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Effects of Different Manures in Combination with Fulvic Acid on the Abundance of N-Cycling Functional Genes in Greenhouse Soils

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Abstract: To investigate the effects of different manures in combination with fulvic acid on the abundance of N-cycling functional genes in greenhouse soils, Chinese cabbage was planted for three growing seasons. A total of six treatments—pig manure (P), pig manure + fulvic acid (PH), chicken manure (C), chicken manure + fulvic acid (CH), sheep manure (S), sheep manure + fulvic acid (SH) and no fertilization (CK)—were set up. The abundance of 13 soil N-cycling functional genes (*gdhA*, *amoA*-1, *amoA*-2, *amoB*, *narG*, *nirK*-1, *nirK*-2, *nirK*-3, *nirS*-1, *nirS*-2, *nirS*-3, *nosZ* and *nifH*) were investigated after the harvest of the third growing season using a gene chip approach. The results showed that fertilization treatments increased the abundance of most N-cycling functional genes in the soil, such as nitrification genes *amoA*-2 and *amoB* as well as denitrification genes *narG*, *nirK*-1, *nirS*-1 and *nirS*-2, with the stronger influence of sheep and pig manure than chicken manure. Fortunately, the additional fulvic acid reduced the increasing effect resulting from pig, chicken and sheep manure application. The abundance of functional genes for nitrogen cycling in soil was positively correlated with the content of soil organic matter, available phosphorus and NO_3^--N, and negatively correlated with electrical conductivity. Overall, fertilization treatments increased soil nitrification and denitrification genes abundance, with a risk of increasing soil nitrogen loss, but the supplementary fulvic acid could limit the increase. In this study, it was concluded that the sheep manure (31.3 t/ha) + fulvic acid (7.5 kg/ha) treatment was more powerful in regulating the abundance of N-cycling functional genes in soil.

Keywords: Chinese cabbage; HT-qPCR; humic acid; N-cycling functional genes

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1. Introduction

Soil nitrogen (N) research has been a hot topic in agriculture [1,2]. Nitrogen is one of the most important nutrients affecting the growth of crops and micro-organisms [3], and different forms of nitrogen undergo a complex transformation process in the soil environment [4]. It has been shown that soil nitrogen transformation is influenced by numerous environmental factors such as moisture [5], temperature [6], reactive substrates [7,8], organic matter (OM) [9,10], pH [11], among others. Most scholars proved that soil micro-organisms related to the nitrogen cycle play a major role in regulating nitrogen transformation processes, including mineralization, nitrification, denitrification and fixation [12–14]. The abundance of soil N-cycle functional genes is an important character of soil N-cycle micro-organisms. Therefore, mining the change of the abundance of soil N-cycle functional genes and the associated microbiological mechanisms is of great significance in clarifying the transformation of soil nitrogen.

The input of different organic fertilizers may shift the content of different forms of soil nitrogen, affecting the abundance of soil N-cycle functional genes and the composition of the microbial community [15]. In previous studies, the response of soil N-cycling functional genes to organic fertilizers was generally compared to that of inorganic fertilizers and that of a combined application with organic and inorganic fertilizers. Ouyang et al. [16] documented through a meta-analysis that the application of nitrogen fertilizers altered the abundance of soil N-cycle functional genes, significantly increasing the abundance of nitrification gene *amoA* and denitrification genes *nirK*, *nirS* and *nosZ*, and that the organic fertilizers exerted a more potent effect than inorganic fertilizers. Ma et al. [15] found that a combined application with organic and inorganic fertilizers decreased the abundance of nitrification genes *amoA*, *amoB* and *Hao*, but increased the abundance of denitrification genes *nirK*, *norB* and *nosZ*. In addition, manures were found to increase mainly the abundance of ammonia-oxidizing bacteria but not ammonia-oxidizing archaea in a separate study [17]. It is also evidenced that organic fertilizers increased soil microbial community diversity and enzyme activity due to an increase in soil nutrients and organic carbon [18]. Liang et al. [19] confirmed that the increased content of carbon and nitrogen in soils was an important reason why organic fertilizers increased the abundance of main N-cycle functional genes. The application of organic fertilizers enhanced the abundance of soil genes for the N-cycle and enriched the composition of the associated microbial communities [20,21]. However, variations in the effects of various types of organic fertilizers (manures) on functional genes of the N-cycle are still unclear.

In addition, it is found that humic acid with strong biological activity can improve soil properties, enhance soil fertility, promote crop growth and regulate soil enzyme activity and the microbial community structure, etc. [22–26]. The genes, as the genetic material, control microbial activities and enzyme synthesis in organisms [27]. The unknowns are how humic acid affects N-cycle functional genes in the soil and whether humic acid has different impacts on different genes. Furthermore, the effects of manure applications coupled with humic acid on the N-cycle functional genes in the soil have not been reported. Considering fulvic acid has a low molecular weight, good solubility and high functional group content [28], it was selected to investigate the effects of a combined application of different manures with humic acid on the abundance of N-cycle functional genes in soils and to depict the main environmental factors determining the abundance of N-cycling functional genes. This study has some implications for regulating each step of the nitrogen cycle at the gene level.

2. Materials and Methods

2.1. Experimental Site

The experiment was conducted in an ordinary arched plastic greenhouse (length 81 m, width 8 m and height 3 m) in Xinxiang City, Henan province, China (35°19′47″ N, 114°0′51″ E), with a typical warm temperate continental monsoon. The average annual temperature and the average annual precipitation in Xinxiang is 14 °C and 582 mm, respectively. The greenhouse was covered by a transparent plastic film and installed with film coilers on both long sides to adjust the temperature and humidity. In the previous 3 years, there were no agricultural activities conducted in the greenhouse, which was suitable for the study of organic agriculture. The soil is a fluvo-aquic soil according to Chinese classification, and a Fluvic Cambisol according to the World Reference Base, and the soil properties of 0–10 cm are shown in Table 1.

Table 1. The original soil properties (0–10 cm).

Parameter	BD (g/cm^3)	FC (%)	OM (g/kg)	pH /	EC (dS/m)	NH_4^+-N (mg/kg)	NO_3^--N (mg/kg)	AP (mg/kg)	AK (mg/kg)	TN (g/kg)
0–10 cm	1.34	27.98	25.24	8.35	0.29	0.98	128.12	34.33	366.00	0.85

Note: BD, bulk density; FC, field capacity; OM, organic matter; EC, electrical conductivity; AP, available phosphorus; AK, available potassium; TN, total nitrogen.

2.2. Experimental Design

Chinese cabbage (*Brassica chinensis* L.) was planted for three growing seasons using the varieties Hanxiu, Shandaoqingcui and Xiadi, respectively. Three types of manures including pig manure, chicken manure and sheep manure with two levels were set up in the first growing season, and no fertilizer control (CK) was set. The treatments with the detailed information of fertilization and the properties of the manures are shown in Tables 2 and 3. Each treatment had three replicate plots scheduled in a completely randomized design. A total of 21 plots (3.2 m × 2.5 m) were conducted with a 0.5 m gap between adjacent plots. All plots were irrigated with shallow groundwater pumped from the experimental site at 1163 m^3/ha on 8 November 2022, the basic properties of which are shown in Table 4. All the manures used in the experiment were applied evenly as basal fertilizers on 3 December 2022 before soil ploughing. The seeds were sown into the 10 furrows of each plot by hand on 4 December with a sowing density of 7.5 kg/ha. In order to meet the water requirements of emergence, 600 mL of water was sprinkled for each furrow before sowing. The experimental indicators detection and other practices such as irrigation and seedlings thinning were stopped due to the outbreak of the novel coronavirus; consequently, the first growing season lasted 94 days. The vegetable yield was measured after the harvest on 7 March 2023.

Table 2. Treatments with the detailed fertilization information.

	The First Growing Season		The Second Growing Season			The Third Growing Season		
Total Treatments	Manure Types—Fertilization (t/ha)	TN Inputs (kg/ha)	Manure Types—Fertilization (t/ha)	Fulvic Acid (kg/ha)	TN Inputs (kg/ha)	Manure Types—Fertilization (t/ha)	Fulvic Acid (kg/ha)	TN Inputs (kg/ha)
CK	-	-	-	-	-	-	-	-
PH	Pig manure—15	70.5	Pig manure—31.9	7.5	150	Pig manure—31.9	7.5	150
P	Pig manure—9	42.3	Pig manure—31.9	-	150	Pig manure—31.9	-	150
CH	Chicken manure—5	29.5	Chicken manure—25.4	7.5	150	Chicken manure—25.4	7.5	150
C	Chicken manure—3	17.7	Chicken manure—25.4	-	150	Chicken manure—25.4	-	150
SH	Sheep manure—11	52.8	Sheep manure—31.3	7.5	150	Sheep manure—31.3	7.5	150
S	Sheep manure—6	28.8	Sheep manure—31.3	-	150	Sheep manure—31.3	-	150

Note: CK, no fertilization; P, pig manure; C, chicken manure; S, sheep manure; H, fulvic acid.

Table 3. Manure properties.

Manure Type	pH /	EC (dS/m)	OM (g/kg)	TN (g/kg)	TP (g/kg)	TK (g/kg)
Pig manure	8.68	5.66	32.7	4.7	4.0	9.6
Chicken manure	8.42	7.22	35.9	5.9	5.4	11.3
Sheep manure	8.88	6.77	29.9	4.8	4.1	8.5

Note: EC, electrical conductivity; OM, organic matter; TN, total nitrogen; TP, total phosphorus; TK, total potassium.

Table 4. Water properties.

Water Source	pH /	Electrical Conductivity (dS/m)	NO_3^--N (mg/L)	NH_4^+-N (mg/L)
Groundwater	7.25	2.81	0.22	1.94

After the first season, the treatments for the experiments were appropriately adjusted to arrange a long-term experiment to investigate the effects of different manures in combination with fulvic acid on N-cycling functional genes in soil. In the following growing seasons, six treatments were set up—pig manure (P), pig manure + fulvic acid (PH), chicken manure (C), chicken manure + fulvic acid (CH), sheep manure (S), sheep manure + fulvic acid (SH). CK was still there. The corresponding relationships of treatments in the different growing seasons are shown in Table 2. The manures used were the same as the first season. Potassium fulvic acid of a mineral source (Wujin999, fulvic acid contents $\geq$ 50%, $K_2O \geq 8\%$, pH 10.08) was purchased from Xinjiang Shuanglong Humic Acid Co. The fulvic acid (7.5 kg/ha) was dissolved in 2 L of water and the manures (150 kg N /ha) were applied evenly to the plots. After the fertilization and ploughing of plots on 31 March 2023, the seeds were sown on 1 April using the same procedure with the first season. Irrigation was arranged based on local farmers' established practice on 9 April 2023 (2126 m^3/ha), 22 April (1376 m^3/ha) and 6 May (1376 m^3/ha), respectively. Other agronomy practices were carried out according to the field managements of organic vegetables. The vegetables were harvested on 11 May 2023; eventually, the second season lasted a total of 41 d.

The design and management of the vegetables for the third growing season were in the same way as the second season. The plots were fertilized and ploughed on 10 June 2023, and the seeds were sown on 11 June. Irrigation was carried out on 17 June 2023 (1001 m^3/ha), 27 June (1126 m^3/ha) and 8 July (875 m^3/ha), respectively. The vegetables were harvested on 18 July 2023.

2.3. Collection and Analysis of Soil Samples

2.3.1. Soil Samples Collection

After the harvest of the third growing season, the soil samples (0–10 cm) collected using an auger with a diameter of 5 cm from three randomly selected sites in each plot were composited together as a single sample. Each sample was divided into four parts: the first part was oven-dried to determine the soil's water content (WC); the second part was stored at −80 °C for gene determination; the third was stored at 4 °C for analyzing the NH_4^+-N and NO_3^--N content; the remaining was air-dried for the determination of pH, electrical conductivity (EC), organic matter (OM), available phosphorus (AP), available potassium (AK), total nitrogen (TN), etc.

2.3.2. Determination of Soil and Manure Properties

The properties of soil and manures were determined according to the procedure of "Analysis Method of Agricultural Chemistry in Soil" [29]. The soil's water content was determined by drying method. pH and EC were determined using the soil/water ratio of 1:5 with a pH meter (ORION STAR A211) and an EC meter (DDB-303A), respectively. NH_4^+-N and NO_3^--N were determined by indophenol blue colorimetry and ultraviolet spectrophotometry, respectively. OM was determined by a low-temperature exothermic potassium dichromate oxidation-colorimetric method. AP was determined by the sodium bicarbonate method. AK was determined by ammonium acetate extraction method; TN was determined by the Kjeldahl method.

2.3.3. Soil DNA Extraction

A FastDNA SPIN Kit for soil (MP Biomedicals, Santa Ana, CA, USA) was employed to extract total DNA in light of the manufacturer's instructions. The concentration and the

quality of DNA were tested by a spectrophotometer (NanoDrop ND-8000, Thermo Fisher Scientific, Waltham, MA, USA) and 1.5% agar gel electrophoresis.

2.3.4. Quantification of N-Cycle Functional Genes

The WaferGen SmartChip Real-Time PCR System was utilized to perform a high-throughput quantitative PCR (HT-qPCR) of N-cycle functional genes using the 16S rRNA gene as the reference gene in Hefei Yuanzai Biotechnology Co., Ltd, Hefei, China. Samples were loaded onto the SmartChip Multisample Nanodispenser using a 96 (assays) × 54 (samples) array. The HT-qPCR array consisted of 14 primer sets, including 1 primer set targeting nitrogen mineralization gene (gdhA), 3 primer sets targeting nitrification genes (*amoA*-1, *amoA*-2, and *amoB*), 8 primer sets targeting denitrification genes (*narG*, *nirK*-1, *nirK*-2, *nirK*-3, *nirS*-1, *nirS*-2, *nirS*-3 and *nosZ*), 1 primer set targeting the nitrogen fixation gene (*nifH*) and 1 targeting the 16S rRNA gene. Each 100 nL reaction mixture contained 50 nL of 2 × LightCycler 480 SYBR Green I Master Mix (Roche Inc., East Dublin, Ga, USA), 20 nL of a 2 ng/μL DNA template, 1 nL of 0.1 mg/mL bovine serum albumin, 500 nM each of the forward and reverse primers and 19 nL of nuclease-free PCR-grade water. For each primer set, amplification was conducted in triplicate and a non-template control was included. The thermal regimen included an initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 30 s and annealing at 60 °C for 30 s. Finally, the melting curve analysis was autogenerated by the program [30].

Results of the HT-qPCR were analyzed using the SmartChip qPCR software (version 2.7.0.1). Wells with multiple melting peaks or amplification efficiencies outside the range of 1.8–2.2 were not included in the analysis. A limit was set on the number of cycles necessary to observe a significant fluorescence signal (threshold cycle, Ct). Only samples exhibiting Ct less than 31 and having more than two replicates showing amplification were regarded as positive. The primer sequences of the targeted genes were shown in Table 5. Absolute gene abundance is the absolute gene copy number per gram of dry soil. The absolute gene copy number was calculated based on the absolute 16S rRNA gene copy number quantified by conventional qPCR. The absolute gene copy number was calculated as follows:

$$GR = 10^{(31-C_t)/(10/3)} \quad (1)$$

$$GA_{\text{N-cycle gene}} = \frac{GA_{\text{16S rRNA}}.GR_{\text{N-cycle gene}}}{GR_{\text{16S rRNA}}} \quad (2)$$

where *GR* is relative gene copy number, *Ct* is the threshold cycle and *GA* is absolute gene copy number.

Table 5. The primer sequences of the genes.

Target Genes	Sequence (5′-3′)	References
gdhA	GCCATCGGYCCWTACAAGGG ATGTCRCCNGCCGGAACGTC	[31]
amoA-1	STAATGGTCTGGCTTAGACG GCGGCCATCCATCTGTATGT	[32]
amoA-2	GGGGTTTCTACTGGTGGT CCCCTCKGSAAAGCCTTCTT	[33]
amoB	TGGTAYGACATKAWATGG RCGSGGCARGAACATSGG	[34]
narG	TAYGTSGGGCAGGARAAACTG CGTAGAAGAAGCTGGTGCTGT	[35]
nirK-1	GGMATGGTKCCSTGGCA GCCTCGATCAGRTTRTGGTT	[36]
nirK-2	ATGGCGCCATCATGGTNYTNCC TCGAAGGCCTCGATNARRTTRTG	[37]

Table 5. *Cont.*

Target Genes	Sequence (5′-3′)	References
nirK-3	TGCACATCGCCAACGGNATGTWYGG GGCGCGGAAGATGSHRTGRTCNAC	[37]
nirS-1	GTSAACGTSAAGGARACSGG GASTTCGGRTGSGTCTTGA	[38]
nirS-2	ATCGTCAACGTCAARGARACVGG TTCGGGTGCGTCTTSABGAASAG	[37]
nirS-3	TGGAGAACGCCGGNCARGTNTGG GATGATGTCCACGGCNACRTANGG	[37]
nosZ	CGYTGTTCMTCGACAGCCAG CGSACCTTSTTGCCSTYGCG	[39]
nifH	AAAGGYGGWATCGGYAARTCCACCAC TGSGCYTTGTCYTCRCGGATBGGCAT	[40]
16S rRNA	GGGTTGCGCTCGTTGC ATGGYTGTCGTCAGCTCGTG	[41]

2.4. Data Processing and Statistical Analysis

Microsoft Excel 2019 was used for data processing; Origin 2022 was used for principal component analysis (PCA) [42], redundancy analysis (RDA) [43] and drawing. R software (version 4.1.0) was used for the one-factor analysis of variance (ANOVA), Duncan's multiple range test, permutation multivariate analysis of variance (PERMANOVA) [20], Monte Carlo permutation test (999 permutations) [20] and stepwise regression analysis [43]. ANOVA was used to test the differences between treatments and Duncan's multiple range test was used to conduct the comparisons of treatment-means; a probability of $p < 0.05$ was deemed to be significant. PCA and PERMANOVA based on weighted UniFrac phylogenetic distance was used to assess the differences in the abundance of N-cycling genes in soil of different treatments. RDA, the Monte Carlo permutation test (999 permutations) and stepwise regression analysis were performed to investigate the correlation between N-cycling genes and environmental factors. The gene abundance increase rate was calculated as follows:

$$\text{Gene abundance increase rate} = \frac{(\text{Gene absolute abundance}_F - \text{Gene absolute abundance}_{CK})}{\text{Gene absolute abundance}_{CK}} \times 100\% \quad (3)$$

where *F* and *CK* refer to fertilization treatment and no fertilization, respectively.

3. Results and Analysis

3.1. Effects of Different Treatments on Soil Basic Properties

After the harvest of the third growing season, the soil water content fluctuated around 15% (Table 6). The different fertilizations exerted a negligible effect on soil pH. The soil EC value was higher in CK than the original soil, indicating the irrigation led to the accumulation of salt in the soil. Fertilization also increased the soil EC, with the maximum value in the PH treatment. The content of soil OM, NH_4^+-N, NO_3^--N, AP, AK and TN was augmented by fertilization relative to CK, with the highest increase in the SH, SH, S, P, CH and PH treatment, respectively.

3.2. Effects of Different Treatments on the Abundance of 16S rRNA Gene in Soil

Most treatments increased the abundance of the 16S rRNA gene in the soil as compared with CK (Figure 1a). Specially, the abundance of the 16S rRNA gene in pig manure and sheep manure treatments was lifted significantly by 67.05% and 106.97%, respectively, compared to CK, suggesting the more conducive effect of pig manure and sheep manure on the growth and reproduction of bacteria than chicken manure. Exceptionally, the CH treatment lowered the abundance of the 16S rRNA gene by 7.11% relative to CK. When

fulvic acid was introduced, the 16S rRNA gene abundance in the manure-added soils fell, especially for the chicken-manure-added soil.

Table 6. Soil properties after the third growing season.

Treatment /	WC (%)	EC (dS/m)	pH /	OM (g/kg)	NH_4^+-N (mg/kg)	NO_3^--N (mg/kg)	AP (mg/kg)	AK (mg/kg)	TN (g/kg)
OS	20.68 ± 0.01	0.29 ± 0.02	8.35 ± 0.05	25.24 ± 2.93	0.98 ± 0.25	128.12 ± 21.45	34.33 ± 8.01	366.00 ± 63.38	0.85 ± 0.04
CK	14.05 ± 0.56 b	1.25 ± 0.08 ab	8.21 ± 0.24 a	22.18 ± 0.54 c	18.10 ± 1.75 b	71.72 ± 14.17 c	36.90 ± 1.66 b	671.06 ± 34.12 b	1.11 ± 0.01 b
PH	15.73 ± 0.91 ab	1.57 ± 0.06 a	8.15 ± 0.06 a	26.09 ± 1.05 ab	20.14 ± 1.98 b	169.69 ± 8.90 ab	69.04 ± 0.94 a	733.65 ± 20.97 b	1.52 ± 0.08 a
P	15.23 ± 0.72ab	1.07 ± 0.08 b	8.29 ± 0.11 a	25.75 ± 0.85 ab	30.08 ± 2.35 ab	199.53 ± 9.99 ab	69.21 ± 1.00 a	776.01 ± 33.89 b	1.44 ± 0.10 a
CH	13.97 ± 0.70 b	1.52 ± 0.05 ab	8.21 ± 0.11a	25.17 ± 0.95 b	37.25 ± 2.56 a	182.57 ± 4.18 ab	58.24 ± 2.13 a	932.75 ± 42.80 a	1.46 ± 0.06 a
C	15.24 ± 0.54ab	1.40 ± 0.05 ab	8.30 ± 0.13 a	25.53 ± 0.39 b	33.20 ± 3.31 a	118.56 ± 14.96 bc	59.74 ± 0.86 a	781.17 ± 39.07 b	1.42 ± 0.03 a
SH	16.66 ± 0.88 a	1.07 ± 0.07 b	8.31 ± 0.02 a	28.22 ± 0.14 a	37.45 ± 2.24 a	134.73 ± 15.16 bc	66.77 ± 1.73 a	751.71 ± 25.28 b	1.38 ± 0.07 a
S	14.88 ± 0.87 ab	1.27 ± 0.04 ab	8.33 ± 0.11 a	27.21 ± 1.07 ab	26.25 ± 3.00 ab	230.05 ± 7.40 a	66.18 ± 1.73 a	778.49 ± 30.85 b	1.41 ± 0.05 a

Note: OS, original soil; CK, no fertilization; P, pig manure; C, chicken manure; S, sheep manure; H, fulvic acid; WC, water content; EC, electrical conductivity; OM, organic matter; AP, available phosphorus; AK, available potassium; TN, total nitrogen. The data are expressed as the mean ± standard deviation. Lower-case letters in each column indicate significant differences between groups.

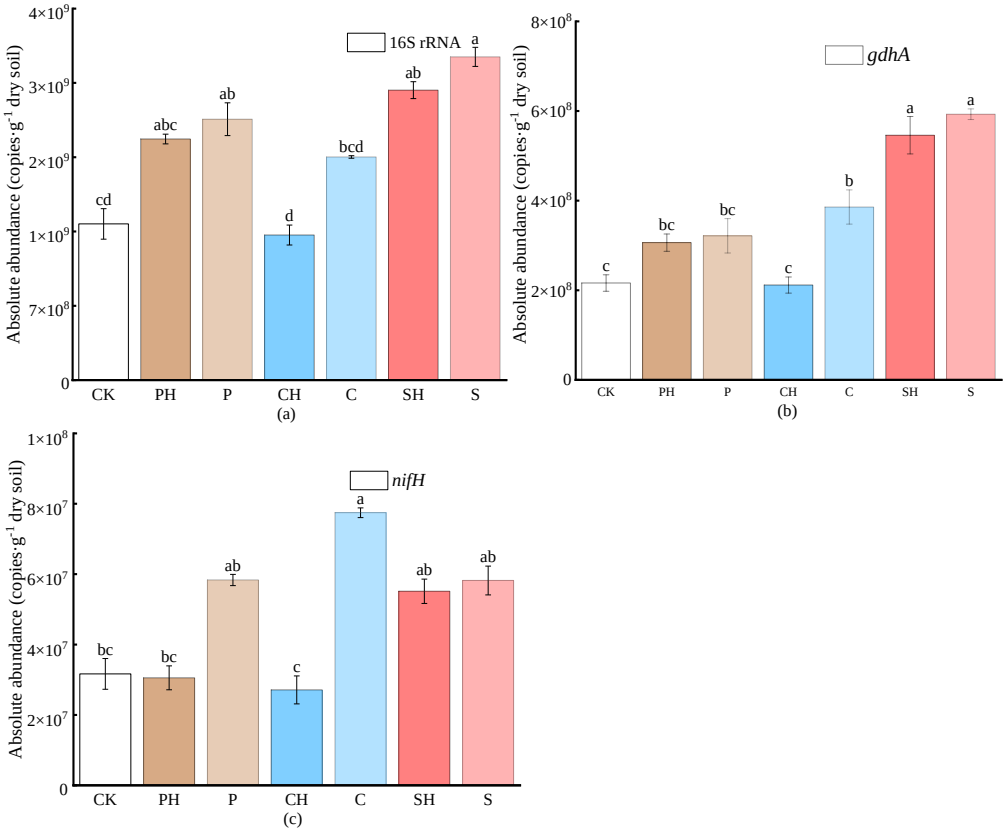

Figure 1. The abundance of the 16S rRNA (**a**), *gdhA* (**b**) and *nifH* (**c**) gene in soil after the third planting of Chinese cabbage. Note: CK, no fertilization; P, pig manure; C, chicken manure; S, sheep manure; H, fulvic acid. The data are expressed as the mean ± standard deviation. Lower-case letters above each column indicate significant differences between groups.

3.3. Effects of Different Treatments on the Abundance of gdhA Gene in Soil

Fertilization (except CH treatment) increased the abundance of the *gdhA* gene in soil (Figure 1b), with the significant improvement in C, SH and S treatments. Sheep manure treatments had a better performance than pig and chicken manure treatments. Similar with the 16S rRNA gene, the additional fulvic acid had a tendency to reduce the abundance of the *gdhA* gene in the manure-added soils, with the most obvious decline (45.15%) in chicken-manure-added soil.

3.4. Effects of Different Treatments on the Abundance of *nifH* Gene in Soil

The abundance of the *nifH* gene was boosted by a single manure application, especially chicken manure; simultaneously, it went down in the soil appended with manure ascribed to the introduction of fulvic acid (Figure 1c). The abundance of the *nifH* gene in the CH treatment was reduced by 65.00% at a significant level compared to the single chicken manure treatment. Though *nifH* abundance was not dramatically altered by the treatments mingling manure and fulvic acid, it descended slightly in the PH and CH treatments in comparison to CK.

3.5. Effects of Different Treatments on the Abundance of Nitrification Genes in Soil

The abundance of nitrification genes in the soil went up under the effect of fertilization (Figure 2). Compared with CK, the chicken manure treatment significantly increased *amoA*-1 gene abundance (Figure 2a), sheep manure increased the abundance of both the *amoA*-1 and *amoA*-2 genes (Figure 2a,b) and the pig manure treatment increased *amoB* gene abundance (Figure 2c). Again, the fulvic acid addition decreased the abundance of nitrification genes in the manure-added soils generally. Take *amoA*-2 gene as an example, its abundance responded to fulvic acid rather sensitively in the sheep-manure-added soils.

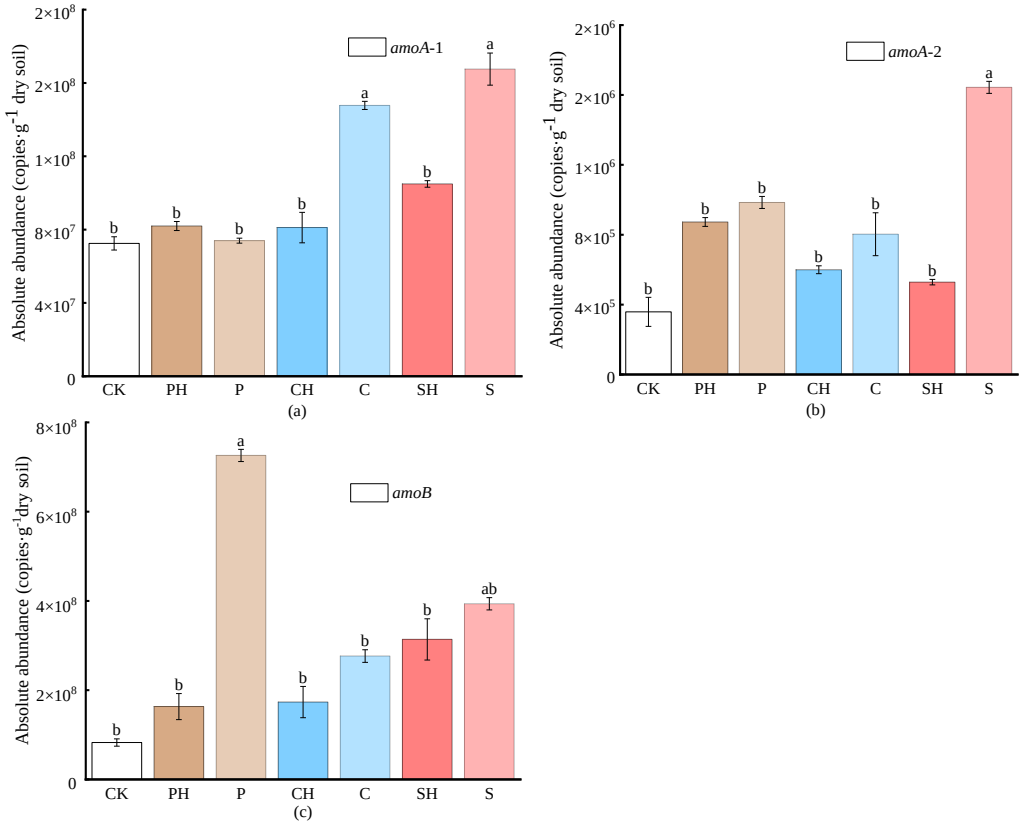

Figure 2. The abundance of nitrification genes *amoA*-1 (**a**), *amoA*-2 (**b**), and *amoB* (**c**) in soil after the third planting of Chinese cabbage. Note: CK, no fertilization; P, pig manure; C, chicken manure; S, sheep manure; H, fulvic acid. The data are expressed as the mean ± standard deviation. Lower-case letters above each column indicate significant differences between groups.

3.6. Effects of Different Treatments on the Abundance of Denitrification Genes in Soil

The distribution of denitrification genes showed a very similar response to fertilization as the nitrification genes studied here. With a few exceptions in the CH treatment, manure application, particularly sheep manure, increased the abundance of the denitrification genes in soil (Figure 3a,c,d). Gene *nosZ* (Figure 3b), encoding nitrous oxide reductase, was more abundant compared with other denitrification genes. For the three *nirK* genes, the

abundance of *nirK*-3 was the highest in most cases in addition to the high abundance of *nirK*-1 in the single pig manure treatment, while the abundance of *nirK*-2 was considerably lower. The abundance of *nirS*, another gene involved in nitrite reductase, was higher than *nirK*, indicating *nirS* played a more essential role in nitrite reduction in our soil. Gene *nirS*-2 was the dominate *nirS* gene among the three *nirS* genes considering their abundance. Due to the fulvic acid input, the abundance of *narG* and *nirS-1* in the soil amended with chicken manure dropped sharply, and the abundance of *nirK-1* and *nirK-3* was cut down remarkably in the soil supplemented with pig manure and sheep manure, respectively; manifesting the extra fulvic acid caused a decrease in the abundance of denitrification genes in the soil applied with manure basically.

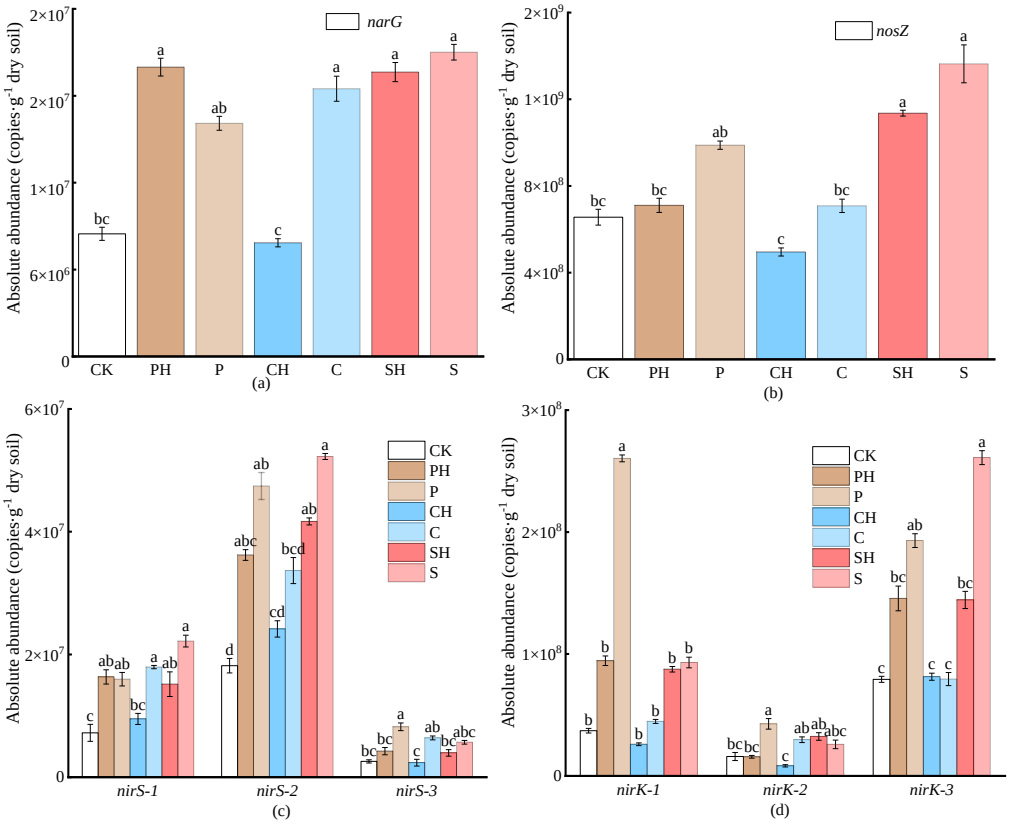

Figure 3. The abundance of denitrification genes *narG* (**a**), *nosZ* (**b**), *nirS* (**c**) and *nirK* (**d**) in soil after the third planting of Chinese cabbage. Note: CK, no fertilization; P, pig manure; C, chicken manure; S, sheep manure; H, fulvic acid. The data are expressed as the mean ± standard deviation. Lower-case letters above each column indicate significant differences between groups.

3.7. Differences of N-Cycling Genes in Different Fertilization Treatments

The results of the principal component analysis (PCA) of soil N-cycle functional genes impacted by different fertilization treatments are presented in Figure 4. Fertilization significantly changed the overall distribution of N-cycle functional genes (PERMANOVA: $F = 69.993$, $p < 0.001$). The treatments could be broadly classified into four groups: CK and CH treatments formed the first group, which was considerably disparate with other treatments along the first PCA axis, accounting for the 64.2% of variance; PH, C and SH treatments formed the second group; S treatments formed the third group; and P treatments stood alone, distinguished with other treatments along the second PCA axis explaining 16.2% of variance. The increase rates of the abundance of N-cycle functional genes in different treatments compared to CK were calculated (Table 7). Fertilization mainly increased the abundance of *amoA*-2, *amoB*, *narG*, *nirK*-1, *nirS*-1 and *nirS*-2, and the increase of different genes varied greatly. The P and S treatments increased the gene abundance

more potently than the PH, C and SH treatments, while the influence of the CH treatment was insignificant.

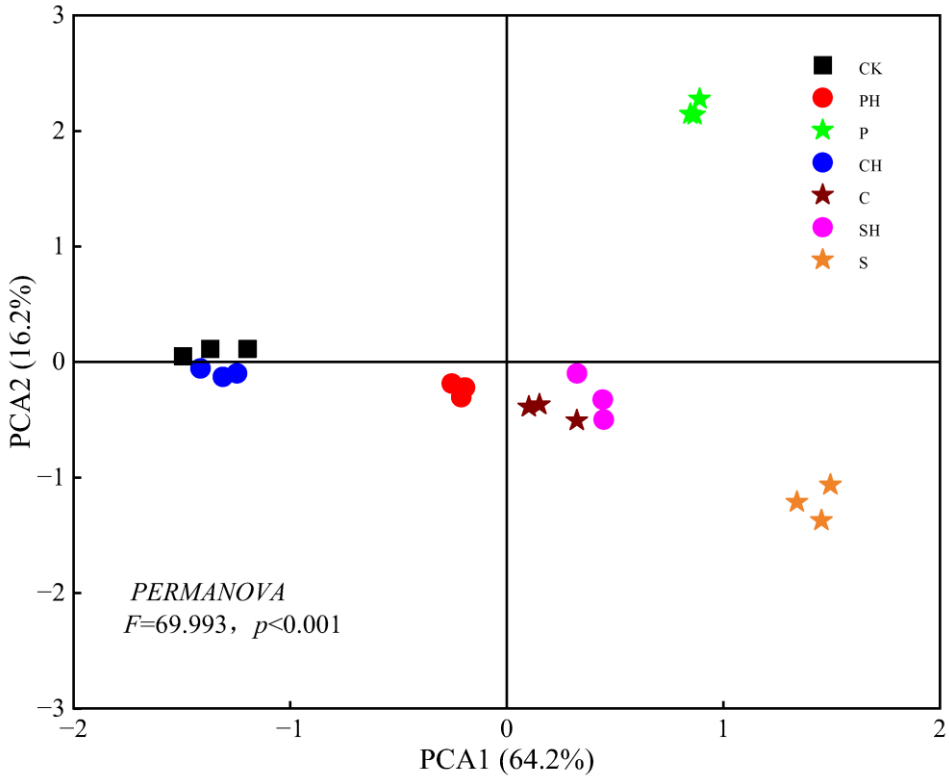

Figure 4. Principal component analysis of N-cycle functional genes. Note: CK, no fertilization; P, pig manure; C, chicken manure; S, sheep manure; H, fulvic acid.

Table 7. Increase rate in the abundance of N-cycle functional genes in different treatments compared to CK (%).

	PH	P	CH	C	SH	S	Average Increase 2
16S rRNA	54.36	67.05	−7.11	42.87	85.65	106.97	58.30
gdhA	41.69	48.73	−2.11	78.47	152.58	174.26	82.27
amoA-1	13.11	2.09	11.92	103.90	44.79	131.24	51.18
amoA-2	143.40	174.50	67.14	123.89	47.35	358.21	152.42
amoB	97.38	777.90	109.63	234.31	279.46	376.06	312.46
narG	135.90	90.10	−7.32	118.31	131.87	148.05	102.82
nirK-1	154.52	601.59	−30.01	20.47	135.71	150.59	172.15
nirK-2	−1.60	167.68	−48.01	86.29	102.90	62.30	61.59
nirK-3	83.76	143.62	2.73	0.27	82.19	229.51	90.35
nirS-1	126.20	121.06	31.39	148.43	109.83	207.01	123.99
nirS-2	99.22	161.03	32.96	85.26	129.31	187.55	115.89
nirS-3	64.77	218.86	−8.22	148.42	53.58	120.84	99.71
nosZ-2	8.33	50.67	−24.47	8.00	73.18	107.89	37.27
nifH	−3.52	84.33	−14.35	144.69	74.17	83.85	61.53
Average increase 1	72.68	193.52	8.16	95.97	107.33	174.60	

Note: P, pig manure; C, chicken manure; S, sheep manure; H, fulvic acid. Average increase 1 is the average of the increase rate in the abundance of all genes in each treatment, and average increase 2 is the average of increase rate in the abundance of 16S rRNA or one N-cycle functional gene in all treatments.

3.8. Effects of Environmental Factors on N-Cycle Functional Genes

A redundancy analysis (RDA) showed that the overall effect of all environmental factors on N-cycle functional genes was significant (p = 0.002) (Figure 5), and OM

($p < 0.01$), AP ($p < 0.01$), NO_3^--N ($p < 0.05$) and EC ($p < 0.05$) influenced significantly the abundance changes of N-cycle functional genes by the Monte Carlo permutation test. This was also confirmed in the stepwise regression analyses (Table 8). The environmental factors (except AK and EC) were mainly positively correlated with the N-cycle functional genes. We also found the *gdhA* gene was more related with OM content than other genes. The denitrification genes *nirS*-2, *nirS*-3 and *nosZ* were closely associated with AP, NO_3^--N content. Genes *nirK*-1, *amoB*, *nirS*-3 and *nirS*-2 formed a cluster, and the increase in their abundance were obviously linked with the decrease in the value of EC.

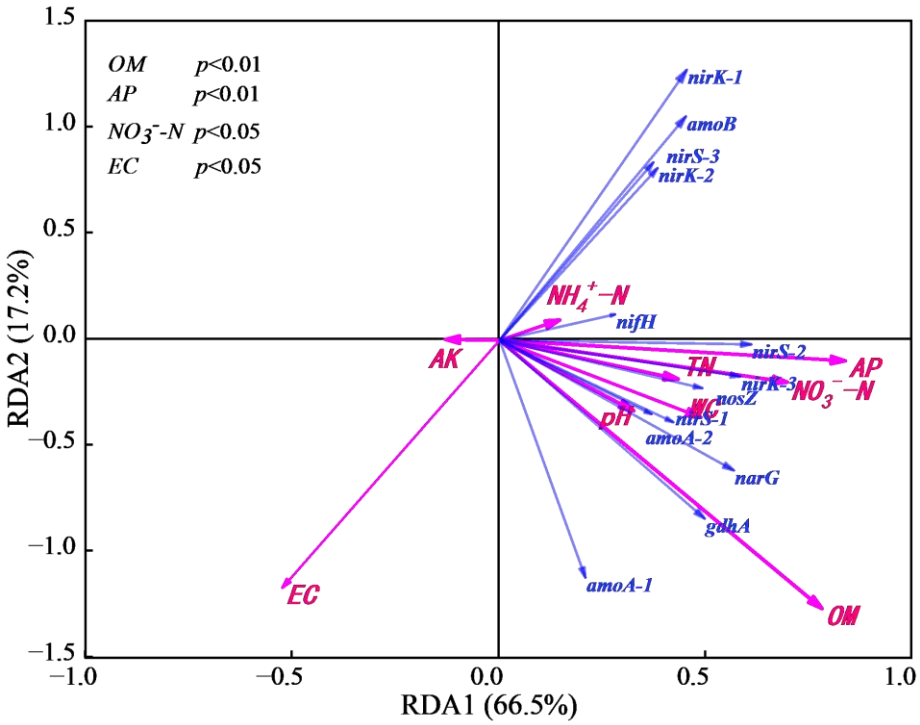

Figure 5. Redundancy analysis of the abundance of N-cycle functional genes and environmental factors. Note: WC, water content; EC, electrical conductivity; OM, organic matter; AP, available phosphorus; AK, available potassium; TN, total nitrogen.

Table 8. Soil properties correlated with N-cycle functional genes analyzed by stepwise regression analysis.

Genes	Explanatory Variables	R^2
16s rRNA	NO_3^--N, OM, AK	0.77 ***
gdhA	pH, OM	0.60 ***
amoA-1	NA	
amoA-2	NO_3^--N	0.59 ***
amoB	WC, EC, OM, AP	0.77 ***
narG	NH_4^+-N, NO_3^--N, OM, AP	0.72 ***
nirK-1	WC, pH, EC, OM, AP, AK	0.85 ***
nirK-2	EC, AP	0.59 ***
nirK-3	NO_3^--N, OM, AK, TN	0.86 ***
nirS-1	AP	0.46 ***
nirS-2	EC, NO_3^--N, AP, AK	0.84 ***
nirS-3	AP	0.26 *
nosZ	EC, OM, AP, AK	0.69 ***
nifH	NA	

Note: WC, water content; EC, electrical conductivity; OM, organic matter; AP, available phosphorus; AK, available potassium; TN, total nitrogen; NA, no optimal fitting model; R^2, the proportion of variance explained by model; ***, $p < 0.001$; *, $p < 0.05$.

4. Discussion

4.1. Effects of Fertilization on N-Cycle Functional Genes

It is known that chemical fertilizers exert a substantial effect on the abundance of N-cycle functional genes in soil, but the effect may be beneficial or detrimental [16,20]. The effects of organic fertilizers on them are usually favorable [19], due to the fact that organic fertilizers are rich in nutrients released slowly which could improve soil fertility for a longer period of time [44], and consequently provide a more stable environment for the growth and reproduction of soil micro-organisms. In this study, different manures increased the abundance of main N-cycle functional genes in soil, consistent with the results of a previous study [45]. Among them, the addition of pig and sheep manure, especially sheep manure, was associated with a more powerful response of N-cycle functional genes than the addition of chicken manure. Additionally, the abundance of these genes showed the weakest response to the chicken manure application with fulvic acid. This is acceptable since the addition of sheep and pig manure improved the content of OM, AP, NO_3^--N and other properties in the soil compared with the addition of chicken manure (Table 6). In other words, different fertilizer treatments created different soil environments [46], leading to the differences in their impacts on the genes. The increase in the abundance of the nitrification and denitrification genes indicated the increased potential of nitrification and denitrification processes in the soil. The complete denitrification reaction releases nitrogen into the atmosphere in the form of gas, resulting in the loss of nitrogen and the nitrification in the soil provided the reaction substrate for denitrification [47]. As a result, the increase in the abundance of nitrification and denitrification genes resulting from manure addition might risk soil nitrogen loss, and similar results were also found in an early study [42].

We also found that fulvic acid reduced the abundance of N-cycle functional genes in soil caused by manure usage. One possible reason is that fulvic acid addition lowered the soil pH (Table 6), affecting the abundance of N-cycle genes [48]. In addition, the supply of fulvic acid could accelerate the nutrient uptake of crops and thereby the crop biomass [49], and increase the competition for nutrients between crops and micro-organisms and between micro-organisms [50,51]. The reduction originating from fulvic acid input might reduce the soil nitrogen loss, which is conducible to the efficient use of nutrients.

4.2. Effects of Fertilization on N-Cycle

Manure addition provided a large amount of organic nitrogen to the soil environment, which can be used by crops and micro-organisms when converted to inorganic nitrogen. This conversion is controlled by mineralizing micro-organisms in soil [52]. In this study, the increase in *gdhA* gene abundance was relatively higher in sheep-manure-applied soils, indicating that micro-organisms containing the *gdhA* gene were more abundant in the soil added with sheep manure than those with pig and chicken manure. Nitrate, the product of nitrification reaction [53], is an important nitrogen nutrient for nitrate-loving crops, but highly mobile and prone to nitrogen losses such as runoff and leaching. The abundance of the nitrification gene *amoA*-1 in this study was higher than that of the *amoA*-2 gene, in line with the study of Xu [54]. Fertilization particularly increased the abundance of the nitrification genes *amoA*-2 and *amoB*, meaning that *amoA-2* and *amoB* genes possessed a greater effect on soil nitrification than the *amoA*-1 gene in this study. This is understandable, because the *amoA*-2 and *amoB* genes dominate nitrification under alkaline conditions [55,56].

Denitrification genes are important indicators of soil denitrification [57]. In this study, manures addition significantly increased the abundance of the *narG* gene involved in the first step of denitrification (nitrate reduction). The *nirK* and *nirS* genes associated with the second step of denitrification (nitrite reduction) were also enriched by the addition of manures. However, the response of the targeted *nirK* or *nirS* genes with different primers was distinct: the abundance of the *nirK*-1 and *nirK*-3 genes were higher than that of *nirK*-2 genes, and the abundance of the *nirS*-1 and *nirS*-2 genes were higher than the *nirS*-3 gene. Whether genes with high abundance contribute more to soil denitrification remains to be further studied. Soil nitrogen fixation converts atmospheric N_2 by nitrogen-fixing

micro-organisms into ammonia that can be utilized by crops [10]. Here, the increase in the abundance of the *nifH* gene was comparable between the SH and S treatments. Whereas, for the pig and chicken manure treatments, the addition of fulvic acid had a significant inhibitory effect on *nifH* gene abundance. Overall, the effects of a combined application with manure and fulvic acid on the soil nitrogen cycle under a long-term positioning test need to be confirmed by further research.

4.3. Effects of Environmental Factors on N-Cycle Functional Genes

In this study, both the redundancy analysis and stepwise regression analysis confirmed that OM, AP, NO_3^--N and EC were the main factors affecting N-cycle functional genes, in accord with the results of previous studies [8,58]. OM, the basis of soil fertility, can improve the soil environments [59] and provide nutrients and energy for soil micro-organisms. It is believed that AP content and the abundance of N-cycle functional genes were closely linked [58], in consistency with this study. This suggests the strong coupling between soil phosphorus and N-cycle micro-organisms, since nitrogen fertilization promotes the demand for phosphorus by soil micro-organisms [60], and the abundant phosphorus in manure is the guarantee for the increase of soil nitrogen transformation genes [61]. Lan [62] proposed that NO_3^--N as a reaction substrate for soil denitrification is closely linked to denitrification genes. The relationship between EC and gene abundance is expected [63], because the higher the salt content, the stronger the salt stress soil micro-organisms are subjected to.

In addition, this study also concluded that WC, pH, NH_4^+-N, AK and TN exerted less effect on the abundance of functional genes for the soil nitrogen cycle. However, it is proven that soil pH could affect the abundance of soil N-cycle micro-organisms and functional genes significantly [11,64]. The weaker correlation in this study might be due to the small fluctuating range of pH (8.15–8.33) and the fact that manures had a longer duration of interaction with micro-organisms and a greater regulation of soil acidity and alkalinity compared to chemical fertilizers. Many studies had found that NH_4^+-N and TN were also important factors influencing N-cycle functional micro-organisms [19,64]. The present study observed a minor effect of them on the abundance of N-cycle functional genes, probably because manures were able to provide balanced and stable nutrients [65], all of which could satisfy the requirements of soil micro-organisms. In alkaline soil, the content of NH_4^+-N is always low (Table 6), which also leads to its weak correlation with N-cycle functional genes. TN in soil could not be easily altered in a short time; therefore, the association between TN and N-cycle functional genes was not constructed apparently here. What is more, soil temperature [6], soil type [66,67], crop diversity [68] and other factors also affect the abundance of N-cycle functional genes expressively. Due to the limited environmental factors determined and the short experimental period in this study, the effects of multiple environmental factors on soil N-cycle functional genes under the long-term positioning experiment need to be further explored.

5. Conclusions

In this study, fertilization increased the abundance of N-cycle functional genes in the soil, particularly nitrification and denitrification genes, such as *amoA*-2, *amoB*, *narG*, *nirK*-1, *nirS*-1 and *nirS*-2, and increased the risk of soil nitrogen loss. The response of sheep and pig manure to the N-cycle functional genes were more potent than chicken manure. Additional fulvic acid tended to reduce the abundance of main N-cycle functional genes in the manure-added soils, especially for nitrification and denitrification genes. OM, AP, NO_3^--N and EC were the main environmental factors affecting the abundance of N-cycle functional genes. Considering the changes in the abundance of main N-cycle functional genes, it was concluded that the fertilization with sheep manure (31.3 t/ha) and fulvic acid (7.5 kg/ha) was more effective in regulating the N-cycle functional genes in soil.

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