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Differential Responses of Soil Microbial N-Cycling Functional Genes to 35 yr Applications of Chemical Fertilizer and Organic Manure in Wheat Field Soil on Loess Plateau

Yinyan Liang [1], Caidi Yang [1], Upendra M. Sainju [2], Nannan Zhang [1], Fazhu Zhao [1], Weizhen Wang [3] and Jun Wang [1,4,*]

[1] Shaanxi Key Laboratory of Earth Surface System and Environmental Carrying Capacity, College of Urban and Environmental Science, Northwest University, Xi'an 710127, China; lyinyan@stumail.nwu.edu.cn (Y.L.); 11714043@zju.edu.cn (C.Y.); nanzhangstu@163.com (N.Z.); zhaofazhu@nwu.edu.cn (F.Z.)
[2] Northern Plains Agricultural Research Laboratory, USDA-ARS, 1500 North Central Avenue, Sidney, MT 59270, USA; upendra.sainju@usda.gov
[3] College of Life Sciences, Northwest University, Xi'an 710069, China; wwz870220158@126.com
[4] State Key Laboratory of Soil Erosion and Dryland Farming on the Loess Plateau, Institute of Soil and Water Conservation, Chinese Academy of Sciences and Ministry of Water Resources, Xianyang 712100, China
* Correspondence: wangj@nwu.edu.cn

Abstract: Fertilization changes nitrogen (N)-cycling processes and associated microbial communities in agricultural ecosystems. However, the long-term responses of N-cycling potential and microbial functional genes to different fertilization sources remain unclear. Soil samples were collected to a depth of 20 cm under winter wheat applied annually with N fertilizer (NF), organic manure (OM), N fertilizer plus organic manure (NM), and a control without fertilization (CK) for 35 yr, and they were analyzed for microbial functional genes involved in soil N cycling using metagenome sequencing in the Loess Plateau of China. Soil N fractions were greater with OM and NM than NF and CK. The total abundances of N-cycling genes were 9.3% ($p < 0.05$) greater with NM than CK, and 8.2% ($p < 0.05$) and 12.2% ($p < 0.01$) higher with OM and NM than NF, respectively. Compared to CK, OM and NM increased the abundance of genes associated with nitrification, denitrification, dissimilatory nitrate reduction, and assimilatory nitrate reduction, but decreased the abundance of genes related to organic N metabolism. However, NF increased the abundance of genes involved in nitrification. Both OM and NM also enhanced the relative abundance of *Proteobacteria* carrying N-cycling genes but reduced those of *Firmicutes* and *Cyanobacteria*. Soil organic carbon, total N, and potential carbon mineralization were the dominant factors affecting the abundances of N-cycling genes. Long-term application of OM and NM can promote N cycling by enhancing gene abundance due to increased soil organic matter and microbial biomass compared to NF and CK.

Keywords: fertilizer application; N transformation; functional genes; microbial community

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1. Introduction

Nitrogen (N) cycling in agroecosystems is driven primarily by microbial abundance and activity which release enzymes encoded by functional genes [1,2]. For instance, *amoA* encoding ammonium monooxygenase is involved in nitrification, but *narG* encoding nitrate reductase, *nirK* encoding nitrite reductase, *norB* encoding nitric oxide reductase, and *nosZ* encoding nitrous-oxide reductase are responsible for denitrification [3]. Long-term application of N fertilizers and organic manure can change microbial community structure and functional gene abundance, which regulate N transformation and availability, thereby affecting crop growth and yield [4–6]. Therefore, information on functional genes related to N cycling is needed for N management practices to sustain crop yields.

The source of N fertilizer can influence functional genes related to nitrogen cycling. Chemical N fertilizer has high effective nutrient contents, which can rapidly promote crop

growth and directly stimulate the microbial community and related genes [7,8]. A meta-analysis suggested that N fertilization markedly increased the abundance of genes related to nitrification (*amoA*) and denitrification (*nirK*, *nirS*, and *nosZ*) [9]. Organic fertilizers provide continuous and balanced nutrients for soil, which increase the number and diversity of most soil microorganisms related to N cycling, thus promoting soil N-cycling rates [10,11]. Application of organic manure and compost significantly increased the abundance of all N-cycling genes, affecting N transformation processes stronger than N fertilization [9,12]. The combined application of inorganic N fertilizer and organic manure can further enhance N-cycling genes, thereby improving the sustainability of crop production systems [13,14].

Previous studies focused only on specific genes and lacked a comprehensive understanding of microbial functional genes and metabolic potential responsible for N cycling under long-term fertilization. Recent advances in metagenomics sequencing can provide more information on functional genes related to N cycling and can comprehensively and accurately reflect the composition, structure, and functional potential of microbial communities that release enzymes encoded by various functional genes [15,16]. As information on functional genes related to N cycling is lacking, we evaluated the long-term effects of N fertilizer sources (inorganic N fertilizer and organic manure) on N-cycling genes under winter wheat in the Loess Plateau of China. We hypothesized that long-term application of a combination of inorganic N fertilizer and organic manure would enhance the abundances of N-cycling functional genes compared to either source of fertilizer alone or the control without fertilization. The objectives of the study were as follows: (1) to examine the differential responses of N-cycling gene abundance and functional potential to long-term fertilization; and (2) to explore the mechanisms responsible for N cycling due to functional genes affected by microbial communities and activities.

2. Materials and Methods

2.1. Site Description

A long-term field experiment was established in 1984 at the Changwu State Key Agro-Ecological Experimental Station in China (35°12′ N, 107°40′ E, altitude 1220 m). The site has a sub-humid continental monsoon climate with mean annual air temperature and precipitation of 9.1 °C and 584 mm, respectively. The soil is Heilutu silt loam (Calcarid Regosol, FAO Soil Classification System) with sand, silt, and clay concentrations of 4.5, 65.6, and 30.9%, respectively. The soil at a depth of 0–30 cm had pH of 8.4, bulk density (BD) of 1.30 g·cm^{-3}, soil organic carbon (SOC) of 6.50 g·kg^{-1}, total N (STN) of 0.80 g·kg^{-1}, and $CaCO_3$ of 105 g·kg^{-1} at the beginning of the experiment in 1984. A detailed description of the long-term field experiment was previously reported [17].

2.2. Experimental Design

The experiment included four fertilization treatments under winter wheat: (1) CK, the control with no N fertilizer; (2) NF, chemical N fertilizer; (3) OM, organic manure; and (4) NM, chemical N fertilizer plus organic manure. The treatments were arranged in a randomized block design and replicated three times. The plot size was 10.3 m × 6.5 m with a strip of 0.5 m between plots. Following local practices, urea (46% N) at a rate of 120 kg·N·ha^{-1} for NF and NM treatments and cattle manure at a rate of 2205 kg·ha^{-1} (equivalent to 800 kg·C·ha^{-1}, 87 kg·N·ha^{-1}, and 44 kg·P·ha^{-1}) for OM and NM were applied to winter wheat every year. Winter wheat (*Triticum aestivum* L., cultivar Changwu 134) was sown at 180 kg seeds ha^{-1} with 20 cm row spacing in late September each year. At planting, all fertilizers were broadcast and incorporated into the soil at a depth of 15 cm using a rotary tiller. Weeds were controlled using manual weeding and pesticides (Bifenthrin) were applied before, during, and after winter wheat growth as needed. No irrigation was applied. Winter wheat was harvested in late June each year.

2.3. Soil Sampling

Soil samples were collected at 0–20 cm after wheat harvest in June 2018. Ten soil cores (2.5 cm inside diameter) were randomly sampled from each plot in a zig-zag pattern. Samples were placed in a sterile plastic bag, homogenized, placed in a cooler containing ice, and immediately transported to the laboratory for analysis. A total of 12 soil samples (4 treatments × 3 replicates) were obtained. In the laboratory, samples were sieved (2.0 mm) and all crop residues, rocks, and other foreign materials were removed. Each sample was divided into two parts: one part was air-dried and stored for analysis of physical and chemical properties, and the other part was stored at −80 °C for DNA extraction and metagenomic sequencing.

2.4. Laboratory Analysis

SOC and STN concentrations were measured using the dry combustion method with a C and N analyzer (Euro Vector EA3000, Manzoni, Italy) [18]. The C:N ratio represents the ratio of the concentrations of SOC to STN. Particulate organic C and N (POC and PON) were determined using the particle separation method with sodium hexametaphosphate [19]. Potential C and N mineralization (PCM and PNM) was measured using the incubation method, measuring CO_2 flush and mineralizable N, respectively [20]. The microbial biomass of C and N (MBC and MBN) was measured using the chloroform fumigation method [21]. The concentrations of NH_4^+-N and NO_3^--N were determined using an autoanalyzer (CleverChem380, DeChem-Tech, Hamburg, Germany) by the modified Griess–Illosvay method [18]. Soil pH (soil: water at 1:2.5 ratio) was measured using a PHS-3C pH meter (Rex, Shanghai, China). Gravimetric soil water content (SWC) and BD in the field were measured by collecting five undisturbed cores (2.5 cm inside diameter) randomly within a plot. Samples were oven-dried at 105 °C for 24 h to determe SWC and BD, which were calculated by dividing the weight of oven-dried soil by the volume of the core.

2.5. Soil DNA Extraction and Sequencing

Total genomic DNA was extracted from 0.5 g soil samples using the FastDNA® Spin Kit (MP Biomedicals, Santa Ana, CA, USA) following the manufacturer's instructions. The concentration and purity of extracted DNA were measured using a TBS-380 Mini-Fluorometer and a NanoDrop2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA), respectively, and the quality was measured on 1% agarose gel electrophoresis. DNA extracts were fragmented using Covaris M220 (Gene Company Limited, Hongkong, China) to an average size of approximately 400 bp for paired-end library construction using the NEXTflex™ Rapid DNA-Seq Kit (Bioo Scientific, Austin, TX, USA). Paired-end sequencing was performed on the Illumina Hiseq4000 platform (Illumina Inc., San Diego, CA, USA) at Majorbio Bio-Pharm Technology Co., Ltd., (Shanghai, China) using HiSeq X Reagent Kits (Illumina, USA). The raw sequence data relevant to this project were deposited in the National Center for Biotechnology Information (NCBI) database with accession number PRJNA898982 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA898982, accessed on 9 May 2023).

2.6. Metagenomic Analysis

The basic metagenomic sequencing information is shown in Table S1. The raw reads from metagenomic sequencing were quality-controlled using fastp (https://github.com/OpenGene/fastp, accessed on 9 May 2023, version 0.20.0) to generate clean reads after removing adaptor sequences, trimming, and removing low-quality reads with a length < 50 bp, an average quality < 20, and nitrogen bases. The high-quality reads were assembled to contigs using Megahit (https://github.com/voutcn/megahit, accessed on 9 May 2023, version 1.1.2) with the optimal k mer parameter (k mer_min = 47, k mer_max = 97, step = 10). Open reading frames (ORFs) were predicted on contigs with lengths > 300 bp using MetaGene (http://metagene.cb.k.u-tokyo.ac.jp/, accessed on 9 May 2023). The

predicted ORFs with length > 100 bp were translated into amino acid sequences. All predicted gene sequences with 90% identity and 90% coverage were clustered to construct a non-redundant gene catalog using CD-HIT (http://www.bioinformatics.org/cd-hit/, accessed on 9 May 2023, version 4.6.1). Reads after quality control were mapped to the non-redundant gene catalog with 95% identity using SOAPaligner (http://soap.genomics.org.cn/, accessed on 9 May 2023, version 2.21). The number of reads corresponding to functional genes was counted for each sample. The non-redundant gene catalog was searched against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/keeg/, accessed on 9 May 2023) using Diamond (https://github.com/bbuchfink/diamond, version 0.8.35) for functional annotation where we selected N-cycling functional genes. Genes were taxonomically annotated based on the NCBI NR database (ftp.ncbi.nlm.nih.gov/blast/db/, accessed on 9 May 2023) using blastp (ftp.ncbi.nlm.nih.gov/blast/executables/blast+/, accessed on 9 May 2023, version 2.3.0) for taxonomic annotation.

For statistical analysis of gene abundances, we normalized the abundance values using trans per million [TPM: (Reads Number/Gene Length) _Relative × 1,000,000] [22]. The response of N-cycling functional genes due to the addition of different fertilizers was described using 24 genes. These functional genes encode 5 pathways belonging to N-cycling processes that include nitrification, denitrification, dissimilatory nitrate reduction (DNRA), assimilatory nitrate reduction (ANRA), and organic N metabolism (ONM). Detailed information on these functional genes is listed in Table S2.

2.7. Statistical Analysis

One-way analysis of variance (ANOVA) with least significant difference (LSD) tests to separate means was performed to assess the effects of fertilization treatment on soil properties, functional genes, and microbial abundances at a significant level of $p < 0.05$ using SPSS 25.0. These treatment differences were visualized using Origin 9.5. Based on the Bray–Curtis distance, nonmetric multidimensional scaling analysis (NMDS) and analysis of similarities (ANOSIM) were used to illustrate the overall differences in microbial functional gene composition for N cycling among treatments, which were performed with the "vegan" package in R 4.1.2. Mantel test and Spearman's correlation analysis were conducted to reveal the relationships between soil properties and functional genes or microbial abundances using the "vegan" and "pheatmap" packages in R 4.1.2. The graphs were drawn using the "ggplot2" package in R 4.1.2. The schematic diagram was drawn using Adobe Illustrator 2020.

3. Results

3.1. Soil N Fractions

The STN and PNM were significantly greater with OM and NM than CK and NF (Figure 1). PON was greater with OM than CK, NF, and NM and MBN was greater with NM than other treatments. NH_4^+-N was greater with OM and NM than CK, and NO_3^--N was greater with OM and NM than NF. SWC, SOC, PCM, and MBC were greater with OM and NM than CK and NF (Table S3). The Soil C:N ratio was lower with CK than other treatments. Soil pH, BD, and POC had no significant changes among treatments.

3.2. The Abundances of N-Cycling Functional Genes

NMDS analysis indicated that long-term fertilization significantly changed the microbial community structure responsible for soil N cycling (ANOSIM, r = 0.623, $p = 0.001$; Figure 2a). The total abundance of N-cycling genes was 9.3% greater ($p < 0.05$) with NM than CK, and 8.2% ($p < 0.05$) and 12.2% ($p < 0.01$) greater with OM and NM than NF, respectively (Figure 2b). All functional genes involved in N cycling were classified into five pathways (Figure S1). Genes related to DNRA accounted for 28.4% of NF to 30.0% of NM of total N-cycling genes. Genes related to denitrification and ANRA were greater for OM and NM than CK and NF, but genes related to ONM were lower for NM than CK (Figure S2).

Genes related to nitrification contributed a small proportion of the total N-cycling genes for all treatments where genes were greater with NF and NM than CK.

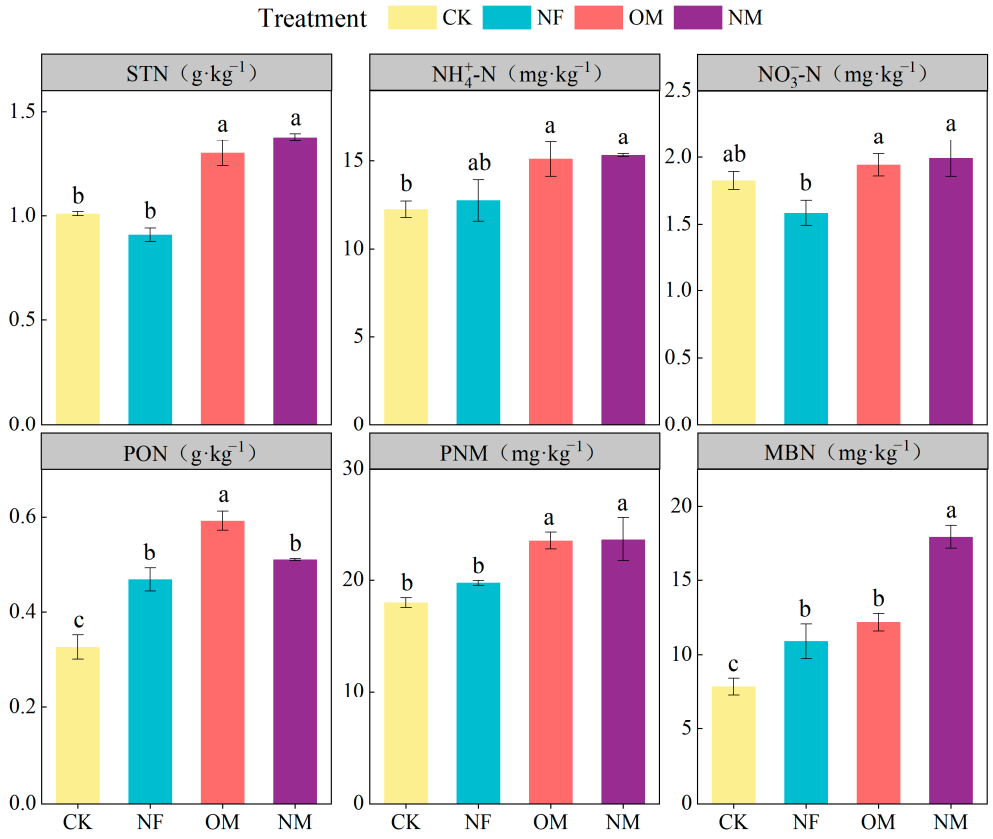

Figure 1. Effects of organic manure and chemical N fertilization on soil N fractions. Treatments were CK, control; NF, chemical N fertilization; OM, organic manure application; and NM, combination of chemical N fertilizer and organic manure application. Soil N fractions were STN, soil total N; PON, particulate organic N; PNM, potential N mineralization; MBN, microbial biomass N; NH_4-N; and NO_3-N. Error bars represent standard error of the means (n = 3). Different lowercase letters above the bar indicate significant difference ($p < 0.05$) among treatments.

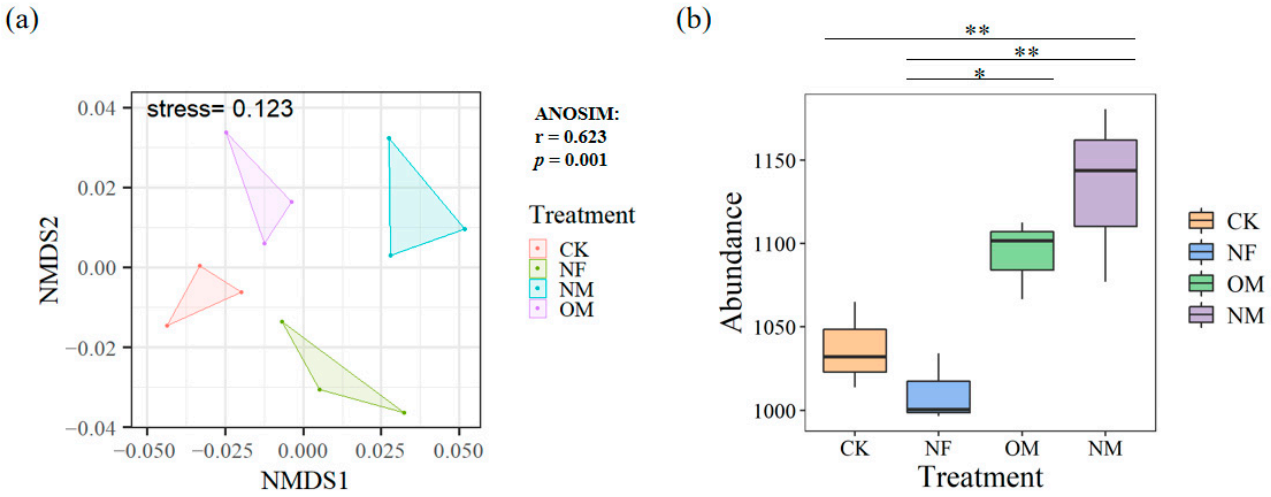

Figure 2. Nonmetric multidimensional scaling plots (NMDS) of N-cycling genes due to different fertilization treatments (**a**) and the total abundance of N-cycling genes under different fertilization treatments (**b**). *—$p < 0.05$, **—$p < 0.01$.

For nitrification, the abundance of *amoA* (encoding ammonia monooxygenase) was greater with NM than OM (Figure 3 and Table S4). The abundance of *amoC* (encoding ammonia monooxygenase) was lower with CK than other treatments. Similarly, the abundance of *hao* (encoding hydroxylamine dehydrogenase) was lower with CK than NF and NM. For denitrification, the abundance of *narGI* (encoding nitrate reductase) and *nosZ* (encoding nitrous-oxide reductase) was greater with NM than other treatments. Similarly, the abundance of *narH* (encoding nitrate reductase) was greater with NM than CK and NF. The abundance of *nirK* (encoding nitrite reductase) was greater with OM and NM than CK and NF. For DNRA, the abundance of *napA* (encoding nitrate reductase) was greater with NM than CK and NF, and the abundance of *napB* (encoding nitrate reductase) was greater with OM and NM than CK and NF. The abundance of *nirB* (encoding nitrite reductase) was greater with OM than NF and the abundance of *nrfH* (encoding nitrite reductase) was greater with NM than NF. For ANRA, the abundance of *nasA* (encoding assimilatory nitrate reductase) was greater with OM and NM than CK and NF. In contrast, for genes involved in ONM, the abundance of *gdh* (encoding glucose 1-dehydrogenase) was lower with OM and NM than CK.

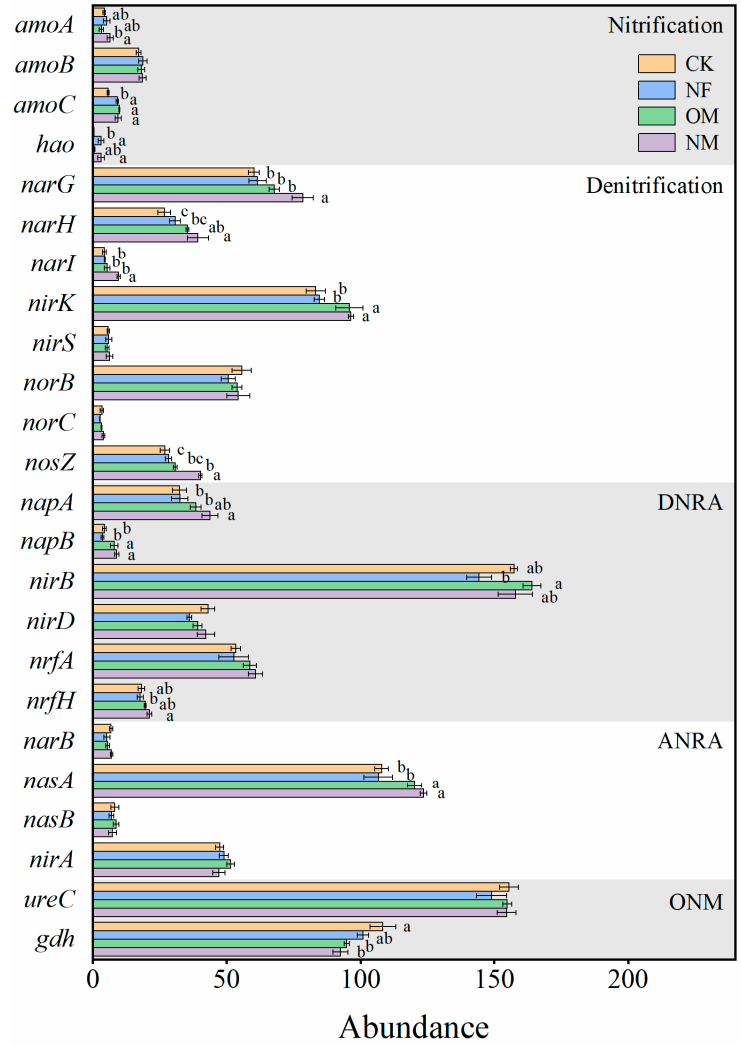

Figure 3. The abundances of N-cycling genes as affected by fertilization treatments. Treatments were CK, control; NF, chemical N fertilization; OM, organic manure application; and NM, combination of chemical N fertilizer and organic manure application. Error bars represent standard error of the means (n = 3). Different lowercase letters above the bar indicate significant difference ($p < 0.05$) among treatments.

Among the microbial phyla related to soil N cycling (Figure 4), *Proteobacteria* exhibited the highest relative abundance, accounting for 33% in NF to 36% in OM of all microbial phyla, followed by *Actinobacteria*, *Thaumarchaeota*, and *Acidobacteria*. The abundance of *Proteobacteria* was greater with OM and NM than NF, but the abundances of *Cyanobacteria* and *Firmicutes* were lower with OM and NM than CK (Table S5).

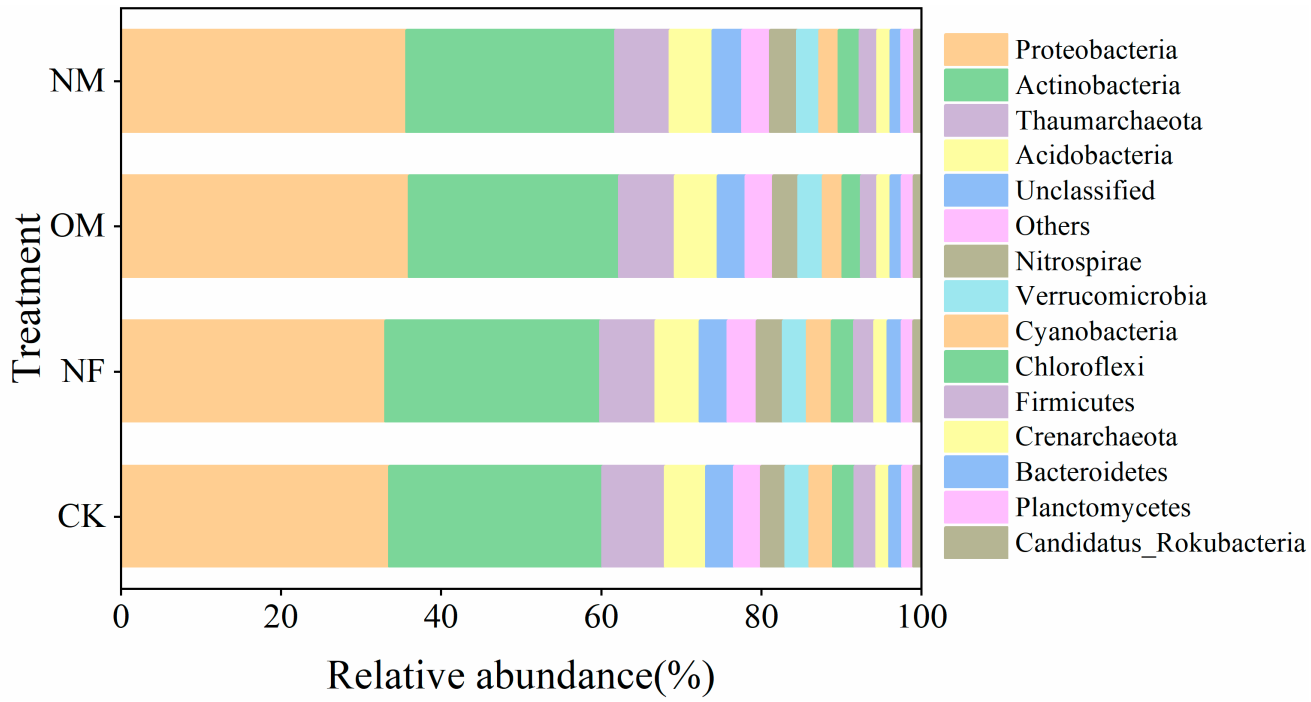

Figure 4. Relative abundances of microbial dominant phyla (relative abundance > 1%) involved in soil N cycling under different fertilization treatments. Treatments were CK, control; NF, chemical N fertilization; OM, organic manure application; and NM, combination of chemical N fertilizer and organic manure application.

3.3. Linkages between Soil Properties and N-Cycling Functional Genes

Mantel test and Spearman's correlation analysis indicated that soil C and N fractions were significantly related to the microbial functional genes involved in N cycling (Figure 5). The total abundance of genes related to N cycling was positively correlated with soil properties, except for pH, C:N ratio, POC, PON, and NO_3^--N (Table S6). The microbial functional genes involved in different N-cycling pathways were related to different extents with soil properties (Figures S3 and S4). There were no correlations between the abundance of nitrification genes and soil properties. The abundance of genes involved in denitrification (e.g., *narGH*, *nirK*, and *nosZ*) was positively correlated with SWC, SOC, STN, PCM, PNM, MBC, and MBN. Additionally, the abundances of genes associated with DNRA (e.g., *napB*) were positively correlated with SOC, STN, PCM, NH_4^+-N, and NO_3^--N. Strong positive correlations were also observed among SOC, STN, and PCM, as well as the abundance of genes involved in ANRA (*nasA*). However, SOC, STN, and PCM were negatively correlated with the abundance of genes involved in ONM (*gdh*).

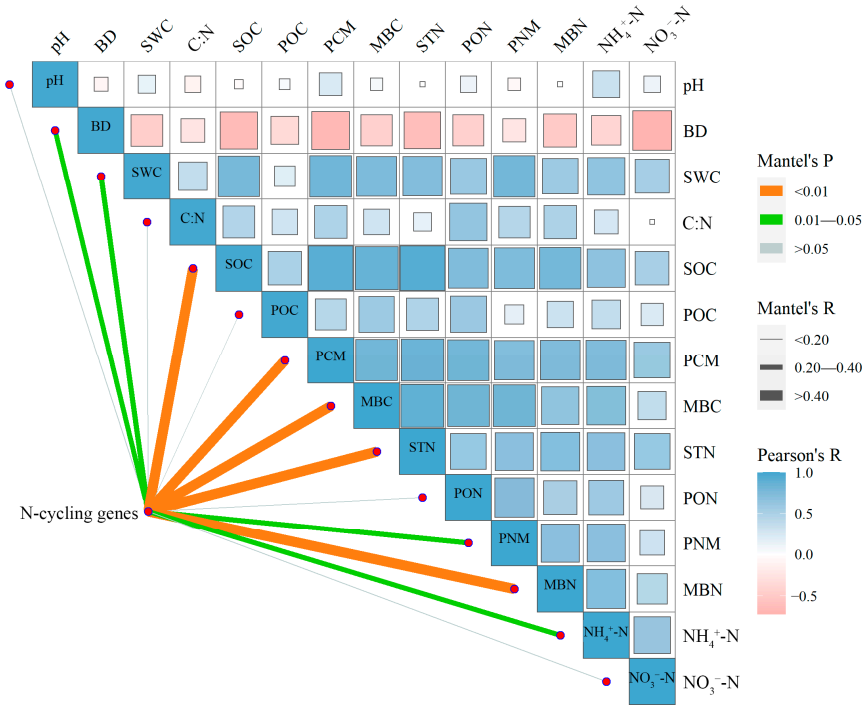

Figure 5. Relationships among N-cycling genes and soil properties. Treatments were CK, control; NF, chemical N fertilization; OM, organic manure application; and NM, combination of chemical N fertilizer and organic manure application. Soil properties were pH; BD, bulk density; SWC, soil water content; C: N, the ratio of soil organic C to total N; SOC, soil organic C; POC, particulate organic C; PCM, potential C mineralization; MBC, microbial biomass C; STN, soil total N; PON, particulate organic N; PNM, potential N mineralization; MBN, microbial biomass N; NH_4^+-N; and NO_3^--N.

4. Discussion

4.1. Soil N Fractions Response to Long-Term Fertilization

The greater N fractions with OM and NM than CK and NF (Figure 1) suggest that long-term application of OM with or without N fertilization increased N storage and availability compared to N fertilization alone or no fertilization. Because the mineralization and availability of N from OM occur slowly compared to NF, it is likely that some N released by OM during the N transformation process may have converted to STN and PON, while others became available to plants [12,23]. As a result, long-term application of OM may have increased all N fractions in contrast to NF, which may have increased NH_4^+-N and NO_3^--N immediately after N fertilization due to the rapid availability, some of which not taken up by plants can be lost to the environment due to leaching, denitrification, and volatilization [24,25]. Reduced N inputs with lack of fertilization may have reduced N fractions with CK [26]. Our findings are consistent with those observed by numerous others, who reported that manure application had stronger effects on N fractions than N fertilization [27–29]. NF and OM may also have promoted the growth of crop and root systems, thereby increasing crop residues and root exudates in soil and enhancing PON and MBN [14,30,31]. Increased soil organic matter caused by the application of manure and crop growth can promote microbial metabolism, stimulating the mineralization of labile organic N pools [32,33].

4.2. N-Cycling Functional Genes Response to Long-Term Fertilization

The plots received different N input annually in this long-term experiment, which may lead to a dose effect on soil N-cycling genes. The study found that the abundance of some N-related functional genes would increase with the increase in N dose level [34]. However, the results of this study were different, that is, the level of N input under NF treatment (120 kg·N·ha^{-1}) was higher than that under OM treatment (87 kg·N·ha^{-1}),

while the abundance of N-cycling genes under NF treatment was lower than that under OM treatment (Figure 2b). This may be due to the long-term application of mineral N fertilizers causing N loss in farmland soil, resulting in low N utilization efficiency [24]. Furthermore, this may be due to the small differences in N input levels under different treatments, so the fertilizer form is the most important factor affecting the abundance of N-related genes. In a meta-analysis study, Ouyang et al. (2018) found that fertilizer form (inorganic vs. organic) rather than dose strongly affected the response of most N-cycling genes to N fertilization [35]. This is also confirmed by this study. The greater abundances of N-cycling genes involved in nitrification, denitrification, DNRA, and ANRA with OM and NM than NF and CK (Figures 2 and 3) was likely due to enhanced energy supplied by C substrate and food supplied by the N substrate to microorganisms that increased their activity and abundance of genes associated with N transformations. Manure has many stable organic compounds that provide energy and nutrients to stimulate microbial growth and reproduction [9,36]. A similar increase in N-cycling genes with OM and NM suggests that organic manure had a greater effect on the abundance of genes compared to inorganic N fertilizer. In contrast, the lower abundance of *gdh* associated with ONM with OM and NM was likely due to reduced N mineralization due to the greater C:N ratio of manure compared to inorganic N fertilizer [37,38]. Application of manure provided both C and N inputs compared to N input, only by inorganic N fertilizer [14]. Amendments with a greater C:N ratio mineralize more slowly than those with a lower ratio [38]. This is contrary to the findings reported by several researchers who observed that application of organic manure increased the abundance of *gdh* [39,40]. The greater abundance of nitrification genes (*amoC* and *hao*) with NF than CK was likely due to the increased availability of N substrate (Figure 2 and Table S4). As urea, which is rich in NH_4^+-N, was the main source of N fertilizer, long-term application of this fertilizer may have enriched NH_4^+-N concentration in the soil, which may activate genes associated with nitrification, thereby enhancing the abundance of these genes [5,41,42].

Consistent increase in *Proteobacteria* with OM and NM compared to CK and NF (Figure 4) suggests that the greater abundance of N-cycling genes was associated with enhanced activity of this bacteria induced by application of organic manure. Previous research has observed greater abundance of *Proteobacteria* in soils applied with organic manure [43]. The *Proteobacteria* generally had high capacity to utilize nutrients from manure and is considered to be the main driver of changing soil functions with manure application [44]. However, the reduced activities of *Firmicutes* and *Cyanobacteria* with OM and NM was likely due to increased suppression by *Proteobacteria* with manure application [45]). *Proteobacteria* includes many species containing denitrification genes, which explains the increase in denitrification genes with the application of manure [3].

4.3. Driving Factors of N-Cycling Functional Genes

Strong positive correlations among N-cycling genes and SOC, STN, PCM, MBC, and MBN (Figure 5) suggest that the abundance of N-cycling genes was enhanced by increased soil C and N storage and rich microbial biomass and activity. All of these parameters were greater with OM and NM than CK and NF (Figures 1 and 2). It is likely that increased availability of C and N substrates due to greater SOC and STN increased microbial biomass and activity and therefore promoted the abundance of N-cycling genes [46]. Numerous studies have reported that increased soil organic matter increased N-cycling microbial communities and functional gene distribution [12,39,47]. Weak correlations among N-cycling genes and BD, SWC, and NH_4^+-N indicate that soil physical and chemical properties may have less influence on N-cycling genes. Soil pH has been identified as a pivotal factor for shaping N-cycling genes [3,9], which was not seen in our study due to a notably narrow range of soil pH (8.20–8.39; Table S3).

In addition, N-cycling genes involved in different metabolic functions responded differently to soil properties (Figure S3). The abundance of *narGH*, *nirK*, *nosZ*, *napB*, and *nasA* was positively correlated with SOC, STN, and PCM (Figure S4), indicating that the

genes involved in denitrification, DNRA, and ANRA were strongly dependent on soil C and N content and availability [39,48]. However, the abundance of *gdh* was negatively correlated with SOC, STN, and PCM. We surmised that a soil environment with high C and N contents may be unfavorable for survival of microorganisms related to organic N metabolizing, which needs to be verified in subsequent studies. In addition, we observed that the abundance of nitrification genes was not significant to all soil properties, which might be because wheat roots exudate inhibited soil nitrification, thereby weakening the association between the abundances of nitrification genes and soil properties [35,49].

4.4. A Model of N-Cycling Genes Induced by Long-Term Fertilization

Based on the results above, we developed a model for genes associated with N transformation processes due to the application of N fertilizer and organic manure. NF increased MBN and OM increased SOC, STN, PCM, MBC, and MBN compared to CK, and the pathways for genes associated primarily with N cycling are shown in Figure 6. The lower abundance of *gdh* genes with the application of manure slowed down the release of organic N to NH_4^+ during the ONM process, which would be beneficial to soil N retention and sustainable utilization [50]. Part of NH_4^+ is used by crops and microorganisms, and the rest is transformed into NO_3^- by nitrification [1]. Both the application of chemical N fertilizer and organic manure increased the abundance of *amoAC* and *hao* genes related to nitrification and accelerated NO_3^- accumulation, which could lead to soil N loss by runoff and leaching [51]. The application of manure enhanced N transformation from NO_3^- to NO and from N_2O to N^2 by *narGHI*, *nirK*, and *nasZ* genes involved in denitrification, which may be lost via nitrogenous gas [48]. However, DNRA and ANRA directly reduce NO_3^- to NH_4^+ by *napAB*, *nasA*, *nirB*, and *nrfH* genes, and the genes involved in DNRA account for the largest proportion of N cycling. This suggested that DNRA and ANRA may largely compensate for N loss caused by nitrification and denitrification and help for the retention of soil-available N.

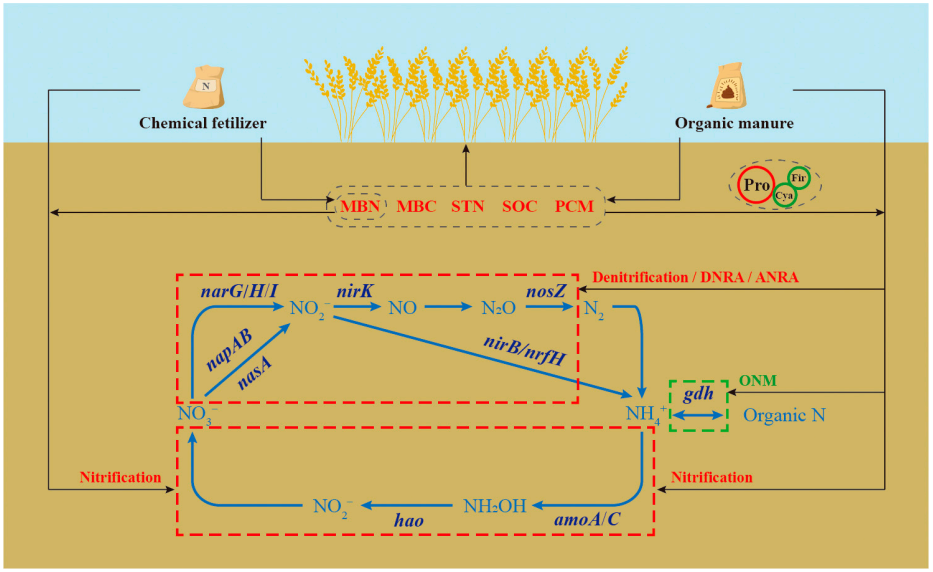

Figure 6. Schematic diagram of N-cycling genes associated with N transformation processes due to chemical N fertilization and organic manure application. Red words indicate soil C and N fractions and N transformation processes. Blue lines indicate N-cycling pathways and blue italics words indicate N-cycling genes. Red dotted lines indicate that gene abundance significantly increased during the processes and green dotted lines indicate that gene abundance significantly decreased during the process. Red circles indicate increased abundance of *Proteobacteria* (Pro) and green circles indicate decreased abundance of *Cyanobacteria* (Cyn) and *Firmicutes* (Fir). Soil C and N fractions were STN, soil total N; SOC, soil organic C; PCM, potential C mineralization; MBC, microbial biomass C; MBN, microbial biomass N; NH_4^+-N; and NO_3^--N.

5. Conclusions

Soil microbial functional genes related to N cycling responded differentially to 35 yr annual fertilization with chemical N fertilizer and organic manure. The application of N fertilization increased the abundance of genes related to nitrification, while organic manure with or without N fertilizer increased the abundances of genes involved in nitrification, denitrification, DNRA, and ANRA, but decreased those associated with ONM. Moreover, manure with or without N fertilization also increased the contents of SOC, STN, PCM, MBC, and MBN, and there were strong correlations between N-cycling genes and these parameters. Therefore, the abundance of N-cycling genes was enhanced by increased soil C and N storage and microbial biomass and activity due to organic manure application. Long-term manure application can enhance the sustainability of agroecosystems by promoting N-cycling genes that increase N storage and availability compared to chemical N fertilization in the Loess Plateau of China.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13061516/s1, Table S1: Basic information of metagenomic sequencing; Table S2: The information of microbial functional genes involved in N-cycling processes identified in this study referring to KEGG database; Table S3: Effects of organic manure and chemical N fertilization on soil properties; Table S4: The abundances of genes involved in soil N cycling under different fertilization treatments; Table S5: Relative abundances of microbial dominant phyla (relative abundance > 1%) involved in soil N cycling under different fertilization treatments; Table S6: Correlation coefficients of Mantel tests between the functional gene composition responsible for soil N cycling and soil properties; Figure S1: The relative abundances of genes involved in different N metabolism pathways under different fertilization treatments; Figure S2: The abundances of genes involved in different N metabolism pathways under different fertilization treatments; Figure S3: Relationships between the functional gene composition involved in different N metabolism pathways and soil properties; Figure S4: Spearman's correlations between the abundances of N-cycling genes and soil properties.

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