.02a	7.723 ± 0.02c	8.03 ± 0.01b	7.74 ± 0.02c
Organic matter (g/kg)	19.86 ± 0.18b	23.84 ± 0.64a	22.71 ± 0.22a	23.65 ± 0.51a
Total N (g/kg)	1.08 ± 0.08b	1.435 ± 0.05a	1.21 ± 0.01b	1.40 ± 0.05a
Available N (mg/kg)	81.36 ± 1.70c	203.51 ± 4.40a	91.07 ± 1.56b	202.02 ± 5.21a
NO_3^--N (mg/kg)	9.69 ± 1.29b	81.07 ± 0.85a	12.45 ± 0.53b	80.43 ± 1.55a
NH_4^+-N (mg/kg)	3.43 ± 0.23b	8.68 ± 0.75a	3.23 ± 0.15b	3.96 ± 0.39b
Urease (mg/kg)	0.06 ± 0.00c	0.08 ± 0.00ab	0.07 ± 0.00b	0.09 ± 0.00a
Alkaline proteinase (U/kg)	0.30 ± 0.02c	0.39 ± 0.01b	0.32 ± 0.01c	0.44 ± 0.02a
Total Cd (mg/kg)	1.30 ± 0.04a	1.28 ± 0.06a	1.38 ± 0.23a	1.24 ± 0.17a
Available Cd (mg/kg)	0.42 ± 0.02a	0.41 ± 0.01a	0.45 ± 0.04a	0.43 ± 0.03a

The values represented as means ± SD, different lowercase letters indicate significant differences ($p < 0.05$)

Changes of nitrogen cycle gene relative abundances

The copy numbers of critical genes invovled in soil N cycle were determined by absolute quantitative method. Results showed that the number of *nif*H, AOA-*amo*A, AOB-*amo*A, *nir*S, *nir*K and *nos*Z copies/μg DNA ranged from 2.16×10^8 to 4.45×10^8, 3.49×10^7 to 6.83×10^7, 2.08×10^7 to 5.23×10^8, 3.48×10^7 to 5.21×10^7, 1.36×10^4 to 5.20×10^4, and 1.35×10^4 to 1.23×10^5, respectively. As shown in *Figure 1,* N fertilization enlarged the abundances of AOB-*amo*A and *nir*k, while decreased that of AOA-*amo*A, *nir*S and *nos*Z. CMV treatment improved the abundances of *nif*H, AOA- *amo*A and *nir*S ($p > 0.05$); however, CMVN treatment significantly increased the abundances of *nif*H, AOA-*amo*A and *nos*Z.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2303_43514367
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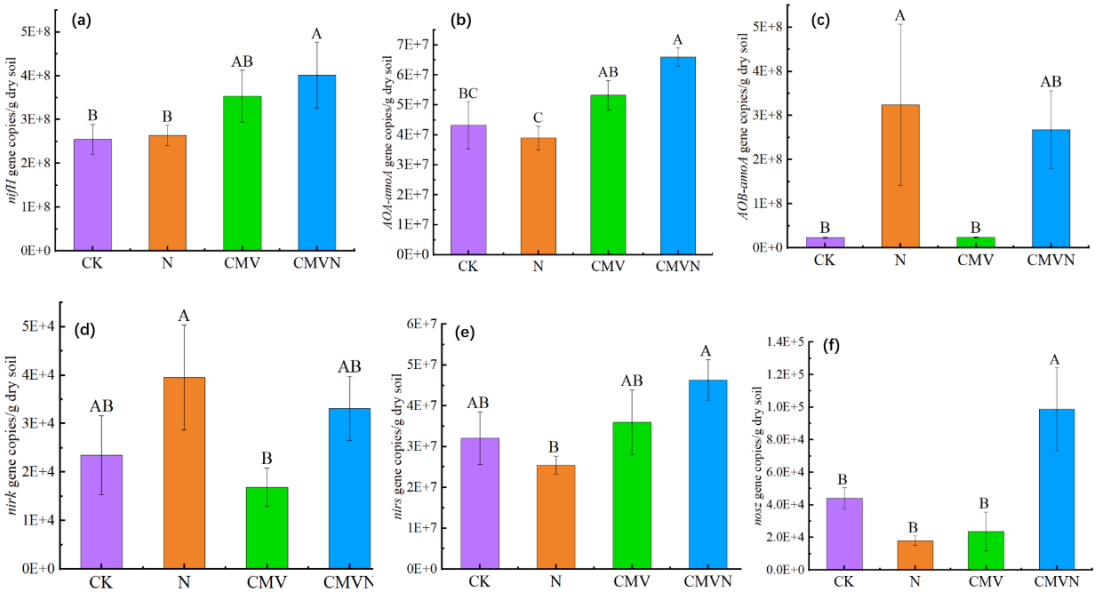

Figure 1. *Effect of different treatments on gene abundances. (a) nifH; (b) AOA-amoA; (c) AOB-amoA; (d) nirK; (e) nirS; (f) nosZ, the different letters (A–B) indicate significant differences ($p < 0.05$)*

Effects of different treatments on gene copy number

AOA and AOB are the major microorganisms regulating ammonia-oxidizing during the nitrogen cycle. To explore the response of AOA and AOB to different treatments, the ratios of AOA to AOB copy number were compared among four groups. As indicated in *Table 3*, the ratios in CMV and CK group were 2.25 and 1.90. This suggested that AOA was the principal community in CMV and CK. However, the AOA/AOB ratio in the N and CMVN groups were 0.146 and 0.265, implying that AOB was the superior community in these two groups. All of these results suggested that combined practics of Chinese milk vetch growth and N fertilization haved influenced the population size of rhizospheric AOA and AOB.

Table 3. *The ratio of nitrogen-cycle gene copy number*

Gene name	Treatment			
	CK	N	CMV	CMVN
AOA-*amo*A/AOB-*amo*A	1.90 ± 0.51a	0.15 ± 0.07b	2.25 ± 0.18a	0.26 ± 0.08b
(*nir*S + *nir*K)/*nos*Z	749.79 ± 232.21bc	1449.19 ± 363.48ab	1683.36 ± 528.67a	485.14 ± 100.14c

The values represented as means ± SD, different lowercase letters indicate significant differences ($p < 0.05$) between treatments

To reveal the response of divergent denitrifying bacteria to different agronomic parctices, the ratio of (*nir*S + *nir*k) to *nos*Z gene copy numbers were compared. As shown in *Table 3*, the ratio ranged from 485.14 to 1683.36, when compared with CK, the ratio decreased to 485.14 in CMVN group, on the contrary, the ratio value increased to 1449.193 and 1683.355 in N and CMV group. The results suggested that *nos*Z-denitrifying bacteria were more sensitive in CMVN group.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2303_43514367
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Effect of different treatments on the relative genus abundances of N functional bacteria

As seen in *Table 4*, the relative abundance of each genus involved in N cycle accounted less than 1%. Urea fertilizer increased the richness of *Nitrosospira* and *Nitrospira* ($p < 0.05$), Chinese milk vetch application significantly enhanced the *Hyphomicrobium* ($p < 0.05$), Chinese milk vetch combined with urea increased the abundances of *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Hyphomicrobium*, *Neorhizobium* and *Nitrospira* ($p < 0.05$).

Interrelations between N-cycling genes and soil physiochemical properties

Multiple regression was carried out to reveal interrelations between N-cycling genes and soil properties. As indicated by *Figure 2*, the *nif*H copy number was only negatively correlated with pH ($p < 0.05$); AOA-*amo*A copy number was only negatively correlated with pH ($p < 0.05$) but significantly positively correlated with the contents of TN, OM, available N, NO_3^--N, urease activity, and ALP activity ($p < 0.01$); the AOB-*amo*A copy number was very negatively correlated with pH and negatively correlated with total Cd level ($p < 0.05$), but significantly positively correlated with the levels of total N, available N, NO_3^--N, and alkaline protease activity ($p < 0.01$), and significantly positively correlated with the levels of NH_4^+-N and urease activity ($p < 0.05$); the *nir*S copy number was remarkably negatively correlated with pH, and was significantly positively correlated with available N, NO_3^--N, and alkaline protease activity; the *nir*K copy number was significantly positively correlated with available nitrogen, NO_3^--N and NH_4^+-N; the *nos*Z copy number was remarkably negatively correlated with pH and significantly positively correlated with total N, NH_4^+-N and alkaline protease activity, and very significantly positive correlated with the level of available N and NO_3^--N. These results suggested that pH, TN, available N and NO_3^--N were the top four factors leading to the changes of nitrogen cycle gene copy number.

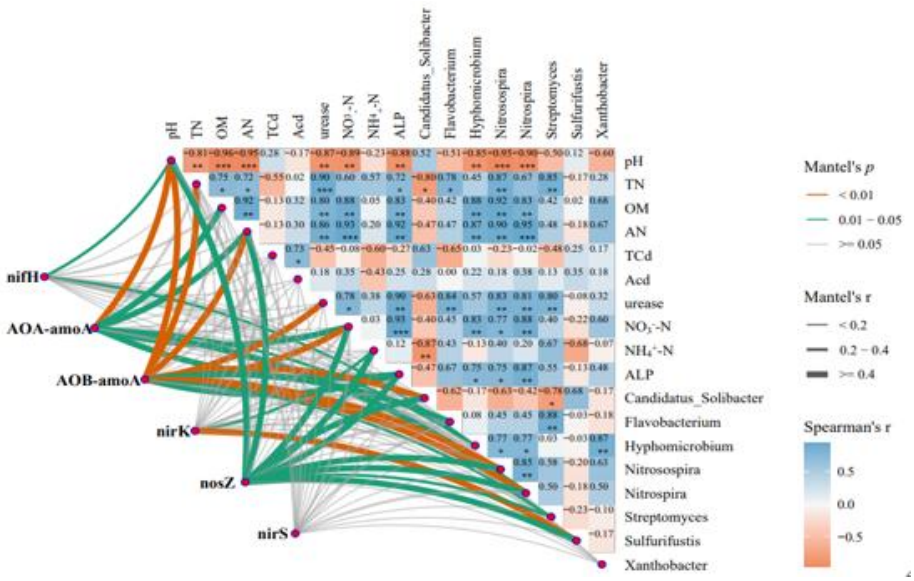

Figure 2. *Correlation analysis of soil physicochemical factors, N cycle gene abundances and bacterial community densities by Mantel statistics method. *, **and ***indicate that the correlation coefficient of all indicators is significant difference at the 0.05, 0.01 and 0.001 levels, respectively*

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DOI: http://dx.doi.org/10.15666/aeer/2303_43514367
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Table 4. *Comparison of relative abundances of N functional bacterial community at genus level*

Genus	Groups			
	CK	**N**	**CMV**	**CMVN**
Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium	0.000431967 ± 0.0000614727b	0.000646654 ± 0.000254107b	0.00074699 ± 0.0000864593b	0.00127 ± 0.0000577137a
Candidatus_Solibacter	0.00188 ± 0.000189854ab	0.00169 ± 0.000461986ab	0.00235 ± 0.000195182a	0.00136 ± 0.000191609b
Hyphomicrobium	0.00081029 ± 0.0000448014c	0.000910812 ± 0.0000681285bc	0.00119 ± 0.0000964092ab	0.00129 ± 0.000186426a
Neorhizobium	0.0000499423 ± 0.000032645b	0.000289241 ± 0.00026607ab	0.0000357379 ± 0.00000613316b	0.000563576 ± 0.000206959a
Nitrosospira	0.000242891 ± 0.000143157b	0.00345 ± 0.00196a	0.000382429 ± 0.0000642232b	0.00222 ± 0.00028765ab
Nitrospira	0.00358 ± 0.000194094c	0.00578 ± 0.000828819ab	0.00478 ± 0.000923863bc	0.00724 ± 0.000401282a
Streptomyces	0.00184 ± 0.000941979ab	0.0036 ± 0.000662852a	0.00143 ± 0.000515983b	0.00287 ± 0.000554723ab
Sulfurifustis	0.000153574 ± 0.0000762803b	0.0000214157 ± 0.0000185157b	0.000314432 ± 0.0000530463a	0.000106902 ± 0.000066556b
Azotobacter	0.0000285266 ± 0.0000269182a	0.0000178521 ± 0.0000123468a	0.0000856166 ± 0.000130086a	0 ± 0a
Flavobacterium	0.000239223 ± 0.0000649232a	0.000228983 ± 0.000211264a	0.000407215 ± 0.000392753a	0.000752844 ± 0.000124376a
Mesorhizobium	0.000435369 ± 0.000198591a	0.00045745 ± 0.00012544a	0.000339561 ± 0.0000588773a	0.000514332 ± 0.000162038a
Mycobacterium	0.000410464 ± 0.0000524263a	0.000806992 ± 0.000425145a	0.000278927 ± 0.0000780211a	0.00036763 ± 0.000101091a
Thiobacillus	0.000153422 ± 0.000101061a	0.0000749186 ± 0.0000641691a	0.000175135 ± 0.0000964787a	0.0000678017 ± 0.0000246641a
Xanthobacter	0.00000356529 ± 0.00000617527a	0 ± 0a	0 ± 0a	0.0000250915 ± 0.0000434597a

The values represented as means ± SD, different lowercase letters indicate significant differences ($p < 0.05$) between treatments

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2303_43514367
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Correlation analysis of soil physicochemical factors, N cycle gene abundance and bacterial community

Pairwise comparisons was performed to explore the correlation between soil physicochemical factors and nitrogen cycle genes, and the microbial community. As shown in *Figure 2*, Mantel's statistic result showed a certain correlation nitrogen cycle genes and the dominant bacterial genera involved in N metabolism. For instance, *nif*H abundance showed a significant correlation with the richness of *Hyphomicrobium*. AOA-*amo*A abundance exhibited a robust correlation with the richness of *Hyphomicrobium* and *Nitrosospira*. AOB-*amo*A abundance was correlated significantly with the abundance of *Candidatus_Solibacter Nitrosospira* and *Nitrospira*. *nir*K was significantly correlated with the richness of *Sulfurifustis*. Additionally, *nos*Z abundance displayed a significant correlation with the abundances of *Candidatus_Solibacter*, *Nitrosospira*, *Nitrospira*, *Streptomyces*, and *SulphurIfustis*.

Furthermore, the abundance of *Candidatus_Solibacter* correlated negatively with levels of TN and NH_4^+-N. Conversely, *Flavobacterium* showed a positive correlation with TN and urease activity. *Hyphomicrobium* was positively associated with organic matter, available nitrogen, NO_3^--N, and alkaline phosphatase activity, while negatively correlated with pH levels. Similarly, an increase in the population of *Nitrosospira* demonstrated a positive correlations with TN, organic matter, available nitrogen, urease activity, NO_3^--N levels as well as alkaline phosphatase activity but a negative associations with pH. Likewise, *Nitrospira* exhibited similar patterns by showing positive correlations with organic matter, available nitrogen, urease activity, and NO_3^--N as well as alkaline phosphatase activity but had negative associations pH values. Finally, the presence *Streptomyces* showed negative correlations only for total N content along with urease activities.

Discussion

Variation of soil physiochemical properties and enzyme activity

Soil pH has a marked effect on soil nutrient availability, microbial activities, crop development, and heavy metal availability (Hou et al., 2019; Wang et al., 2020a; Zhang et al., 2019). In this paper, the pH value decreased with the addition of nitrogen, which is consistent with the results of most nitrogen addition experiments (Bijay-Singh, 2018). Urea fertilizer application caused soil acidification (Dal Molin et al., 2020; Shen et al., 2010). In addition, urease also produces ammonia during the hydrolysis of urea, then NH_4^+ can be oxidized to NO_3^- and H^+ can be produced during nitrification (Sahrawat, 2008), which promotes soil acidification. We found rhizosphere acidification in CMV and CMVN groups, it is good agreement with previous studies, for the excretion of organic acids by plant roots leads to a reduction in the pH (Adeleke et al., 2017). Nevertheless, the joinly impact of organic acids and denitrification on acidification did not manifest in the CMVN group. In comparison, the minimal reduction of soil pH in this may be caused by plant varieties, soil and experimental conditions.

The levels of TN as well as available N and NO_3^--N in the N and CMVN groups were similar, but were remarkable higher than that of the control and CMV groups, suggesing that these changes were mainly due to urea addition, which was consistent with the previous results (Shang and Yi, 2015). For instance, the accumulation of TN and NO_3^--N was promoted by urea addition (Gu et al., 2023; Han et al., 2015); the

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DOI: http://dx.doi.org/10.15666/aeer/2303_43514367
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increase of soil NO_3^--N and NH_4^+ N in the CMVN group was less than that of the single application of urea, which was because the growth of Chinese milk vetch also needed nutrients. In addition, the agronomic practices have significantly increased the organic matter content, which confirmed the previous results (Xie et al., 2016).

Three agronomic practices increased the levels of TN, OM, available N, NO_3^--N, NH_4^+-N and urease activity. The results showed that both treatments were beneficial soil fertility and have promoted nutrient cycling in rhizosphere. The main reason might be that an appropriate amount of N fertilizer can strengthen the metabolism of microorganisms in the soil. In turn, the increase of metabolites secreted by microorganisms was conducive to promoting the mineralization of humus and refractory OM in the soil, thereby increasing soil its level. It may also be due to the symbiosis of Chinese milk vetch with rhizobia during planting, which continuously inputs organic N into the soil to increase N, and the increased N in turn affects the soil C/N ratio, subsequently affects microbial performance, and improves substance decomposition in soil and the release of N.

Soil enzymes are indicators for soil physical and chemical properties (Cheng et al., 2020), and have been widely used as key markers to evaluate soil quality and biological activity (Yuan et al., 2007). The improved soil urease activity in N and CMVN group might be due to nitrogen fertilization or microorganisms participating in N transformation (Liu et al., 2017; Siman et al., 2022). Our study suggested a significant increase in alkaline proteinase activity in the CMVN group. Possible explanation was that practice of CMVN increased the contents of total soil N, organic matter, available N, NO_3^-N and NH_4^+-N, which provided sufficient nutrition for the maintenance of life activities of soil microorganisms, and leads to the increased abundance of soil microorganisms and rhizospheric soil enzyme activities.

The effects of different treatments on N-cycle gene copy number

CMV and CMVN treatments enriched the abundance of *nif*H while urea fertilization hardly had any increase of the *nif*H copy number, suggesting the increased enhancement of *nif*H mainly resulted from Chinese milk vetch. Nitrogen mainly enters the terrestrial ecosystem through biological process. *Nif*H gene is a signature of nitrogen fixation, encoding the Fe-protein of nitrogenase. Soil bacteria, known as rhizobia, often coexist with legumes by providing nutrients for legumes through nodulation and nitrogen fixation, and receiving carbohydrates in exchange to promote themself growth and reproduction in the mutualism pattern (Shamseldin and Abdelkhalek, 2016).

CMVN and N treatments both increased the abundances of AOB-*amo*A and *nir*K while CMV hardly increased AOB-*amo*A copy number, suggesting the increased enhancement of AOB-*amo*A and *nir*K mainly resulted from urea fertilization, which matches well previous studies of Wang et al. (2017), Tan et al. (2013), and Dai et al. (2013).

Both *nir*K and *nir*S are considered as biomarkers of denitrifying microorganisms that encode nitrite reductase (Wang et al., 2020b). However, the abundances of *nir*K and *nir*S in the rhizosphere soil of CMV and CMVN groups increased slightly, indicating that denitrification related with nitrite reductase was not active in rhizosphere soil in CMV and CMVN groups. The number copies of denitrification *nir*S in the rhizosphere soil of CMV and CMVN was considerably higher than that of *nir*K and *nos*Z, indicating that *nir*S-microorganisms were the main denitrification microorganisms in the soil.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Denitrification is a process of gradually reducing NO_3^- to N_2 mediated by microorganisms (Zumft and Kroneck, 2006), and this is a critical step in the whole process that involves with the nitrous oxide reductase (NOS) encoded by *nos*Z. The number of copies of *nos*Z in the CMVN group was highest, which is probaly that the growth of Chinese milk vetch has provided organic fertilizer for denitrifying microorganisms, and nitrogen addition also provided more nutrients that promoted the growth of denitrifying microorganisms.

The effects of different treatments on the N functional bacterial community

The population and community structure of soil AOA and AOB were realted to soil temperature (Tourna et al., 2008), pH (He et al., 2007), soil water content (Hastings et al., 2000) and fertilization measures (Strauss et al., 2014). pH is a critical environmental factor affecting the distribution of AOA (Stempfhuber et al., 2014). AOA is more competitive than AOB in low N or acid soil environment, while AOB is more competitive in alkaline soil or fertilization conditions (Lin et al., 2021). We found that all the soils of the four treatments were alkaline,and that the ratio of AOA to AOB was less than 1 in the CMVN and N groups, suggesting AOB was the dominant population in the urea fertilization group, which may be due to the fact that AOB can use inorganic nitrogen for growth (Zhou et al., 2014). Meantime, the increased level total N in the CMVN group maybe resulted from nitrogen addition, it was lined with the results of a long-term N application experiment. Additionally, the abundance of AOA and AOB was also positively correlated with available N and NO_3^--N at same time, suggesting that the population structure of AOA and AOB was comprehensively regulated of by multiple factors. Finally, we speculated that substances were secreted into the soil during the plants grew, which significantly increased the content of TN and other substances that enhanced the amount of ammonia-oxidizing microorganisms.

The conversion of nitrite (NO_2^-) into NO is the initial step in gas production and the rate-limiting step in denitrification process (Pajares and Bohannan, 2016; Sun et al., 2014). *nir*S- and *nir*K- denitrifying microorganisms are crucial in regulating the denitrification process (Hallin et al., 2018). The copy numbers of *nir*S and *nir*K can be used to measure the intensity of denitrification. In this paper, *nir*S copy number was higher than that of *nir*K in all groups, which aligns with previous researches (Wallenstein and Vilgalys, 2005; Yin et al., 2015; Zhao et al., 2019). Niche differences influence the distribution patterns of *nir*S and *nir*K due to soil environmental factors (Bowen et al., 2018), *nir*K-denitrifying microorganisms exhibit greater sensitivity to environmental changes, *nos*Z-denitrifying microorganisms can convert N_2O into N_2, thereby mitigating greenhouse gas emissions. We demonstrated that Chinese milk vetch alone had an insignificant influence on the copy number of *nir*S, *nir*K and *nos*Z. However, when N fertilizer was added in CMVN, *nos*Z copy number was significantly increased, this might be attributed to N fertilizer that provided an ample organic nitrogen pool for denitrifying microorganisms while increasing reaction substrates availability (Lu et al., 2018). Fertilization measures not only altered soil physiochemical properties but also significantly influenced the abundance of certain denitrifying bacteria. It has been proved that organic fertilizer addition can promote growth among agricultural system's denitrifying bacteria within specific regions (Cui et al., 2016).

In addition, fertilization or combined application of chemical and organic fertilizers can significantly increase the abundance of denitrifying bacteria (Cui et al., 2016; Kleineidam et al., 2010). In our study, the increase of soil nutrients in the CMVN group

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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has positively influenced the richness of denitrifying bacteria. Compared with the milk vetch growth group, the CMVN treatment has significantly reduced the (*nir*S + *nir*K)/*nos*Z value in the soil, and ultimately decreased the N_2O emissions in the soil when combined with N fertilizer.

Soil microorganisms keep the stability of the soil ecosystem by participating in the biogeochemical cycling (Smith et al., 2015). Soil microbial community was influenced by Cd pollution. Many AOB, such as *Nitrosomonas*, *Nitrosospira* and *Nitrosococcus* species can use urea for growth (Koper et al., 2004). *Nitrospira* population abundance significantly increased because of N fertilization (Orellana et al., 2018). Based on the two-step nitrification theory, AOB and nitrite-oxidizing bacteria (NOB) collaborate closely to accomplish the process of ammonia nitrogen oxidation to nitrate. Our findings are consistent with a previous finding that urea fertilization has led to an increase in relative abundance of *Nitrosospira* and *Nitrospira,* as well as, Chinese milk vetch combined with urea increased the relative abundance of *Nitrospira*. Chinese milk vetch growth enhanced the *Hyphomicrobium,* it is consistent with the enhanced richness of *Hyphomicrobium* in the rhizosphere soil of *Legumes* cowpeas (Afkairin et al., 2024). The majority of legumes has the ability to fix atmospheric N_2 via symbiotic rhizobia, legume specificity for Rhizobial symbionts has been summarized (Andrews and Andrews, 2017), CMVN treatment markedly increased the abundance of *Neorhizobium* while the CMV or N fertilizer alone did not, it demonstrates that the two collaborated to led the increase.

Correlation of soil physicochemical factors, N cycle gene abundance and bacterial community

Correlation of soil physicochemical factors and N cycle gene abundance was analyzed, this result supports previous result that pH (Yu et al., 2019), total N (Ni et al., 2023), available N and nitrate N (Ding et al., 2020) were the main factors leading to the changes of nitrogen cycle gene copy number. It was proved that nitrate N content in soil is significantly correlated with *nir*S abundance, Liu et al. (2010) explored that *nos*Z gene abundance was closely related to soil properies such as soil pH and substrate concentration.

In the present study, soil pH negatively correlated with *Hyphomicrobium*, *Nitrosospira* and *Nitrospira*. Meanwhile, other factors, including TN, OM, available N, NO_3^--N and NH_4^+-N correalted positively with *Hyphomicrobium*, *Flavobacterium*, *Nitrosospira* and *Streptomyces*. It is consistent with previous documents that environmental factors have affected bacterial communities involved in N cycling (Campillo-Cora et al., 2022; Diao et al., 2023; Feng et al., 2022). However, the dominant colony species are not completely consistent with previous studies because the effect of N fertilizer treatment on bacterial community structure varies with different research regions (Bai et al., 2021).

Conclusions

In summary, we investigated soil properties and N-cycling gene and bacterial community of Cd-polluted soil under different agronomic practices. CMVN treatment had a better effect in improving the cadmium-containing soil properties compared with milk vetch growth treament or N fertilization. These results suggested that N fertilization was beneficial for Chinese milk vetch to improve soil health, and CMVN

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indirectly increased the abundance of *nif*H, AOA-*amo*A, and AOB-*amo*A by altering pH, total N, and so on. Total N, pH, available N and nitrate N were the main factors to driving the changes of nitrogen cycle gene copy number. Then different agronomic practices indirectly caused changes in abundances of *Nitrosospira*, *Nitrospira*, *Hyphomicrobium*, *Neorhizobium*. This indicates that different soil microorganisms' abundance was greatly affected by agronomic practices. This study provides a mechanism for soil restoration through a combination of green manure plants and fertilization by regulating rhizosphere soil properties and functional genes related to nitrogen cycling. It offers a scientific basis for formulating measures involving the combined application of N fertilizers while providing theoretical support for green manure plant strategies.

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http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2303_43514367
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