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Effects of Nitrogen Fertilizer on *Nitrospira*- and *Nitrobacter*-like Nitrite-Oxidizing Bacterial Microbial Communities under Mulched Fertigation System in Semi-Arid Area of Northeast China

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Abstract: The accumulation of nitrite is frequently overlooked, despite the fact that nitrification is the most essential phase of the entire nitrogen (N) cycle and that nitrifying bacteria play a significant role in nitrification. At present, the effects of different N application rates on soil nitrite-oxidizing bacteria (NOB) abundance, community composition, diversity, and its main influencing factors are still unclear. In this study, five N fertilizer application rates under film mulching and a drip irrigation system were studied in the semi-arid area of Northeast China. The treatments were 0 kg ha^{-1} (N0), 90 kg ha^{-1} (N1), 150 kg ha^{-1} (N2), 210 kg ha^{-1} (N3), and 270 kg ha^{-1} (N4). Fluorescent quantitative PCR and Illumina Miseq sequencing were used to analyze the abundance and community structure of NOB under different amounts of N application. The results showed that the increase in amounts of N application was strongly accompanied by an increase in the content of soil organic matter (SOM), total nitrogen (TN), nitrate nitrogen (NO_3^--N), and ammonium nitrogen (NH_4^+-N), while the pH significantly reduced with an increase in N fertilization. N fertilization significantly increased soil nitrite oxidoreductase (NXR) activity, soil nitrification potential (PNR), and soil nitrite oxidation potential (PNO). A high N application rate significantly heightened the abundance of *Nitrospira*- and *Nitrobacter*-like NOB. N fertilizer considerably raised the Shannon index of *Nitrospira*-like NOB. The N application amount was the key factor affecting the community structure of *Nitrospira*-like NOB, and available nitrogen (AN) had the dominant influence on the community structure of *Nitrospira*-like NOB. N fertilizer can cause soil acidification, which affects NOB abundance and diversity. *Nitrospira*-like NOB may promote nitrite oxidation in different N application rates under a mulched fertigation system. The findings offered a crucial scientific foundation for further investigation into how nitrite-oxidizing bacteria respond to N fertilizer management strategies in farmland soil under film mulching drip irrigation in Northeast China.

Keywords: N fertilizer application rate; nitrite-oxidizing bacteria (NOB); *Nitrospira*-like NOB; *Nitrobacter*-like NOB; nitrite oxidoreductase; nitrite oxidation potential (PNO)

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1. Introduction

China's third most significant crop, maize, is a key component of the country's agricultural growth. One of the key strategies used to raise maize production is fertilization [1]. Pasley et al. found that, for crop production, nitrogen (N) fertilizer application is more important than other major essential nutrients [2]. N fertilization can boost crop quality, productivity, and the rate at which plants absorb CO_2. It can also strengthen crops' resilience to environmental stressors, including scarce water supplies and saline-alkaline

soil conditions [3]. The biogeochemical cycle of N has been greatly impacted by human activity, to the point that just 125 million tons of reactive N are used in natural processes, and the remaining 179 million tons mainly come from N fertilizers [4]. Moreover, 20% of the world's population depends on cultivated land for their livelihoods [5], while the per capita arable land in China is approximately 0.08 ha, far below the world average level of 0.22 ha [6]. In the past 70 years, the average grain yield has increased from 1.09 t ha^{-1} to 6.51 t ha^{-1}. The input of chemical N fertilizer is the main factor for the continuous increase of grain production, thus alleviating food security problems in China [7]. However, excessive application of N fertilizers causes soil eutrophication and acidification. Soil N buildup enhances pathogens and suppresses antagonistic bacteria [8]. Acidified soil directly affects soil ecosystems and microbial diversity, resulting in the frequent occurrence of soil-borne diseases that have devastating effects on plant growth and health.

The ability of soil to supply N is determined by its N cycle, the primary component of which is nitrification. It is the only biological activity that adds inorganically oxidized and reorganized N into nature, which is essential for preserving the global N balance. Nitrification has a significant effect on how N is used in agricultural ecosystems and how the biological ecology of the soil is kept stable [9]. According to conventional theory, ammonia (NH_4^+) is oxidized to nitrite (NO_2^-) by both ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB), whereas nitrite is then converted to nitrate (NO_3^-) by nitrite-oxidizing bacteria (NOB) [10,11]. However, most previous studies focused only on the effects of nitrification on the abundance and community structure of AOA, AOB, and complete ammonia oxidation (comammox) and the response of nitrifiers to environmental factors [12,13]. Since the first step of nitrification is usually rate-limiting [14], nitrous acid salt accumulation is often overlooked, and nitrite oxidation is more likely to be inhibited than ammonia oxidation [15]. Studies have proven that soil with high ammonium concentration inhibits the activity of soil nitrite oxidoreductase through ammonium, which is easy to accumulate nitrite and causes danger to the soil environment [16]. Suppressing NOB activity by operational techniques during complete nitrification is an integral aspect for developing successful nitritation, which is one of the primary explanations for this growing interest [17,18]. Therefore, it is necessary to study the abundance and community structure of NOB under different N application rates.

Sustaining excellent soil quality and a healthy atmosphere is necessary for researching the sustained productivity of black soil. Converting the rich microbiological original data under conservation tillage into an understanding of the functions of soil that are essential to agricultural ecosystems is a beneficial process. This offers a potent instrument for judicious fertilization and can also enhance forecasts regarding the impact of management methods on the make-up and function of soil microbial communities ("Who lives there?" and "What are they doing?"). Soil microorganisms play a major role in agricultural ecosystem health and productivity. Applying chemical fertilizers sensibly is crucial for both resource preservation and all-around agricultural improvement, especially when it comes to N fertilizers. At present, less research has been conducted on the abundance and community structure of NOB compared to typical nitrifying bacteria, which have been the subject of numerous studies under different fertilization regimes [19].

Nitrite oxidoreductase (NXR), the essential enzyme for NO_2^- oxidation, is present in NOB. The genes *nxrA* and *nxrB*, which encode the alpha and beta subunits, respectively, are found in its genome [20]. Recently, there has been widespread usage of the *nxrA* and *nxrB* genes, which were initially investigated by Poly et al. [21] and Pester et al. [22], respectively, as phylogenetic and functional markers for nitrite-oxidizing members of the *Nitrobacter* and *Nitrospira* communities [23]. The most varied and prevalent NOB in the ecological environment are those of the genera *Nitrobacter* and *Nitrospira*, which are members of the phyla Proteobacteria and Nitrospirae, respectively [24]. Long-term fertilization in acid forest soils led to fast changes in the community structure of *Nitrobacter*-like NOB and increased their abundance, while having no effect on *Nitrospira*-like NOB [25]. *Nitrospira* may be the primary cause of nitrite oxidation in surface agricultural soils [26,27], which

suggests that, in the soil of rapeseed–rice rotation, *Nitrospira* may be more sensitive than *Nitrobacter*. Furthermore, *Nitrospira* was considerably impacted by soil pH, moisture, and NH_4^+, but *Nitrobacter* was not.

Therefore, this study used real-time quantitative PCR to analyze the abundance of the *Nitrospira*- and *Nitrobacter*-like NOB under different N application rates with film mulching drip irrigation. Illumina Miseq sequencing technology was performed to investigate the community structure of *Nitrospira*-like NOB. Structural equation modeling (SEM) was used to elucidate the interaction between soil chemical characteristics and biological characteristics of NOB under different N application rates, revealing the impact of different N application rates on the NOB community. We hypothesized that (1) N fertilization may have an impact on the abundance and diversity of NOB; (2) *Nitrospira*-like NOB are the most important nitrite oxidation potential (PNO) predictor; and (3) N fertilizer can cause soil acidification, which negatively affects the NOB community. This will be of great significance for evaluating the ecological impact of different types of fertilization management and for maintaining the stability of agricultural ecosystems.

2. Materials and Methods

2.1. Experimental Design

The experiment location was situated at 45°26′ N and 125°88′ E in Minle Village, Ningjiang District, Songyuan City, Jilin Province (Figure S1). The region experiences a continental monsoon climate that is mid-temperate, with most of region's yearly precipitation falling between May and September. There are approximately 2867 h of average annual sunshine, 135 to 140 days without frost, and an average annual temperature of 5.6 °C. Maize was sown in May and harvested in October. The field experiment with chernozem soil was started in 2017. Maize variety Xiangyu 998 (Hongxiang Agriculture Company, Changchun, Jilin Province, China) was used in the test, and planting density was 70,000 plants per hectare. Each plot included three replicates placed in random blocks over a 40 m^2 (10 m × 4 m) area (Figure S2). The plot used a large monopoly two-way cultivation mode, narrow row spacing was 40 cm, wide row was 80 cm, drip irrigation was placed in the middle of the narrow line when sowing, the inner diameter of the drip drill was 16 mm (Hongtaiweiye Water-saving Irrigation Equipment Company, Jinan, Shandong Province, China), and the distance from the head of the drop was 30 cm. White transparent plastic film (Wanchen Plastic Technology Company, Linqi, Shandong Province, China) was used to cover the maize and drip irrigation, and then artificial covering film was used after fertilizer sowing, with one being film a monopoly. When fertilizing according to the fertilizer requirement of the experiment, the fertilizer could be filled with water and mixed, so that it completely dissolved, before fertilization. The first dripped clean water 30 min, then fertilizing valve drip fertilizers were opened, and after fertilization, it continued to drip clear water for 30 min. Field management practices, such as fertilization method, planting density, and fertilizer dosage, remained the same once the experiment started.

Five levels of N fertilizer application were set, N0, N1, N2, N3, and N4, in which N was applied at a rate of 0, 90, 150, 210, and 270 kg ha^{-1}, respectively. Moreover, 90 kg P_2O_5 ha^{-1} and 90 kg K_2O ha^{-1} were applied under each treatment. The N fertilizer was applied at five different stages (Table S1). Additionally, 30% N and 50% P_2O_5 and K_2O were used as the base fertilizer. The base fertilizer was applied before sowing, and the top dressing was applied with the integrated drip irrigation technology of water and fertilizer.

2.2. Sample Collection

The soil was sampled from the 0–20 cm deep layer on 21 October 2021. To create a composite soil sample, five randomly selected locations from each repeated plot were combined evenly. A total of 15 soil samples were gathered, and they were delivered to the lab in an ice box. Before passing the soil samples through a 2 mm screen, they were cleared of any debris, including stones and tree roots. To assess the activity of soil enzymes, NH_4^+-N, and NO_3^--N, fresh soil was employed. For the purpose of extracting DNA,

some soil samples were placed in a −80 °C freezer. For the investigation of soil chemical parameters, other soil samples were air-dried at ambient temperature.

2.3. Assessment of Soil Chemical Properties and Soil Nitrite Oxidoreductase Activity

The K_2CrO_7 oxidation–external heating method was used to assess soil organic matter (SOM) [28]. Using the PHS-25 pH meter (Raici Corporation, Shanghai, China) to measure soil pH (water-to-soil ratio of 2.5:1) [29], the Kjeldahl method was used to test soil total N (TN) [30], and the alkaline solution diffusion method was used to measure soil accessible N (AN) [31]. Using a 2 M KCL solution, the NH_4^+-N and NO_3^--N were extracted and subsequently quantified using a continuous flow analytical apparatus (AutoAnalyzer3, Bran+Luebbe, Norderstedt, Germany) [32]. Samples of soil (2 g) were dissolved using an ultrasonic extraction method in 5 mL of cell lysis solution (50 mmol L^{-1} Tris HCl (pH 7.5), 300 mmol L^{-1} sodium chloride, and 90 mmol L^{-2} ethylenediaminetetraacetic acid disodium salt) [33]. A 50 mmol L Tris HCl and 5 mmol L MgCl buffer solution (pH 7.8) was used to reduce the extraction volume to 25 mL. The supernatant from the centrifugation of the extract at 12,000 rpm for 20 min was utilized to measure the enzyme activity. The NXR ELISA kit from Saint-Bio Company (Huzhou, Zhejiang Province, China) was used to measure nitrite oxidoreductase (NXR) activity in accordance with the manufacturer's instructions.

2.4. Determination of Soil Nitrite Oxidation Potential (PNO)

A total of 30 mL of sodium nitrite solution (containing 15 mg of NO_2-N) was added to fresh soil samples of 3 g, and the flask was left in a water bath shaker (HH-US-A1, Xinchunlan Scientific Instrument Company, Changzhou, Jiangsu, China) for about 150 revolutions per minute for 30 h at constant temperature. Between constant temperature cultures, 1.5 mL of the suspension was taken at 0, 9, 24, and 30 h and centrifuged at 500 rpm for 3 min. The supernatant was filtered (0.2 mm holes) and stored at −20 °C for the analysis of NO_2^- [34]. NO_2^- was measured at 520 nm using a spectrophotometer (Uvikon 800, Leeds, UK), and it was calculated that the NO_3^--N content increased linearly with time.

2.5. Soil DNA Extraction and Quantification of Nitrospira- and Nitrobacter-like NOB Abundance

When 0.5 g of fresh soil was weighed, all of soil DNA was extracted using the MP Biomedicals, Irvine, CA, USA FastDNA® SPIN Kit for Soil. A Thermo Scientific, Wilmington, DE, USA, NanoDrop 2000 UV-Vis spectrophotometer was used to measure DNA concentration, and 1% agarose gel electrophoresis was used to measure DNA quality. The primer sequences of the *nxrB* gene PCR of soil *Nitrospira*-like NOB were: *nxrB*169-F (5′-ATACATGTGGTGGAACA-3′) and *nxrB*169-R (5′-TACATGTGGTGGAACA-3′) [22]. The primer sequences of the *nxrA* gene of *Nitrobacter*-like NOB were: F1*norA* (5′-CAGACCGACGTGTGCGAAAG-3′) and R2*norA* (5′-TCCACAAGGAACGGAAGGTC-3′) [35]. The PCR amplification system (50 µL) consisted of: 1 µL of *nxrB*169-F (10 µM), 1 µL of *nxrB*169-R (10 µM), 1 µL of SYBR® Premix Ex Taq™ II (TliRNaseH Plus), ROX plus, 25 µL of 2 × TaqMasterMix buffer, and sterile ddH_2O added to a final volume of 50 µL. The PCR amplification conditions were as follows: initial denaturation at 94 °C for 300 s; denaturation at 55 °C for 30 s, annealing at 2 °C for 30 s for 30 cycles; and a final extension at 72 °C for 600 s. Three repetitions of each sample were made, and NOB with known copy numbers were used to create the standard curve.

2.6. High-Throughput Sequencing and Data Analysis Method for nxrB Gene of Nitrospira-like NOB

The extracted DNA from each sample was used for PCR amplification with the primers 169-F/169-R, which target the *nxrB* gene. To discriminate between various samples, 8 bp barcode sequences were inserted to the 5′ ends of the upstream and downstream primers. The PCR amplification system (25 µL) consisted of the following: 1 µL of Forward Primer (5 µM), 1 µL of Reverse Primer (5 µM), 12.5 µL of 2 × Taq Plus Master Mix, 3 µL of

BSA (2 ng/μL), 2 μL of DNA, and sterile ddH_2O added to a final volume of 25 μL. The PCR amplification conditions were as follows: initial denaturation at 95 °C for 300 s; denaturation at 55 °C for 45 s; annealing at 72 °C for 50 s, for 30 cycles; and a final extension at 72 °C for 300 s. The PCR products were detected by 1% agarose gel electrophoresis to amplify the target band size and were purified by the Agencourt AMPure XP Nucleic Acid Purification Kit. At Aoweisen Gene Technology Co., Ltd., (Beijing, China) the PCR results were used to create a sequencing library for microbial diversity. The gene sequence analysis was processed by QIIME (V.1.8.0) software, and the data were filtered and spliced by Pear software (V.0.9.6). Uncertain bases, primer mismatches, and sequences with scores below 20 were eliminated. During splicing, the minimum overlap was 10 bp, and the mismatch rate was 0.1. Vsearch Denovo software (V.2.7.1) was used to remove short sequences and chimeric sequences. The UPARSE algorithm of Vsearch software was used to perform OTU (Operational Taxonomic Units) clustering on high-quality sequences, with a similarity level at 97%. In order to ensure that the coverage of all samples was quite high, the data size of all samples was normalized to 25,223 sequences. Using the Blast algorithm, the taxonomic identity of each phylotype was determined through the GenBank non-redundant nucleotide database (nt). The gene sequences obtained in this study were submitted to NCBI and were assigned the accession number SRP455972.

2.7. Statistical Method and Data Analysis

One-way ANOVA and Pearson correlation analysis were used to examine significant differences in soil physicochemical characteristics, enzyme activities, abundance of nitrifying microorganisms, and soil PNR using SPSS (version 23.0). RDA was performed with the Canoco (V.5.0) program. Heatmaps were made with R (version 3.4.2) using the "pheatmap" and "ggplot2" tools. Sequence alignment was conducted using Mafft's phylogenetic tree analysis, and tree building was performed using Fasttree. Python provided a visual representation of the basic genera's abundance and evolutionary connection. The Bray–Curtis method in the R program (version 3.4.2) was used to perform the Wayne analysis, principal component analysis (PCoA), and analysis of similarities (ANOSIM). Amos (23rd edition) developed a structural equation model chi-square test ($p > 0.05$) and demonstrated the direct and indirect influences between soil chemical characteristics and soil NOB communities using the chi-square ratio of degrees of freedom (CMIN/DF < 3) and fitness index (CFI > 0.9).

3. Results

3.1. Soil Chemical Properties

Soil chemistry (i.e., SOM, TN, NO_3^--N, and NH_4^+-N content) changed dramatically with the increasing N delivery rate (Table 1). SOM in the N4 treatment was 1.33 times higher than in the N0 soil. The TN content increased by 0.05–0.6 g kg^{-1} in treatments with high N input (N3, N4) as opposed to low N (N0, N1, N2). In contrast to the low rates of N application (N0, N1, N2), N4 significantly increased soil AN content by 12.9–57.66 mg kg^{-1}. Compared with N0, the contents of NH_4^+-N in N3 and N4 increased by 0.41 and 0.44 mg kg^{-1}, respectively, and the contents of NO_3^--N in N3 and N4 increased by 7.29 and 6.49 mg kg^{-1}, respectively, while the pH value decreased significantly with increased N fertilizer application (Table 1). In summary, N fertilization can increase soil nutrient content but decreases soil pH.

3.2. Soil NXR Activity and PNO

The high N application rates (N3, N4) considerably enhanced the activity of NXR in comparison to the low N application rates (N1, N2) (Figure 1). Compared with N0, the activities of NXR in N3 and N4 were 1.93 times and 1.95 times higher, while those of N1 and N2 were 1.62 times and 1.63 times higher, respectively. In conclusion, N fertilization increased soil NXR activity. NXR and SOM, AN, TN, NH_4^+-N, and NO_3^--N were significantly positively correlated (Table S2). High N application rates (N3, N4) sig-

nificantly increased soil PNR (Figure 2a) compared to low N application rates (N0, N1, N2). The greatest value of PNO in N4 was 0.32 μg NO_2^--N g^{-1} dry soil h^{-1} (Figure 2b). Soil PNR was significantly positively correlated with pH, TN, AN, NO_3^--N, and NH_4^+-N, while soil PNO was significantly positively correlated with SOM, TN, AN, NO_3^--N, and NH_4^+-N (Figure S3).

Table 1. Soil properties under different N application rates.

	pH	SOM (g kg^{-1})	TN (g kg^{-1})	AN (mg kg^{-1})	NH_4^+-N (mg kg^{-1})	NO_3^--N (mg kg^{-1})
N0	6.89 ± 0.09 a	17.6 ± 1.85 c	1.11 ± 0.01 d	105.64 ± 0.92 d	0.33 ± 0.07 b	2.74 ± 0.22 d
N1	6.79 ± 0.26 ab	21.23 ± 2.15 b	1.42 ± 0.02 c	127.32 ± 4.48 c	0.59 ± 0.09 a	3.54 ± 0.99 d
N2	6.64 ± 0.11 a	23.38 ± 0.33 ab	1.53 ± 0.06 b	150.4 ± 7.88 b	0.7 ± 0.15 a	5.97 ± 0.43 c
N3	6.62 ± 0.31 ab	23.82 ± 0.55 a	1.58 ± 0.02 b	153.83 ± 1.18 b	0.74 ± 0.03 a	7.96 ± 1.47 b
N4	6.45 ± 0.29 b	23.42 ± 1.25 ab	1.71 ± 0.05 a	163.3 ± 11.47 a	0.77 ± 0.13 a	10.03 ± 1.58 a

Values are means ± SD, different letters indicate significant differences among treatments ($p < 0.05$, ANOVA). SOM, soil organic matter; TN, total nitrogen; AN, available nitrogen; NH_4^+-N, ammonium nitrogen; NO_3^--N, nitrate nitrogen. N0, N1, N2, N3, and N4 represent N fertilizer being used at a level of 0, 90, 150, 210, and 270 kg ha^{-1}, respectively.

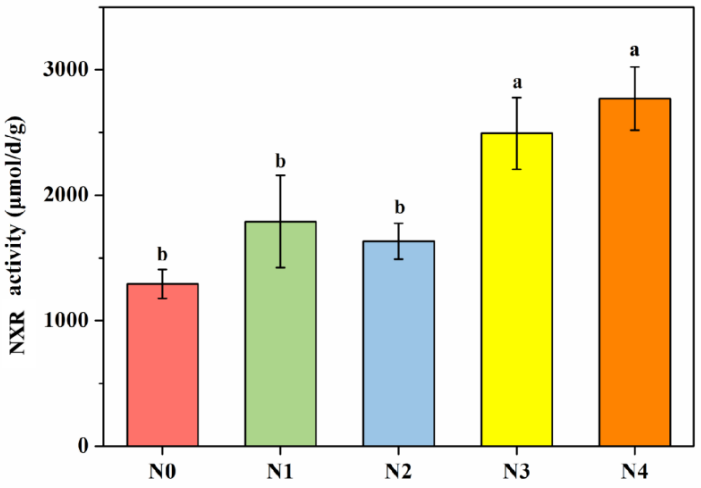

Figure 1. Soil NXR activities under different N application rates. Bars indicate SD, different letters indicate significant differences among samples ($p < 0.05$, ANOVA). NXR, nitrite oxidoreductase; N0, N1, N2, N3, and N4 represent N fertilizer being used at levels of 0, 90, 150, 210, and 270 kg ha^{-1}, respectively.

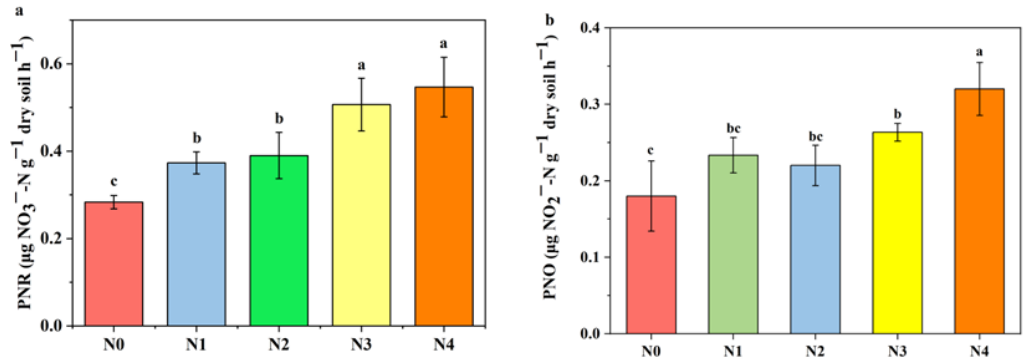

Figure 2. The soil PNR (**a**) [36] and PNO (**b**) under different N application rates. Bars indicate SD, different letters indicate significant differences among samples ($p < 0.05$, ANOVA). PNR, potential nitrification rate; PNO, potential nitrite oxidation; N0, N1, N2, N3, and N4 represent N fertilizer being used at a level of 0, 90, 150, 210, and 270 kg ha^{-1}, respectively.

3.3. Abundance of Soil Nitrospira- and Nitrobacter-like NOB

The abundance of *Nitrospira*- and *Nitrobacter*-like NOB can be increased by applying N fertilizer in comparison to N0 (Figure 3). In contrast to low N content (N0, N1, N2), N4 significantly increased the abundance of *Nitrospira*-like NOB, and N3 and N4 significantly increased the abundance of *Nitrobacter*-like NOB (Figure 3). The abundance of *Nitrospira*-like NOB was significantly higher than that of *Nitrobacter*-like NOB. The ratio of *nxrB*/*nxrA* gene copy numbers ranged from 54.47 to 117.66, with the highest value in N0, followed by N1, N2, N3, and N4, indicating that fertilization had a significant negative effect on *nxrB*/*nxrA* gene copy numbers. The correlation analysis between *Nitrospira*- and *Nitrobacter*-like NOB abundance, PNO, and soil chemical properties under the different N application rates showed that the soil *Nitrospira*- and *Nitrobacter*-like NOB abundances were significantly positively correlated with SOM, AN, TN, NO_3^--N, NH_4^+-N, and PN (Figure S3). However, the ratio of *nxrB*/*nxrA* gene copy numbers was significantly negatively correlated with SOM, AN, TN, NO_3^--N, NH_4^+-N, AN, and PNO (Figure S3).

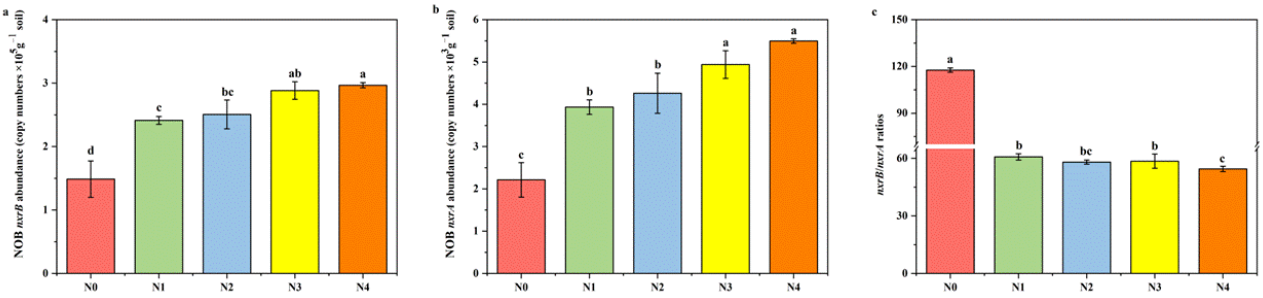

Figure 3. NOB abundance under different N application rates. (**a**) *Nitrospira*-like NOB abundance; (**b**) *Nitrobacter*-like NOB abundance; (**c**) ratios of *nxrB*/*nxrA* copy numbers. Bars indicate SD, different letters indicate significant differences among samples ($p < 0.05$, ANOVA). N0, N1, N2, N3, and N4 represent N fertilizer being used at levels of 0, 90, 150, 210, and 270 kg ha^{-1}, respectively.

3.4. Compositions of Soil Nitrospira-like NOB Taxa

All samples of the NOB *nxrB* gene sequenced with Illumina MiSeq yielded 343,050 optimum sequences, with 25,406–157,473 high-quality sequences per sample (Table S3). The phylogenetic tree displayed that the main phyla of *Nitrospira*-like NOB were Proteobacteria and Actinobacteria (Figure 4b). Venn analysis showed that there were 274 OTUs in the different N application rate treatments (N0, N1, N2, N3, N4), and the 20 OTUs with the highest relative abundance came from the *Nitrospira*-like NOB community. Under the various N application rates for NOB, OTU13 was the predominant bacteria (Figure 4a), and its relative abundance was 16.06–38.70%, respectively. In contrast to N0, N3 and N4 greatly enhanced the relative abundance of OTU13 (CP011801.1), for which BLAST analysis once more indicated that OTU13 (CP011801.1) may represent *Nitrospira moscoviensis* (Table S4).

3.5. Diversities of Soil Nitrospira-like NOB

In contrast to N0, N1, N2, N3, and N4 considerably heightened the Shannon diversity of *Nitrospira*-like NOB, while the Chao1 of *Nitrospira*-like NOB was not significant (Figure 5). The results based on the Bray–Curtis PCoA of *Nitrospira*-like NOB under different N application rates showed that *Nitrospira*-like NOB were significantly divided into two clusters of high-N soil and low-N soil (Figure 6a). The interpretation rate of the first axis of the principal component analysis graph was 38.63%, and the interpretation rate of the second axis was 19.36%. The community structure of *Nitrospira*-like NOB had a significant distance between low N (N0, N1, N2) and high N application rates (N3, N4), indicating that the community structure of *Nitrospira*-like NOB has obvious differences under different N application rates (Figure 6a,b).

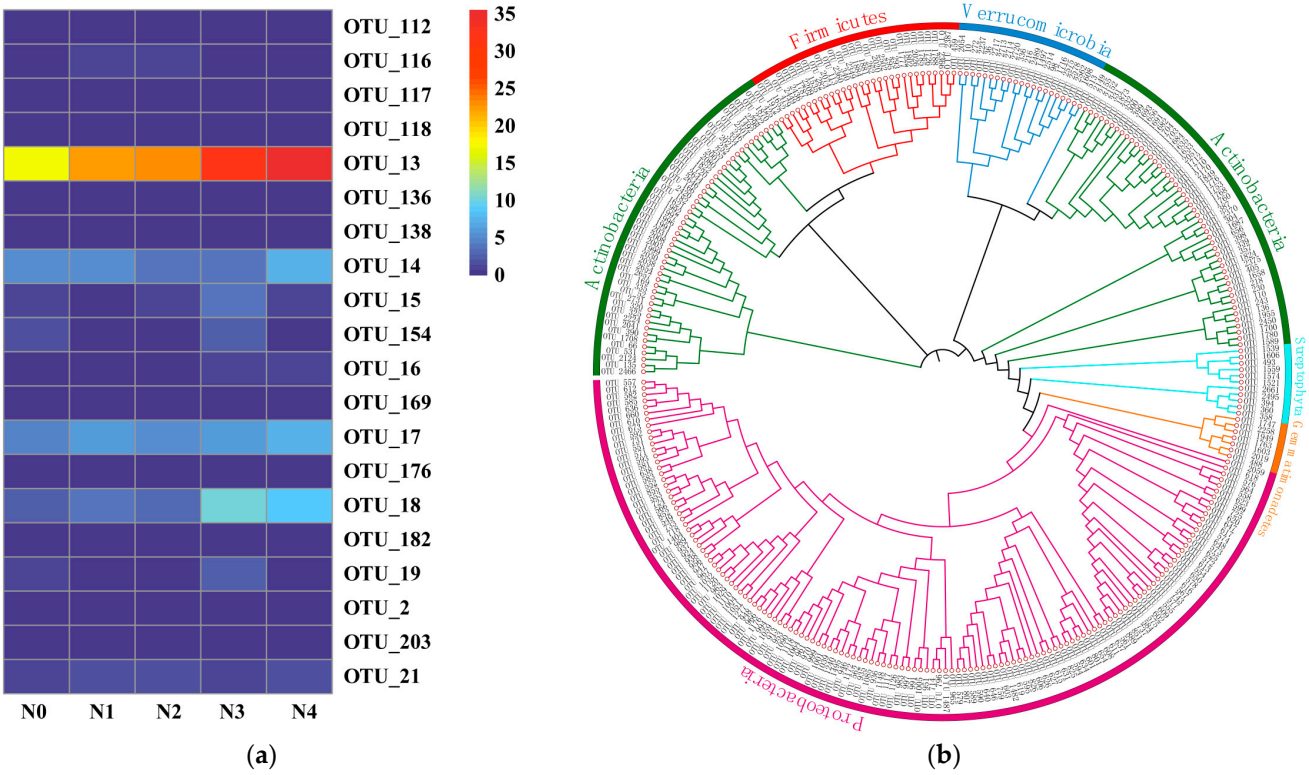

Figure 4. The relative abundance of dominant OTUs (**a**) and phylogenetic tree (**b**) of the NOB community under different N gradients. N0, N1, N2, N3, and N4 represent N fertilizer being used at levels of 0, 90, 150, 210, and 270 kg ha^{-1}, respectively.

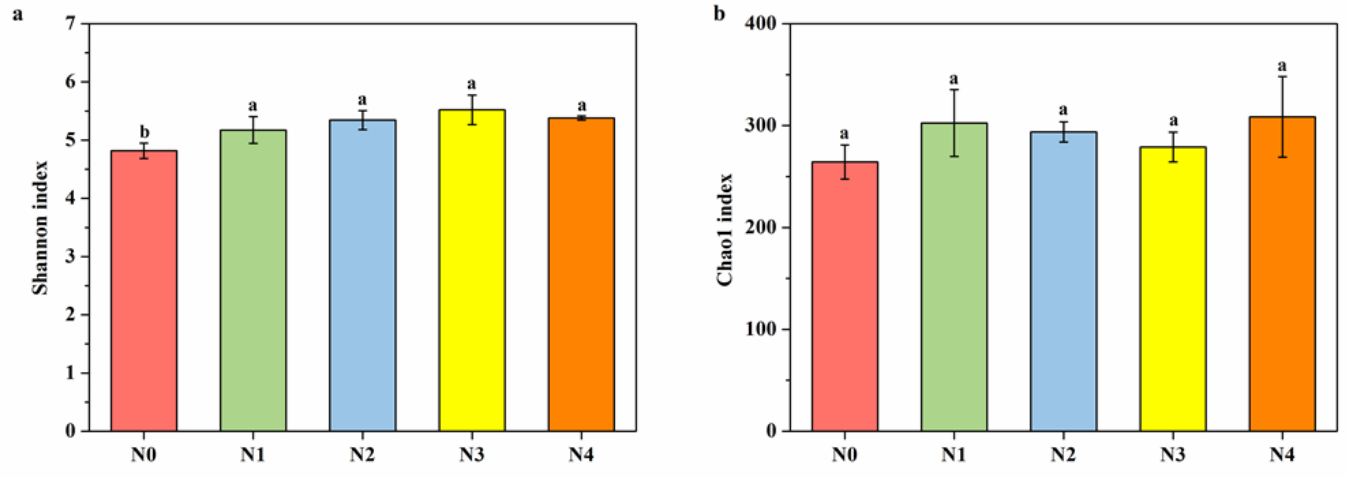

Figure 5. NOB diversity index (**a**) for Shannon index, (**b**) for Chao1 index) under different N application rates. Bars indicate SD, and different letters indicate significant differences among samples ($p < 0.05$, ANOVA). N0, N1, N2, N3, and N4 represent N fertilizer being used at levels of 0, 90, 150, 210, and 270 kg ha^{-1}, respectively.

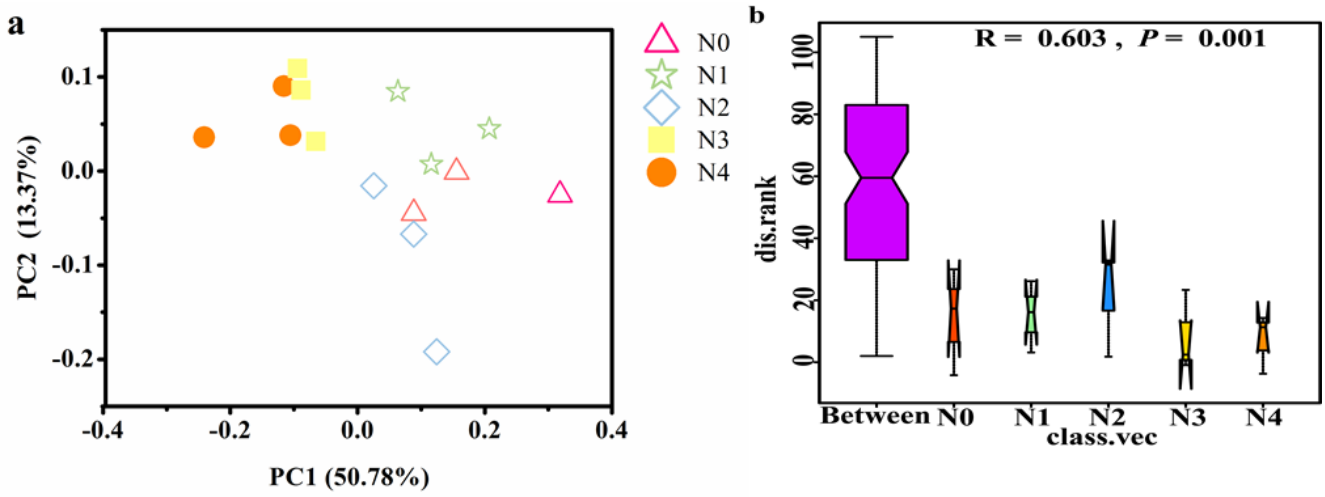

Figure 6. Principal coordinate analysis (PCoA) (**a**) and analysis of similarities (ANOSIM) (**b**) of the NOB community based on OTU. N0, N1, N2, N3, and N4 represent N fertilizer being used at levels of 0, 90, 150, 210, and 270 kg ha^{-1}, respectively.

3.6. Drivering Factors of Changes in Soil Nitrospira-like NOB Community

An RDA study revealed that AN was the primary cause of the shift of the *Nitrospira*-like NOB community structure ($p < 0.05$) (Figure 7, Table 2), and SOM, TN, NO_3^--N, NH_4^+-N, AN, and pH were positively correlated with N0, N1, N2, N3, and N4 (Figure 7). The contribution of *Nitrospira*- and *Nitrobacter*-like NOB abundance and microbial features (alpha, beta diversity) to PNO using the random forest analysis suggested that *Nitrospira*-like NOB abundance (10.26%) had the largest incremental in MSE compared to other components, indicating that *Nitrospira*-like NOB abundance was a major driving factor for PNO (Figure 8). The SEM was constructed to reveal the direct and indirect effects of soil nutrient content and soil enzyme activity on the biological traits of soil *Nitrospira*-like NOB under different N application rates (Figure 9). The SEM fully fitted the data (c2/df = 2.15, = 0.14, CFI = 0.99, c2/df = 0.01, = 0.91, CFI = 1.00), and the SEM explained 43.9% of the soil *Nitrospira*-like NOB community structural changes. AN ($\lambda = 1.71$, $p < 0.01$) had the biggest influence on the structure of *Nitrospira*-like NOB communities. TN ($\lambda = 0.38$), NO_3^--N ($\lambda = 0.58$), NH_4^+-N ($\lambda = 0.027$), and AN ($\lambda = 1.71$) had a direct impact on the *Nitrospira*-like NOB community structure. In addition, NH_4^+-N, NO_3^--N, and SOM indirectly affected the community structure of soil *Nitrospira*-like NOB through soil enzymes.

Table 2. Explanation rate and contribution rate of soil properties for the NOB community.

	Explains %	Contribution %	Pseudo-F	*p*
AN	15.6	31.6	2.4	0.014
NH_4^+-N	9.3	18.7	1.5	0.21
NO_3^--N	7.8	15.8	1.2	0.28
pH	6.3	12.7	1	0.37
TN	5.6	11.4	0.8	0.404
SOM	4.9	9.8	0.7	0.47

SOM, soil organic matter; TN, total nitrogen; AN, available nitrogen; NH_4^+-N, ammonium nitrogen; NO_3^--N, nitrate nitrogen.

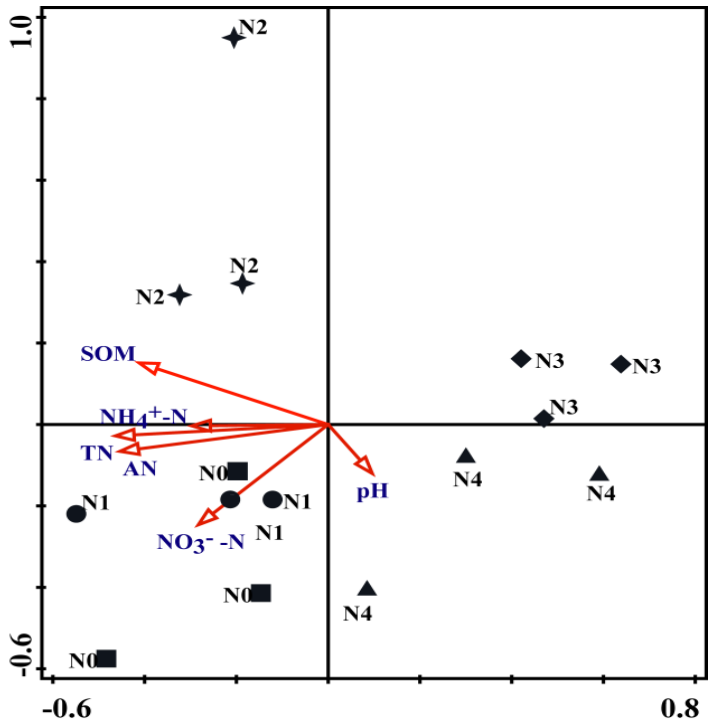

Figure 7. Redundancy analysis (RDA) between soil nutrients and the NOB community under different N application rates. SOM, soil organic matter; TN, total nitrogen; AN, available nitrogen; NH_4^+-N, ammonium nitrogen; NO_3^--N, nitrate nitrogen. N0, N1, N2, N3, and N4 represent N fertilizer being used at levels of 0, 90, 150, 210, and 270 kg ha^{-1}, respectively.

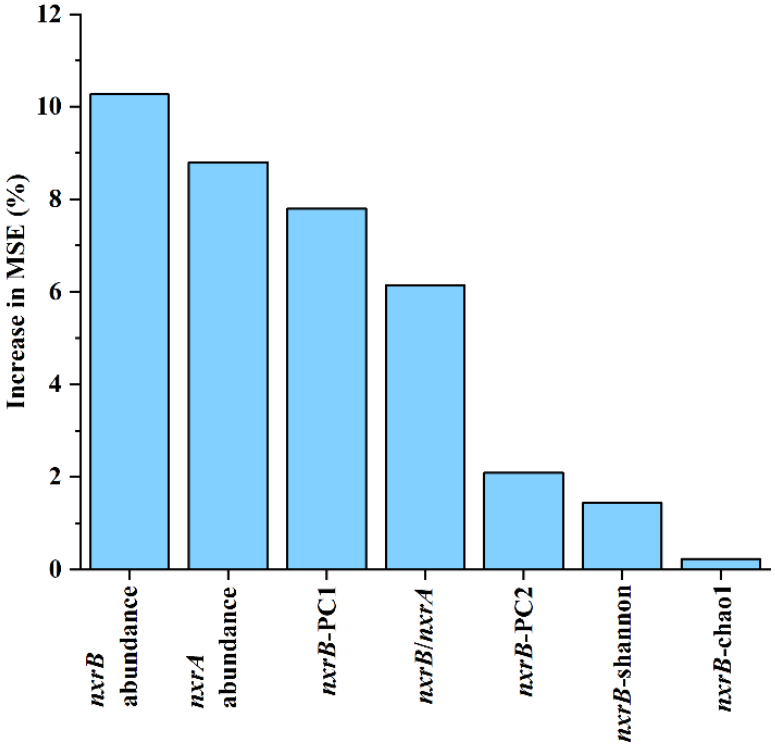

Figure 8. Random forest analysis under different N application rates. The horizontal axis represents NOB abundance; α diversity (Shannon, Chao1 Index) and β diversity (PC1, PC2) were predictive factors for PNO. IncMSE%, increase in mean squared error. N0, N1, N2, N3, and N4 represent N fertilizer being used at levels of 0, 90, 150, 210, and 270 kg ha^{-1}, respectively.

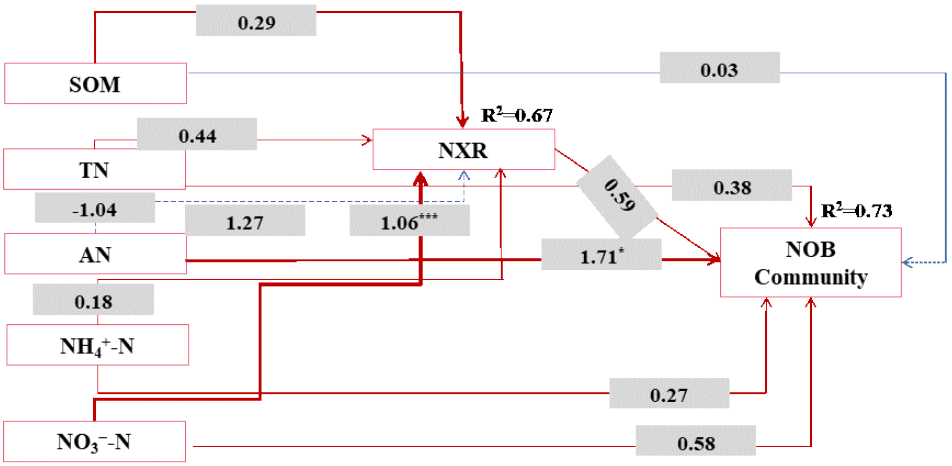

Figure 9. The structural equation model (SEM) between the soil nutrients, NOB community, and NXR. The arrow width is proportional to the strength of the path coefficients. Solid red arrows indicate a positive correlation, while blue dotted arrows indicate a negative relationship. * and *** indicate $p < 0.05$ and $p < 0.001$, respectively. SOM, soil organic matter; TN, total nitrogen; AN, available nitrogen; NH_4^+-N, ammonium nitrogen; NO_3^--N, nitrate nitrogen.

4. Discussion

4.1. Variations in N Application Rates' Impact on the Chemical Properties of Soil and Enzyme Activity

SOM is a key indicator of soil health, and it has important functions used to affect and evaluate soil fertility, maize productivity, and soil environment. The findings of this study showed that N fertilization significantly increases SOM, and Singh et al. [37] also showed that N fertilization can increase SOM. N fertilization may affect SOM in two major ways. First, compared with the soil without N fertilization, by encouraging plant development and raising the amount of litter and root biomass in the soil, N fertilizer may raise the content of SOM [38]. Second, N fertilizer can help to replace the SOM lost to the microbial modification or degradation of organic carbon, as well as the litter (leaves, straw, and manure) existing in the soil. This can improve the SOM content and increase carbon fixation in this era of intensive planting systems [39–44].

Our research also showed that adding N soil lowered the pH of the soil. Additionally, Zhang et al. [45] demonstrated how N treatment might cause soil acidity. The nitrification reaction of ammonium is the primary source of soil acidity following N application. This is because two hydrogen atoms are released for every mole of ammonium ion that is nitrated. Following N fertilization with urea, there was a rise in nitrate content and a reduction in soil pH [46]. Continuous use of ammoniated fertilizers can acidify the soil, especially with high urea applications [47]. However, when NO_3^- is leached or N is not taken up by plants, its nitrification process can cause soil acidification. The increase of NO_3^- and the decrease of NH_4^+ after N fertilization can also lead to soil acidification [48].

NXR is the key enzyme in nitrification and is mainly involved in the second step of nitrification, converting NO_2^- into NO_3^- for plant growth and utilization. In contrast to N0, N3 and N4 considerably elevated the activity of NXR. The results of Chen et al. [49] also revealed that the application of N fertilizer can increase the activity of soil enzymes, and the application of N fertilizer increased the yield of crops and crop residues. The increase of crop residues improves soil fertility, as the activity of soil enzymes directly affects soil nutrients by promoting the decomposition and release of nutrients in litter, thereby increasing the nutrient content in the soil.

4.2. Effects of Different N Application Rates on Soil Nitrospira- and Nitrobacter-like NOB Abundance

Our results showed that the abundance of *Nitrospira*-like NOB was significantly higher than that of *Nitrobacter*-like NOB. According to the research of Han et al. [27], the *nxrB* gene was dominant in soil samples because *Nitrospira*-like NOB had a greater abundance than *Nitrobacter*-like NOB. In natural systems with extremely low NO_2 concentrations, *Nitrospira* typically have a dominant role [23,50]. Following N fertilization, the abundance of *Nitrospira*- and *Nitrobacter*-like NOB was much higher than that of N0, and the abundance of NOB was significantly higher at high N application rates than at low N fertilizer application rates. This suggested that N input may be a key factor in the abundance of *Nitrospira*- and *Nitrobacter*-like NOB. The abundance of *Nitrospira* increased significantly by changing pH with the addition of lime to an acidic agriculture soil in Oregon [51]. Zhou et al. [52] also showed that the abundance of NOB was higher in high-N soils, which was consistent with the results of this study. This is an indication that NOB prefer fertile soil, and N application can increase SOM and improve soil fertility, thereby increasing the abundance of NOB. We found that PNO was strongly positively correlated with the abundance of *Nitrospira*- and *Nitrobacter*-like NOB. Attard et al. also reported that the abundance of *Nitrobacter* NOB was positively correlated with PNO [35]. Since pH influences not only the growth rate of NOB but also the availability of substrate to NOB, it has a significant impact on the NO_2 oxidation process. The impact of pH on NOB activity has been the subject of previous research [53]. *Nitrospira*-like NOB were sensitive to soil pH change [54]. De Boer and Kowalchuk showed that lower soil pH affected the activity of ammonia oxidizers because the ionization of ammonium increased as the pH decreased, thereby reducing the availability of ammonia [55]. However, there was no significantly negative correlation between PNO and soil pH. At the same time, PNO was significantly correlated with soil SOM, TN, AN, NO_3^--N, and NH_4^+-N. These results showed that N fertilizer caused soil acidification, which affected NOB abundance.

According to our research, *Nitrospira* was significantly more abundant in N3 and N4 than it was in N0, suggesting that high N content creates favorable conditions for methylcyclobacteria. N4 considerably raised the relative abundance of *Nitrospira* in comparison to N0. Zhong et al. [56] discovered that the application of N contributed to the relative abundance of *Nitrospira*, a finding that aligned with the findings of this investigation. *Nitrospira* can enhance the plant absorption of N and the N cycle [57]. In summary, the higher the N fertilizer application rate, the richer the nitrifying microorganisms, the stronger the nitrification effect, and the more favorable the process by which plants absorb N.

4.3. Relationship between Environmental Factors and Nitrospira-like NOB Community Structure

Compared with N0, the amount of N application (N1, N2, N3, N4) considerably elevated the diversity of *Nitrospira*-like NOB. Han et al. indicated that the α-diversity of *Nitrospira*-like NOB community changed significantly under different fertilization treatments [27]. NOB are not only completely oligotrophic, but they can also grow through trophic or mixed trophic metabolism [25]. As such, the additional N can provide more metabolic substrates for NOB and increase the diversity of NOB. This study showed that the community structure of *Nitrospira*-like NOB was affected by the amount of N application, which concurred with Liu et al.'s [58] conclusions, which also showed that the community structure of NOB was related to the addition of inorganic N. Han et al. [59] revealed that TN was the important environmental factor that regulated the *Nitrospira*-like NOB community structure under various fertilization regimes. Freitag et al. manifested that the community structure of *Nitrospira*-like NOB under fertilizer management was more diverse than those without fertilizer management [60]. The community structure of *Nitrospira*-like NOB was affected by NO_3^--N and NH_4^+-N, which can provide more substrate for NOB utilization. Different subtypes of NOB responded differently to inorganic N. For example, *Nitrospira* responded most positively to NH_4^+-N, while other lineages had the most obvious impact on NO_3^--N. RDA analysis and the SEM study showed that AN had the biggest influence on the NOB

community structure, as similarly reported by Liu et al. [58]. Inorganic N can meet the needs of different nitrite species and metabolisms, thereby increasing the diversity of soil NOB. The random forest analysis indicated that *Nitrospira*-like NOB abundance was the most important factor affecting PNO. The *Nitrospira*-like NOB community may be the driving factor for the regulation of nitrite oxidation processes in different N application rates under a mulched fertigation system. Han et al. [27] and Attard et al. [35] also considered that *Nitrospira*-like NOB, rather than *Nitrobacter*-like NOB, play a major role in low activity soils. Ouyang and Norton [9] also showed that *Nitrospira* was reported as the dominant NOB (>99.7%) in an agriculture site in Utah, USA. Our results suggest that *Nitrospira*-like NOB were the key functional players of NOB communities under N fertilizer addition in the mulched drip irrigation system and that the variation in PNO was attributed to the *Nitrospira*-like NOB community.

5. Conclusions

The contents of soil nutrients (SOM, TN, NO_3^--N, and NH_4^+-N) rose significantly with an increase in N fertilizer addition among 0–270 kg ha^{-1}; however, the pH decreased significantly. High rates of N application greatly raised PNR, PNO, and NXR activity in the soil. N fertilization significantly increased the abundance of *Nitrospira*- and *Nitrobacter*-like NOB. *Nitrospira moscoviensis* was the dominant bacterium of *Nitrospira*-like NOB, and its relative abundance was significantly higher in the soil with high N fertilization than in the soil without N fertilization. The community structure of *Nitrospira*-like NOB was mainly affected by the amounts of N fertilizer addition. TN, NO_3^--N, NH_4^+-N, and AN had a direct impact on the community structure of *Nitrospira*-like NOB, and AN had the greatest impact on the community structure of *Nitrospira*-like NOB. *Nitrospira*-like NOB played a major role in the variation of PNO in different N application rates under a mulched fertigation system. In conclusion, N fertilizer can cause soil acidification, which affects NOB abundance and community structure. We recommend 210 kg ha^{-1} of N fertilizer as the optimal application rate for spring maize cropping under film drip irrigation in Northeast China. This study presents a theoretical framework for the process and adjustments in the NOB community caused by various N fertilizer management practices in cropland under film drip irrigation in Northeast China.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13122909/s1, Table S1. Drip fertigation methods. Table S2. The correlation analysis among soil properties and soil enzymes under different N application rates. Table S3. Illumina MiSeq sequenced NOB data (at 97% sequence similarity) based on the *nxrB* gene. Table S4. The BLAST of NOB at NCBI. Figure S1. The map of experiment site. Figure S2. The plot experiment design. Figure S3. The correlation analysis among soil PNR, PNO, nitrification microbial abundance and soil properties under N application rates. SOM, soil organic matter; TN, total nitrogen; AN, available nitrogen; NH_4^+-N, ammonium nitrogen; NO_3^--N, nitrate nitrogen; PNR, potential nitrification rate. PNO, potential nitrite oxidation.

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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