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Effects of combined application of animal slurry and mineral fertiliser on rice yield and soil nitrogen cycle microbes

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Abstract: This paper studied the response of rice yield and soil nitrogen (N) cycling microorganisms to the combined application of animal slurry and mineral fertiliser. A pot experiment was conducted on typical yellow-brown paddy soil. There were five treatments: (1) CK – no N fertilisation; (2) S0 – mineral fertilisation; (3) S30 – 30% slurry with 70% mineral fertilisation; (4) S60 – 60% slurry with 40% mineral fertilisation; and (5) S100 – slurry application. Rice yield, yield components, and soil properties were investigated at harvest. The abundance of soil N cycle functional genes abundance was quantified *via* quantitative real-time PCR. The rice yield reached a high level when the proportion of slurry used to replace mineral fertiliser was 30–50%. The yield in response to mineral fertiliser (S0) was equivalent to that in response to no N fertilisation since the formation of effective panicles was inhibited. With the slurry replacement ratio increase, the available phosphorus and potassium contents in the soil improved, but the nitrate content decreased. Considering the entire soil N cycle, nitrogen-fixing microbes (*nifH*), ammonia-oxidising archaea (AOA *amoA*) and nitrite-reducing microbes (*nirS* and *nirK*) had greater abundances, reaching 10^8 copies. Compared with those in the S0 treatment, the abundances of most N cycle functional genes in the S30 treatment, except for *napA*, significantly increased from 31.2% to 100.9%, and the increase in the abundance of *nirS* and *nosZ* in the S100 treatment reached 4 times, which was obviously greater than that of the other genes. Correlation analysis revealed that high soil pH promoted N fixation and nitrification, while NH_4^+-N had the opposite effect on N fixation and nitrification, and available phosphorus and potassium actively influenced denitrification. These results showed that a 30–50% slurry application ratio was recommended for rice, which was beneficial for maintaining high yields and high abundances of soil N cycle functional genes.

Keywords: animal manure; waste treatment; yield response model; recycling; soil nutrient cycling

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Large-scale farming produces a large amount of livestock and poultry manure. Unlike solid manure, liquid slurry must occur in large quantities and be difficult to treat, and there is a high risk of returning to the field (Kinyua et al. 2016). This restricts the sustainable development of planting and breeding recycling (Zhang et al. 2021). Slurry plants, which are rich in organic matter and mineral nutrients, are an important source of nutrients in farmland systems. However, unreasonable direct return of slurry to a field may cause harm to the soil, crops, and environment (Möller 2015). The detoxification methods used for breeding wastewater mainly include storage, anaerobic fermentation, ectopic fermentation beds, and other industrial methods. In the Yangtze River Basin, the proportion of the adoption of stored fertilised water returned to the field reached 53.3% (Zhou et al. 2022). Adequate storage time and sufficient airtight storage facilities are the keys to wastewater detoxification. Additionally, the nutrient preservation rate of stored slurry is higher, especially for nitrogen (N) and sulfur (S), which could be volatilised (Ding et al. 2020). However, technologies such as the amount of stored slurry returned to the field and the proportion of alternative fertilisers are still dominated by experience and lack corresponding technical standards (Wei et al. 2021). The scientific application of slurry is an important measure for popularising the return of manure to the field, promoting the reduction of mineral fertiliser and realising green planting and breeding circulation (Hou et al. 2021).

Considering the bearing capacity of the land, a large amount of slurry is applied to crops such as vegetables and fruit trees (Insam et al. 2015). To reduce costs, slurries are applied to field crops locally (Du et al. 2020). In a dryland system, slurry returned to the field significantly affects the soil's physical structure, chemical properties and microbial activity (Bosch-Serra et al. 2015). Based on comprehensive consideration of environmental benefits such as nitrate leaching and ammonia volatilisation, the rational application of slurry (which replaces 50–75% of mineral fertilisers or 160–240 kg N/ha by irrigation) can achieve multiple goals, such as high grain yield, water and fertiliser integration, and environmental protection (Du et al. 2015). Biogas slurry after anaerobic fermentation was the main research focus in paddy systems. At present, the impacts of the application of biogas slurry in paddies on crop yield and quality, N transformation and utilisation, ammonia volatilisation, greenhouse effects and runoff have been basically clarified (Yin et al. 2019, Rahaman et al. 2021, Tang et al. 2021). A scientific application method is necessary because the stored slurry has a high nutrient content (N, P and K). N cycling, including N fixation, nitrification and denitrification, is an important component of farmland ecosystems (Kuypers et al. 2018). These processes are particularly sensitive to soil environmental changes caused by fertilisation (Ouyang et al. 2018, Li et al. 2020). Returning slurry to a field can not only provide energy and substrate for the N cycle but also change soil moisture, pH and other conditions, indirectly affecting N cycle functional microbes (Verdia et al. 2019, Coelho et al. 2020). However, most of the related studies have focused mainly on a certain process or a certain functional microbe of the N cycle, and a quantitative understanding of the functional genes related to the whole N cycle has been lacking. When dairy slurry was applied to the soil, the community compositions of denitrifying bacteria and ammonia-oxidising bacteria exhibited significant differences. The abundances of the *nirS*, *nirK* and *nosZ* genes are sensitive to slurry rate and concentration (Wang et al. 2017, Gao et al. 2019). The application of slurry increased the diversity and richness of ammonia-oxidising archaea in soil (Wang et al. 2015).

The Yangtze River Basin is a specified location with a serried water system with an apparent separation of planting and breeding. There is a strong contradiction between water environment protection and pollution from breeding wastewater. Scientific and reasonable suggestions for returning slurry to the field to break through the key blocking points of the planting and breeding cycle are urgently needed. In this study, with typical yellow-brown soil as the research object, pot experiments were carried out to evaluate the effects of slurry replacement with mineral fertiliser on (A) rice yield; (B) soil physicochemical properties and (C) the abundance of N cycling functional genes. We proposed the following hypothesis: the safety threshold value of applied slurry to a rice field and the key factors affecting the N cycling process were explored. Thus, the results of the present study may provide a reference for the safe utilisation of breeding slurries.

MATERIAL AND METHODS

Site and soil description. A pot experiment was established at the experimental farm of the Hubei

Academy of Agricultural Sciences (114.32°E, 30.49°N) from May to October 2021. The climate type is a subtropical monsoon climate with hot summers (from July to September). During the rice growth period, the average monthly temperature, rainfall, and humidity were 18–29 °C, 75–242 mm and 77–81%, respectively. The yellow-brown soil belongs to the Albic Luvisol according to FAO classification (Hu et al. 2019). The summer rice in this region is generally cultivated in rotation with winter wheat, rapeseed, or fallow.

Experimental design and treatments. The soil was collected from the topsoil (approximately 0–25 cm) in a paddy field. These soils were air-dried and sieved through 5.0 mm mesh to remove rock. The initial soil properties were as follows: pH, 6.4; soil organic carbon, 9.7 g C/kg; available phosphorus (P), 20.4 mg/kg; and available potassium (K), 89.6 mg/kg. All the soils were stored in separate plastic containers (with a height of 32.1 cm and a diameter of 27.8 cm). There were 16 kg of air-dried soil in each container, with a bulk density of 1.2 g/cm^3 and a depth of 25 cm.

The slurry used in this study was produced by storage at a waste treatment plant (Tianyuanmuge (Zhijiang) Urban and Rural Environmental Complex Management Co., Ltd.). The main components of the slurry were as follows: water-insoluble, 2.5%; pH, 7.5; organic carbon, 14.3%; total N, 4 180 mg/L; total P, 307 mg/L; and total K, 334 mg/L.

The rice cultivar used was Yexiangyoulisi. Seedlings were sown on 19 May, transplanted on 23 June, and harvested on 9 October. The growing period was 143 days.

Five treatments were used: (1) CK – no N fertiliser; (2) S0 – mineral N fertiliser; (3) S30 – slurry replace 30% mineral N fertilisers; (4) S60 – slurry replace 60% mineral N fertilisers; and (5) S100 – slurry replace 100% mineral N fertilisers. A total of 20 pots were arranged in a randomised block design with 4 replicates per treatment. Two plants were transplanted into each pot. The application rates of N fertiliser (urea, 46% N content) are shown in Table 1. The same amounts of P (superphosphate, 5.2% P content) and K (potassium chloride, 28.2% K content) fertilisers were applied in all the treatments, and the application rates were 0.61 g P/pot and 1.74 g K/pot, respectively.

Sampling and measurement. The rice yield of each pot was harvested at the fully mature stage, after which it was sun-dried to a constant weight. The plant samples were divided into straw and grain components.

At the rice maturation (10-09) stage 2021, 5 soil samples (0–20 cm depth) collected 5 cm from the root zone were taken from each pot. After removing plant debris and stubbles, these samples were divided into 2 parts: one part was stored at 4 °C for physicochemical property analysis, and the other was stored at –80 °C for real-time quantitative PCR.

The soil properties were analysed as previously described by Hu et al. (2019). The potassium dichromate volumetric method determined the soil organic carbon (SOC) content. The soil NH_4^+ and NO_3^- contents were extracted with a 2 mol/L KCl solution and then measured using an automatic intermittent chemical analyser (AA3, Bran and Luebbe, Norderstedt, Germany). The soil available P (available P) was extracted with 0.5 mol/L $NaHCO_3$ and measured *via* the Olsen method. The available K (available K) soil was extracted with 1 mol/L NH_4Ac and measured *via* flame photometry. The soil pH was measured with a 0.01 mol/L $CaCl_2$ slurry with a glass electrode.

According to the manufacturer's instructions, soil DNA was extracted from a 0.25 g soil sample using a FastDNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, USA). The concentration and purity of the extracted DNA were independently checked using an ND-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA) at 260 nm. The abundance of N-cycling functional genes (NFGs) was determined by using a real-time quantitative PCR system (CFX96, Bio-Rad, Hercules, USA). The target genes included *nifH*, AOB *amoA*, AOA *amoA*, *hao*, *nxrB*, *narG*, *napA*, *nirS*, *nirK*, *norB*, and *nosZ*. The 15 μL PCR mixture contained 7.5 μL of SYBR Green Mix, 0.7 μL of each primer, and 2 μL of the DNA template. The specific primers used for the target genes are shown in Table 2.

Table 1. Type and amount of nitrogen (N) fertiliser (g N/pot) applied in different treatments

Treatment	Basal fertiliser		Topdressing	
	mineral	slurry	mineral	slurry
CK	0	0	0	0
S0	1.26	0	0.84	0
S30	0.63	0.63	0.84	0
S60	0	1.26	0.84	0
S100	0	1.26	0	0.84

CK – no N fertiliser; S0 – mineral N fertiliser; S30 – slurry replace 30% mineral N fertilisers; S60 – slurry replace 60% mineral N fertilisers; S100 – slurry replace 100% mineral N fertilisers

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Table 2. Primer sequences for functional genes involved in the nitrogen (N) cycle considered in the study

Function	Catalyse process	Gene	Primer sequences (5'–3')
N fixation	$N_2 \rightarrow NH_4^+$	*nifH*	F (AAAGGYGGWATCGGYAARTCCACCAC) R (TTGTTSGCSGCRTACATSGCCATCAT)
Nitrification	$NH_4^+ \rightarrow NH_2OH$	AOB *amoA*	1F (GGGGTTTCTACTGGTGGT) 2R (CCCCTCKGSAAAGCCTTCTTC)
		AOA *amoA*	23F (ATGGTCTGGCTWAGACG) 616R (GCCATCCATCTGTATGTCCA)
	$NH_2OH \rightarrow NO_2^-$	*hao*	F (GTMGGHTGYATYGACTGYCAC) R (GRCGRTTGGTBKTYTGDCC)
	$NO_2^- \rightarrow NO_3^-$	*nxrB*	F (TACATGTGGTGGAACA) R (CGGTTCTGGTCRATCA)
Denitrification	$NO_3^- \rightarrow NO_2^-$	*narG*	1960M2F (TAYGTSGGGCAGGARAAACTG) 2050M2R (CGTAGAAGAAGCTGGTGCTGTT)
		napA	V17F (TGGACVATGGGYTTYAAYC) 4R (ACYTCRCGHGCVGTRCCRCA)
	$NO_2^- \rightarrow NO$	*nirS*	cd3AF (GTSAACGTSAAGGARACSGG) R3cd (GASTTCGGRTGSGTCTTGA)
		nirK	FlaCu (ATCATGGTSCTGCCGCG) R3Cu (GCCTCGATCAGRTTGTGGTT)
	$NO \rightarrow N_2O$	*norB*	454F (TACTAYGARCCCTGGACTTACRA) 710R (ATGCGYGGSAWRTAGAAGWAMAMSA)
	$N_2O \rightarrow N_2$	*nosZ*	2F (CGCRACGGCAASAAGGTSMSSGT) 2R (CAKRTGCAKSGCRTGGCAGAA)

AOB – ammonia-oxidizing bacteria; AOA – ammonia-oxidizing archaea (AOA)

Data analysis. Microsoft Excel (version 2013, Redmond, USA) was used for data processing. Origin (version 2021, Northampton, USA) was used for image rendering. Significant differences were analysed *via* ANOVA and Duncan's multiple comparison test to compare the means for each variable based on SPSS Statistics (version 20.0). A significance level of 0.05 was used for all the statistical analyses.

RESULTS AND DISCUSSION

Rice yield. As shown in Table 3, the rice yield of all the treatments could be divided into three grades: the highest yield was in S30, reaching 83.0 g/pot; the second was in S0 and S60, which were significantly lower than that in S30; and the lowest yield was in CK and S100, at only 56.0 and 54.0 g/pot, respectively. Compared with those in the CK treatment, the proportion of mineral fertiliser replaced by slurry fertiliser was within 60%, and the increase in yield could reach more than 30%. In contrast, the risk of yield reduction may occur at total replacement. Compared with that of S0, the yield of S30 increased by 12.2%, while the yield of S100 decreased by 27.0%. The replacement of mineral fertiliser with slurry has various effects on yield components to different

Table 3. The rice yield and yield components were observed in the different treatments

Treatment	Yield (g/pot)	Effective panicle number (/pot)	Grains per panicle	Grain filling percentage (%)	1 000-grain weight (g)
CK	56.0^{c}	25.3^{e}	175^{b}	70.7^{b}	17.3^{b}
S0	74.0^{b}	29.6^{d}	162^{c}	60.3^{c}	15.7^{b}
S30	83.0^{a}	36.7^{a}	184^{ab}	90.0^{a}	21.9^{a}
S60	73.3^{b}	34.0^{b}	178^{b}	87.1^{a}	21.2^{a}
S100	54.0^{c}	31.6^{c}	191^{a}	67.8^{b}	16.9^{b}

Different letters within the same column indicate significant differences among treatments at $P = 0.05$. CK – no nitrogen (N) fertiliser; S0 – mineral N fertiliser; S30 – slurry replace 30% mineral N fertilisers; S60 – slurry replace 60% mineral N fertilisers; S100 – slurry replace 100% mineral N fertilisers degrees (Table 3). The effective panicle size of S30 reached 36.7/pot, which was significantly greater than that of the other treatments. This may be attributed to two reasons. First, mineral fertiliser was applied to the basal fertiliser in S30 in combination with slurry, which promoted the early growth and rapid development of rice tillers (Pan et al. 2022). Second, the fertiliser applied to the panicles in S100 was slurry, which led to an untimely supply of nitrogen. The grain number was relatively high in S30 and S100, reaching more than 180 grains per panicle. The grain filling percentage and 1 000-grain weight were the highest in both S30 and S60, 87.1–90.0% and 21.2–21.9 g, respectively.

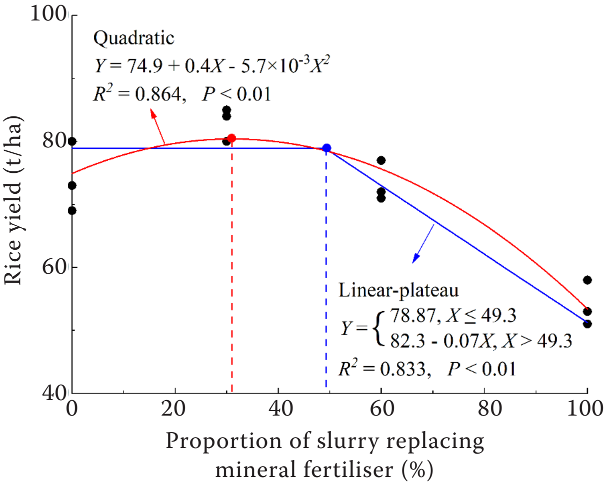

Figure 1. Relationship between rice yield and proportion of slurry replacing mineral fertiliser

The relationship between rice yield and the proportion of slurry (which replaces a portion of mineral fertiliser) is shown in Figure 1. This result was consistent with the yield response of mineral fertiliser with solid manure application (Hou et al. 2022). According to the fertiliser response model, the quadratic and linear plateau model correlation coefficients increased significantly ($P < 0.01$). According to the quadratic model, the recommended proportion of slurry to replace mineral fertiliser was 31.0%, which was calculated based on the highest yield. Moreover, the recommended proportion from the linear plateau model was 49.3%, which was calculated based on yield reduction. This result suggested that 30–50% could be an optimal replacement proportion, which consumes enough slurry and ensures a high rice yield. The proportion of solid manure applied can be adjusted according to soil fertility, and 40–60% of solid manure is generally believed to be effective (Ren et al. 2022). For liquid slurry, the recommended proportion for paddies in different ecological areas and under different soil conditions varies greatly (Chen et al. 2022). The main reasons include unstable slurry components, unclear ecological effects of long-term application, and a lag in supporting application technology (He et al. 2021). In addition, the slurry produced by storage and biogas may differ to some extent, as the nutrient content of the former may increase. Therefore, appropriate dilution is required before application (Meng et al. 2022). The dilution factor can be adjusted according to the crop water demand and the ammonium nitrogen concentration in the field surface water to avoid runoff and seedling burning (Zhang et al. 2021).

Soil properties. As shown in Table 4, the effects of replacing mineral fertiliser with a slurry on various soil properties were somewhat different. From the results of soil inorganic N, the change of NH_4^+-N and NO_3^--N content in each treatment was relatively small, with an average content of 2.56–3.13 and 0.80–0.99 mg/kg, respectively. With increasing slurry replacement proportion, NH_4^+-N exhibited no obvi-

Table 4. Description of the soil physicochemical properties before rice harvest

Treatment	NH_4^+-N	NO_3^--N	SOC	Available P	Available K	pH
	(mg/kg)		(g/kg)	(mg/kg)		
CK	3.13^a	0.87^{abc}	9.98^a	17.1^c	84.1^c	6.07^a
S0	2.92^{ab}	0.99^a	10.03^a	17.1^c	91.8^{bc}	6.15^a
S30	2.56^b	0.96^{ab}	9.93^a	19.9^b	96.4^{bc}	6.17^a
S60	3.06^a	0.82^{bc}	10.09^a	21.9^a	103.6^b	6.12^a
S100	2.84^{ab}	0.80^c	10.21^a	22.8^a	123.8^a	6.15^a

Different letters within the same column indicate significant differences among treatments at $P = 0.05$. SOC – soil organic carbon; CK – no nitrogen (N) fertiliser; S0 – mineral N fertiliser; S30 – slurry replace 30% mineral N fertilisers; S60 – slurry replace 60% mineral N fertilisers; S100 – slurry replace 100% mineral N fertilisers ous change, whereas NO_3^--N gradually decreased. There were no significant differences in SOM or pH among the different treatments, while the contents of available P and K increased with increasing volume of slurry.

Soil N cycling functional genes. The impact of slurry replacement of mineral fertiliser on soil N cycling processes mainly involves N fixation, nitrification and denitrification (Figure 2), which are the main functional pathways of soil N cycling (Wang et al. 2018). The functional genes involved in N fixation were *nifH*; those involved in nitrification were AOB *amoA*, AOB *amoA*, *hao* and *nxrB*; and those involved in denitrification were *narG*, *napA*, *nirS*, *nirK*, *norB* and *nosZ*. Quantifying the abundance of functional genes involved in the N cycle can directly reflect the activity and potential for the occurrence of this process, as well as the response of functional microorganisms to environmental changes (Khanal and Lee 2020). This will be conducive to realising the potential of N-cycling microorganisms to maintain soil health (Zhu et al. 2021).

Compared with that in the soil before rice planting, the abundance of the *nifH* gene after rice planting increased by a relatively large margin as the soil structure changed (Fan et al. 2023). According to the results of the different treatments, the abundance of the *nifH* gene in S30 was the highest, reaching 3.4 × 10^8 copies/g soil, increasing by 70% compared with that in CK, twice as much as that in S60. Similarly, the abundances of the *nifH* gene in S0 and S100 were relatively close, at 2.6 and 2.4 × 10^8 copies/g soil, respectively. With the replacement proportion of slurry increasing from 0% to 100%, the N storage capacity may gradually decrease because of the decreasing NO_3^--N concentration (Tang et al. 2018).

Compared with that in the soil before rice planting, the abundance of the ammonia oxidation functional gene (*amoA*) decreased to varying degrees after rice planting, and the abundance of the hydroxylamine oxidoreductase functional gene (*hao*) increased by a relatively large margin, whereas the abundance of the nitrite oxidoreductase functional gene (*nxrB*) showed no significant change. In addition, the abundance of the AOA *amoA* gene involved in the ammonia oxidation process was one order of magnitude greater than that of the AOB *amoA* gene, which might be associated with the soil C/N ratio and nutrient content (Dai et al. 2021). Among the different treatments, the abundances of the AOB *amoA*, AOA *amoA* and *hao* genes were the highest in the CK, 1.6 × 10^7, 3.6 × 10^8 and 7.4 × 10^6 copies/g soil, respectively. Among the treatments with different replacement proportions of slurry, the *amoA* and *nxrB* genes' abundances were significantly greater in S30 and S100 than in S0 and S60. S30 had the highest abundance of the *hao* gene, and S60 had the lowest abundance of nitrification functional genes compared with the other treatments.

Before rice planting, the abundance of genes related to soil denitrification function was relatively

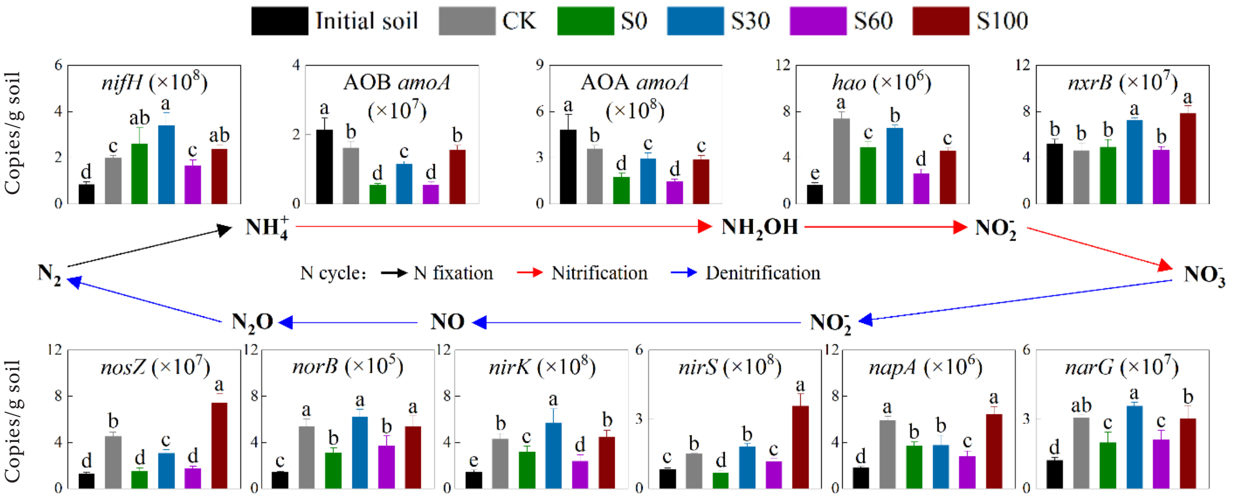

Figure 2. The abundance of the different functional genes (copies/g of soil) involved in the soil nitrogen (N) cycle was measured in the different treatments. Different letters indicate significant differences among treatments at $P = 0.05$. CK – no N fertiliser; S0 – mineral N fertiliser; S30 – slurry replace 30% mineral N fertilisers; S60 – slurry replace 60% mineral N fertilisers; S100 – slurry replace 100% mineral N fertilisers; AOB – ammonia-oxidizing bacteria; AOA – ammonia-oxidizing archaea (AOA)

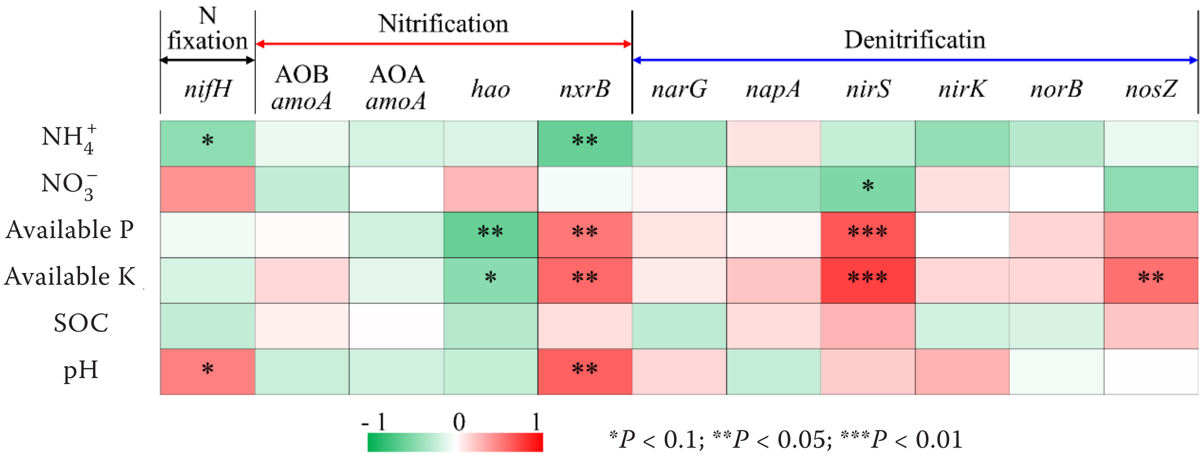

Figure 3. Correlation analysis between soil properties and nitrogen (N) cycle functional genes. SOC – soil organic carbon; AOB – ammonia-oxidizing bacteria; AOA – ammonia-oxidizing archaea (AOA)

low, but after rice planting, it increased to varying degrees (Chen et al. 2021). Similarly, the abundances of the *narG*, *nirK* and *norB* genes exhibited similar trends: the highest abundance was in S30, followed by CK and S100, and the lowest was in S0 and S60. The abundance of the *napA*, *nirS* and *nosZ* genes showed another trend: the abundance in S100 was significantly greater than that in the other treatments. Overall, the abundances of all denitrification functional genes were relatively high in S30 and S100, and the abundances were lower in both S0 and S60 than in CK. The total abundance of *nirS* + *nirK* was relatively high in S30 and S100, indicating that either too high or too low a replacement proportion of slurry may cause N loss (Song et al. 2019, Li et al. 2022).

Correlation. The correlation analysis results between the soil physicochemical properties and N cycle functional genes are shown in Figure 3. The *nifH* gene was significantly negatively and positively correlated with NH_4^+-N and pH during nitrogen fixation. During nitrification, the *hao* gene presented a significant negative correlation with available P and K; the *nxrB* gene presented a significant negative correlation with NH_4^+-N, and a significant positive correlation with available P and K and pH. During denitrification, the *nirS* gene presented a significant negative correlation with NO_3^--N, a significant positive correlation with available P and K, and the *nosZ* gene presented a significant positive correlation with available K.

Consequently, as the proportion of mineral fertiliser replaced by slurry increased to 30%, the rice yield reached its highest value. Then, a downward trend occurred because the rice filling was inhibited. The replacement proportion should be controlled within 30–50% to obtain a high yield. With the increase in the replacement proportion, the soil NO_3^--N content exhibited a decreasing trend, and the available P and K contents exhibited an increasing trend. The abundances of N fixation-, nitrification- and denitrification functional-related genes were relatively high when the replacement proportion of the slurry was 30%. With the slurry replacing 30% of the mineral fertiliser, the nutrient supply could be adequate and effective throughout the growth period. When affected by slurry, NH_4^+-N inhibits N fixation and nitrification, while available P and K promote denitrification.

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