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The Long-Term Application of Controlled-Release Nitrogen Fertilizer Maintains a More Stable Bacterial Community and Nitrogen Cycling Functions Than Common Urea in Fluvo-Aquic Soil

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Abstract: Controlled-release nitrogen fertilizer (CRNF) has been proven to surpass common urea by mitigating nutrient losses, enhancing soil quality, and improving crop productivity. However, the long-term effects of CRNF on soil biological properties are not well understood. Here, a 12-year field experiment was conducted with five treatments: no N fertilizer (PK); the split application of urea at the farmer's practice rate (FP) and the optimal rate (OPT); the one-time application of CRNF at the same rate as the OPT (CRNF); and a 20% reduced rate of the OPT (0.8CRNF). Soil samples were collected during the maize tasseling and filling stages; high-throughput sequencing and the PICRUSt2 method were employed to determine the bacterial community and its functional potential. The results showed that CRNF significantly increased alkaline hydrolysis N by 14.10% and 9.45% compared to OPT during the tasseling and filling stages, respectively. This increase in soil available N resulted in a significant increase in bacterial diversity of 2.09% and 2.35% compared with the FP and OPT, respectively. The bacterial community in the FP and OPT changed markedly between the tasseling and filling stages, with many bacterial species at the ASV and genus levels showing variations in relative abundance. In contrast, CRNF and 0.8CRNF exhibited stable N-cycling functions, as indicated by the lower variations in nitrate reductase and predicted N-cycling functional genes between the tasseling and filling stages. The obtained results suggest that CRNF application can enhance soil N supply, promote the formation of stable bacterial communities, and maintain stable N-cycling functions.

Keywords: bacterial community; controlled-release nitrogen fertilizer; fluvo-aquic soil; long-term fertilization; maize cultivation; nitrogen-cycling functions; urea

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1. Introduction

Nitrogen (N) fertilization is a prevalent agricultural practice used to increase crop yield, as N is considered as the essential element for plant productivity in terrestrial ecosystems [1]. However, the efficacy and sustainability of N fertilizers are questionable, especially for urea, which is the most common and cost-effective N fertilizer [2,3]. Once applied to soil, urea undergoes rapid hydrolysis, leading to a sudden increase in mineral N levels that often exceed the nutritional requirements of crops. As a result, approximately 70% of applied urea is lost to the environment through various pathways, causing low N use efficiency (NUE), soil acidification, and environmental pollution [4,5].

A possible alternative to urea is controlled-release nitrogen fertilizer (CRNF), which is designed to release N gradually and in sync with the specific nutrient demands of different crop growth stages [6]. This approach has been proven to be a safe, economical,

and efficient way to enhance NUE, reduce environmental damage, and lower labor/time costs [7–9]. For example, a recent meta-analysis covering 120 maize studies revealed that CRNF increased NUE by 24.1% and decreased N_2O emissions, N leaching, and ammonia volatilization by 23.8%, 27.1%, and 39.4%, respectively [10]. Despite its high price, the application of CRNF increased net profits by 8.5% to 15.2% due to reduced labor and fertilizer frequency for maize production in northeast China [11]. Moreover, previous studies have confirmed the benefits of CRNF on soil quality, such as adjusting the soil pH, increasing the soil nutrient content and availability, and improving soil aggregate structure and water-holding capacity [12–14]. Additionally, improved crop growth and yields by CRNF such as maize [10,11], wheat [15], and rice [16] have also been widely reported. However, most of the existing studies concentrated on the effects of CRNF on environmental impacts, agronomic effects, or nutrient content, ignoring its impact on soil biological traits, such as microbial community composition and function.

Soil microbes are responsible for multiple ecosystem services, such as nutrient cycling, organic matter decomposition, and soil aggregate stabilization [17]. However, the application of common urea often exerts adverse effects on microbial diversity and community structure due to its rapid hydrolysis and acidification impact [18,19]. In contrast, CRNF offers a slow and steady release of N without altering its form, potentially exerting a positive influence on the soil microbial community and enhancing N-cycling processes. However, the impact of CRNF application on microbial communities is still uncertain. For example, some studies have reported the enhancement of bacterial diversity and the stimulation of certain bacterial groups involved in N-cycling, in response to CRNF [20,21]. Other studies have found a decrease in bacterial diversity and reduced abundance of some bacterial groups, such as *Acidobacteria* and *Actinobacteria* [22]. Yet, some studies have found no significant difference in bacterial diversity between CRNF and urea [23].

Despite these findings, there remains a necessity to examine the temporal dynamics of microbial communities subsequent to CRNF and urea application, as many studies have primarily examined microbial communities at specific growth stages [20–23]. Given the distinctive N release patterns of CRNF and urea, these variations will invariably influence the N content and form in the soil, subsequently affecting the underground microbial community structure and N-cycling functions. The objectives of this study were to examine the dynamic variations of soil N availability, bacterial community, and N-cycling functions under the long-term application of CRNF and common urea during the critical growth stages of maize. Our hypothesis posited that compared with urea, long-term CRNF application could: (1) maintain a stable N supply and increase the available N content of maize throughout the growth stage (especially in the later growth stage); (2) increase bacterial diversity and maintain stable bacterial community structure and composition between the tasseling and filling stages; and (3) keep soil N-cycling enzymes and functions stable.

2. Materials and Methods

2.1. Site Description and Sampling

The long-term field experiment commenced in 2010 in the Huang-Huai-Hai plain, situated in Dezhou, Shandong Province, China (116°20′35.7 E, N37°21′24.5 N). This region experiences a warm and temperate semi-humid monsoon climate, with an average annual air temperature of 12.9 °C and annual precipitation ranging from 439.5 to 593.5 mm. The soil, originating from Yellow River alluvial sediments, was classified as Calcaric Fluvisol by the FAO [24]. At the beginning of the experiment, the soil properties were determined as follows: soil bulk density at 1.43 g cm^{-3}, organic matter at 10.94 g kg^{-1}, total nitrogen at 1.44 g kg^{-1}, available phosphorus at 16.24 mg kg^{-1}, and available potassium at 77.24 mg kg^{-1}.

The experiment was conducted on a winter wheat (Jimai 22)-summer maize (Zhengdan 958) double-cropping system using a randomized complete block design with three replicates; each plot was 40 m^2 (9.5 m × 4.2 m). The treatments included in this study were: (1) no N fertilizer (PK); (2) the split application of urea at the farmer's practice rate (FP); (3) the split application of urea at the optimal fertilizer rate (OPT); (4) the one-time application

of CRNF at the same rate as the OPT (CRNF); and (5) the one-time application of CRNF at a 20% reduction in the OPT (0.8CRNF). The CRNF was resin polymer-coated urea (44% N, with a longevity of 120 d; the coating is biodegradable). For the FP treatment, the urea application ratio was 2:3 (basal:bell-mouth) in the maize season and 1:2 (basal:jointing) in the wheat season. For the OPT treatment, the urea application ratio was 1:2 (basal:bell-mouth) in the maize season and 1:1 (basal:jointing) in the wheat season. For the CRNF and 0.8CRNF treatments, polymer-coated urea was applied once before crop sowing. For all of the treatments, triple superphosphate and potassium sulfate were applied as synthetic P and K basal fertilizers, respectively. Details of the fertilizer application are presented in Table 1.

Table 1. Fertilizer dosage of each treatment in a maize-wheat rotation system.

Crop	Treatment	Urea (kg ha^{-1})		Polymer-Coated Urea (kg ha^{-1})	Superphosphate (kg ha^{-1})	Potassium Sulfate (kg ha^{-1})
		Base	Topdressing			
Maize	PK	0	0	0	228	270
	FP	235	352	0	98	90
	OPT	174	348	0	228	270
	CRNF	0	0	545	228	270
	0.8CRNF	0	0	436	228	270
Wheat	PK	0	0	0	228	150
	FP	174	348	0	225	225
	OPT	261	261	0	228	150
	CRNF	0	0	545	228	150
	0.8CRNF	0	0	436	228	150

PK: no N fertilizer; FP: split application of urea at the farmer's practice rate; OPT: split application of urea at the optimal fertilizer rate; CRNF: one-time application of controlled-release nitrogen fertilizer; 0.8CRNF: one-time application of CRNF at a 20% reduced rate.

Soil samples were collected during the maize tasseling (BBCH 54) and filling stages (BBCH 75) of the year 2022, meaning that the experiment had been conducted for 12 years before sampling. Seven random soil cores (with a diameter of 5 cm and a depth of 20 cm) were collected from each plot and composited to represent one replicate. All of the samples were sealed in sterile plastic bags, kept on ice, and transported immediately to the laboratory. Large particles such as fronds, roots, and stones were removed by means of passage through a 2 mm sieve. Each sample was then divided into three fractions: one was stored at room temperature for chemical analysis, another was stored at 4 °C for extracellular enzyme analysis, and the third was stored at −80 °C for DNA extraction.

2.2. Soil Chemical and Biological Analyses

Soil chemical properties were analyzed according to the methods described by Lu (2000) using air-dried soil [25]. Soil pH was measured at 1:5 (*w*/*v*) soil: water mixture on an electronic pH probe (FE20, Mettler Toledo, Giessen, Germany). Soil organic matter (SOM) was determined using the potassium dichromate volumetric method with external heating. Soil total nitrogen (TN) was measured by Kjeldahl digestion. Ammonium (NH_4^+-N) and nitrate (NO_3^--N) were extracted with 2 M KCl at a soil-to-solution ratio of 1:10 (*w*/*v*) for 30 min and determined by a continuous flow analytical system (San++ System, Skalar, Breda, The Netherlands). Alkaline hydrolysis N (AN) was assayed by the alkali-hydrolyzed diffusion method. Available phosphorus (AP) was extracted by 0.5 M $NaHCO_3$ and determined using the molybdenum blue method. Available potassium (AK) was extracted by 1 M ammonium acetate and determined by atomic absorption spectrometry. Urease were determined by the indigo colorimetry method and calculated based on the content of NH_3^+-N released from one gram of soil within the cultivated time [26]. Nitrate reductase activity was determined using colorimetric methods, and the amount of reduced NO_3^--N was measured to represent the activities of soil nitrate reductase [27].

2.3. Soil DNA Extraction and High-Throughput Sequencing Analysis

Soil DNA was extracted from 0.5 g fresh soil using the FastDNA Spin Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's instructions. The content of extracted DNA was measured by a Nano Drop™ 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). To assess the bacterial communities, the V4–V5 regions of bacterial 16 s rRNA genes were amplified by using the primers 515F/907R [28]. The PCR amplicons were purified and quantified. Sequencing was performed using the Illumina MiSeq platform (Shanghai Personal Biotechnology Co., Ltd., Shanghai, China).

The high-throughput reads were analyzed with the UPARSE pipeline [29]. In brief, paired-end reads were merged into single sequences after low-quality bases were end-trimmed. The high-quality sequences were clustered with simultaneous chimera removal using the UNOISE3 algorithm into amplicon sequence variants (ASVs) with a 100% similarity threshold. Bacterial ASVs were then annotated against the RDP database. After the sequences assigned to chloroplasts were removed from the dataset, the remaining sequences of all samples were rarefied to 27,285 sequencing depth for downstream analysis.

2.4. Statistical Analysis

One-way ANOVA was used to test for treatment differences in soil variables, bacterial diversity, genus, and enzyme activities; paired comparison of treatment means was achieved by Fisher's LSD at $p < 0.05$. Linear regression analysis was carried out to examine the relationship between bacterial diversity and AN. The above analyses were implemented using SPSS 20. Principal coordinates analysis (PCoA), redundancy analysis (RDA), and PERMANOVA analysis were calculated in the R package "vegan". Different abundance analyses were performed in the package "DESeq2", and *p*-values were adjusted for multiple testing using the procedure of Benjamini and Hochberg. Enriched and depleted ASVs at different maize stages were defined as ASVs with absolute differential abundance >1.0 and adjusted $p < 0.05$. The functional genes involved in N-cycling were predicted by PICRUSt2 [30]. The content of TN, NH_4^+-N, NO_3^--N, and AN, the activity of urease and nitrate reductase, and the predicted N functional genes were first standardized (z-score transformation) and then averaged to acquire the N-cycling index for each sample. Random forest analysis was employed to identify the main bacterial genus to predict N-cycling functions using the package "randomForest", and the significance of each predictor on the response variable was assessed by using the package "rfPermute".

3. Results

3.1. Soil Physiochemical Properties

The fertilization regimes had a significant impact on the soil's physiochemical properties (Table 2). The PK treatment, which did not include N fertilizer for 12 years, resulted in a significant decrease in soil organic matter (SOM), total nitrogen (TN), and alkaline hydrolysis nitrogen (AN) while increasing available phosphorus (AP) and available potassium (AK). The CRNF treatment, which applied controlled-release N fertilizer, had different effects on soil N forms at different growth stages. At the tasseling stage, the CRNF treatment enhanced NH_4^+-N and AN by 125.53% and 25.35%, respectively, compared to the OPT treatment. The CRNF treatment also increased NH_4^+-N by 103.85% compared to the FP treatment, which applied conventional N fertilizer. However, the CRNF treatment reduced NO_3^--N by 25.35% and 28.41% compared to the FP and OPT treatments, respectively. At the tasseling stage, the CRNF treatment increased NH_4^+-N by 125.53% and AN by 25.35% compared to the OPT treatment and NH_4^+-N by 103.85% compared to the FP treatment, respectively. However, the CRNF treatment reduced NO_3^--N by 25.35% and 28.41% compared to the FP and OPT treatments, respectively. At the filling stage, the CRNF treatment increased AN and NO_3^--N by 5.36% and 13.99%, respectively, compared to the FP treatment and by 9.45% and 13.99%, respectively, compared to the OPT treatment.

Table 2. Soil physiochemical properties under different fertilization treatments.

Stage	Treatment	pH	Organic Matter (g kg^{-1})	Total N (g kg^{-1})	NH_4^+-N (mg kg^{-1})	NO_3^--N (mg kg^{-1})	Available N (mg kg^{-1})	Available P (mg kg^{-1})	Available K (mg kg^{-1})
Tasseling	PK	8.76 ± 0.01 a	12.93 ± 0.44 b	0.74 ± 0.03 b	1.84 ± 0.61 ab	16.97 ± 0.88 b	66.73 ± 2.32 b	35.04 ± 2.12 a	209.00 ± 16.82 a
	FP	8.82 ± 0.04 a	15.05 ± 0.64 a	0.94 ± 0.06 a	1.04 ± 0.43 b	24.85 ± 1.35 a	76.22 ± 3.38 a	12.00 ± 3.09 c	107.00 ± 6.00 c
	OPT	8.87 ± 0.03 a	13.95 ± 1.33 ab	0.88 ± 0.07 a	0.94 ± 0.15 b	25.91 ± 2.92 a	70.66 ± 3.23 b	14.66 ± 5.33 b	120.67 ± 19.66 bc
	CRNF	8.76 ± 0.08 a	13.46 ± 1.00 ab	0.90 ± 0.09 a	2.12 ± 0.50 a	18.55 ± 1.95 b	80.62 ± 0.14 a	16.88 ± 1.74 b	139.33 ± 23.50 bc
	0.8CRNF	8.74 ± 0.12 a	14.97 ± 0.70 a	0.86 ± 0.02 a	1.17 ± 0.54 b	16.80 ± 0.69 b	75.91 ± 3.96 a	14.48 ± 2.02 bc	155.67 ± 29.50 b
Filling	PK	8.74 ± 0.03 a	14.19 ± 1.20 b	0.79 ± 0.02 b	2.82 ± 0.83 a	7.12 ± 1.06 c	55.62 ± 0.62 d	26.56 ± 2.77 a	179.00 ± 23.58 a
	FP	8.66 ± 0.02 a	17.18 ± 0.28 a	1.01 ± 0.01 a	2.58 ± 0.40 a	9.08 ± 0.42 ab	69.36 ± 2.08 c	19.65 ± 2.16 bc	115.00 ± 7.00 c
	OPT	8.64 ± 0.06 a	15.93 ± 1.96 ab	0.92 ± 0.13 a	2.43 ± 0.39 a	8.29 ± 0.47 b	66.77 ± 0.18 c	23.14 ± 0.33 ab	154.00 ± 31.51 ab
	CRNF	8.67 ± 0.06 a	16.53 ± 0.76 a	0.91 ± 0.07 a	2.52 ± 0.38 a	9.45 ± 0.17 a	73.08 ± 1.86 a	14.54 ± 3.60 c	130.00 ± 7.55 bc
	0.8CRNF	8.67 ± 0.07 a	16.20 ± 0.56 ab	0.90 ± 0.02 a	2.59 ± 0.43 a	9.33 ± 0.31 ab	71.82 ± 2.22 ab	16.67 ± 1.37 c	152.67 ± 2.52 ab

Values within the same column followed by different letters indicate significant differences at $p < 0.05$ according to Fisher's LSD test. PK: no N fertilizer; FP: split application of urea at the farmer's practice rate; OPT: split application of urea at the optimal fertilizer rate; CRNF: one-time application of the controlled-release nitrogen fertilizer; 0.8CRNF: one-time application of CRNF at a 20% reduced rate.

3.2. Soil Bacterial Community

A total of 9679 bacterial amplicon sequence variants (ASVs) were detected after quality control; these ASVs belong to 23 phyla, 51 classes, 109 orders, 218 families, and 461 genera. Long-term application of CRNF has a significant positive impact on bacterial alpha diversity (Figure 1). Specifically, during the tasseling stage, the CRNF treatment increased bacterial diversity by 2.09% and 2.35% compared to the FP and OPT treatments, respectively. Similarly, the 0.8CRNF treatment resulted in increases of 3.81% and 4.08%. Moving to the filling stage, the CRNF and 0.8CRNF treatments showed bacterial diversity increases of 4.84% and 3.31%, respectively, compared to the PK treatment. Linear regression analysis revealed a significant positive correlation between soil bacterial alpha diversity and soil AN content (r = 0.48, p = 0.008).

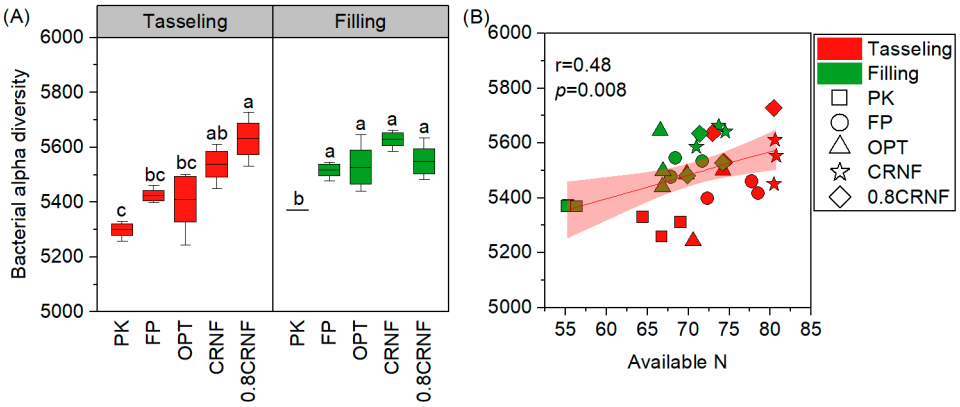

Figure 1. Bacterial alpha diversity under different fertilization treatments (**A**). Error bars represent the standard deviations of three replicates. Boxes with different letters (shown above each) are significantly different (p < 0.05) according to Fisher's LSD test. The regression relationships between the soil available N and bacterial alpha diversity (**B**). PK: no N fertilizer; FP: split application of urea at the farmer's practice rate; OPT: split application of urea at the optimal fertilizer rate; CRNF: one-time application of controlled-release nitrogen fertilizer; 0.8CRNF: one-time application of CRNF at a 20% reduced rate.

The dominant phylum was Proteobacteria, with relative abundances ranging from 34.60% to 42.00% (Figure 2). Other high-abundance species include the phyla *Actinobacteria* (14.40% to 20.60%), *Planctomycetes* (4.96% to 6.22%), *Acidobacteria* (15.90% to 19.90%), *Firmicutes* (6.52% to 8.14%), *Bacteroidetes* (3.60% to 5.06%), *Chloroflexi* (3.76% to 5.06%), *Gemmatimonadetes* (1.45% to 2.11%), *Verrucomicrobia* (0.07% to 0.53%), and *Nitrospirae* (0.63% to 1.15%).

Principal coordinate analysis (PCoA) was conducted based on Bray–Curtis dissimilarity at the ASV level (Figure 3). The first two axes including PCo1 and PCo2 accounted for 20.91% and 10.89% of the community variations, respectively. It is worth noting that the bacterial communities of the PK, FP, and OPT treatments at the tasseling stage were distinctly separated from the filling stage along PCo1, while there were no significant differences between the two growth stages in the CRNF and 0.8CRNF treatments. These results indicate that the application of CRNF resulted in a more stable bacterial community structure than common urea. Redundancy analysis showed that AN, TN, NO_3^--N, NH_4^+-N, and AP played a vital role in structuring the bacterial communities.

Long-term application of common urea resulted in a larger amount of differentially abundant ASVs than CRNF between the tasseling and filling stages (Figure 4). There were 144, 186, and 150 ASVs that showed significant changes for PK, FP, and OPT treatment, while only 84 and 43 ASVs were strongly affected by the CRNF and 0.8CRNF treatments between the tasseling and filling stages. Specifically, there were 80, 81, 69, 64, and 20 ASVs significantly enriched in the tasseling stage and 66, 105, 81, 20, and 23 ASVs significantly enriched in the filling stage for PK, FP, OPT, CRNF, and 0.8CRNF treatments, respectively. Similarly, among the bacterial genera with the highest relative abundance, there were 6, 9,

and 9 genera that showed significant changes for PK, FP, and OPT treatments, while only 4 and 0 ASVs were strongly affected for the CRNF and 0.8CRNF treatments between the tasseling and filling stages. Specifically, for the OPT treatment, the relative abundance of *Luteitalea*, *Arboricoccus*, *Baekduia*, *Skermanella*, *Aggregatilinea*, and *Thermoanaerobaculum* was higher in the tasseling stage, while the relative abundance of *Thermanaerothrix*, *Brevitalea*, and *Arthrobacter* was higher in the filling stage. For the CRNF treatment, the relative abundance of *Aggregatilinea* was higher in the tasseling stage, while the relative abundance of *Vicinamibacter*, *Skermanella*, and *Thermoanaerobaculum* was higher in the filling stage.

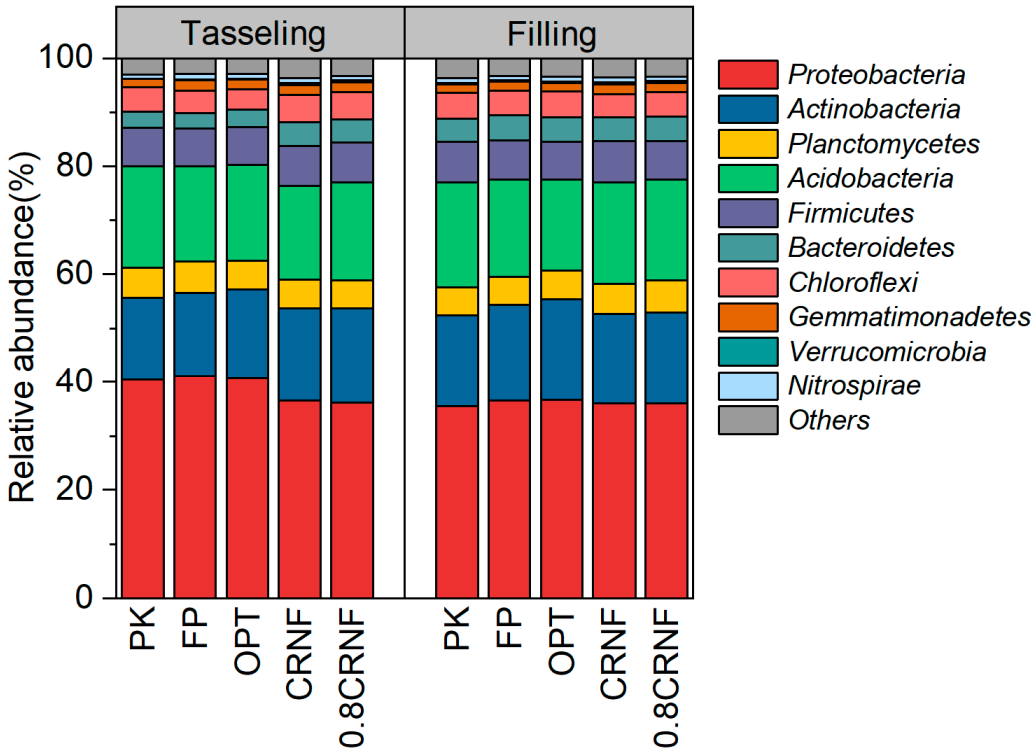

Figure 2. Relative abundance of major bacterial communities at the phylum level under different fertilization treatments. PK: no N fertilizer; FP: split application of urea at the farmer's practice rate; OPT: split application of urea at the optimal fertilizer rate; CRNF: one-time application of controlled-release nitrogen fertilizer; 0.8CRNF: one-time application of CRNF at a 20% reduced rate.

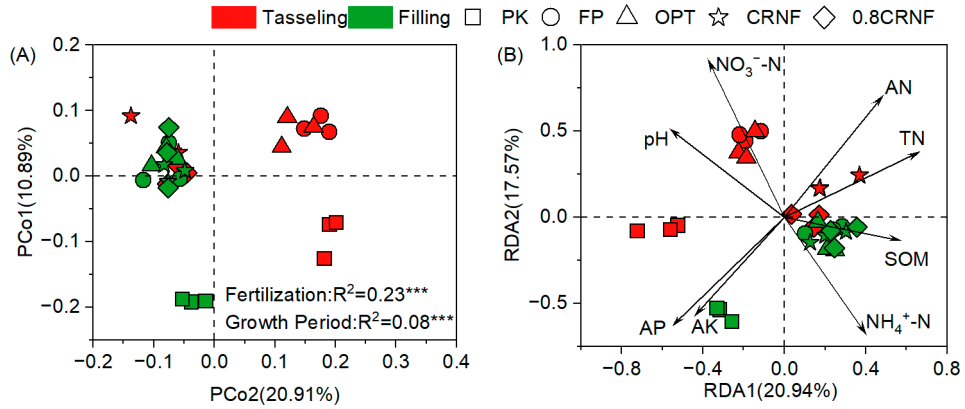

Figure 3. Principal coordinates analysis (**A**) and redundancy analysis (**B**) of ASV-based Bray–Curtis distance. *** mark significant differences at $p < 0.001$ based on PERMANOVA analysis. PK: no N fertilizer; FP: split application of urea at the farmer's practice rate; OPT: split application of urea at the optimal fertilizer rate; CRNF: one-time application of controlled-release nitrogen fertilizer; 0.8CRNF: one-time application of CRNF at a 20% reduced rate.

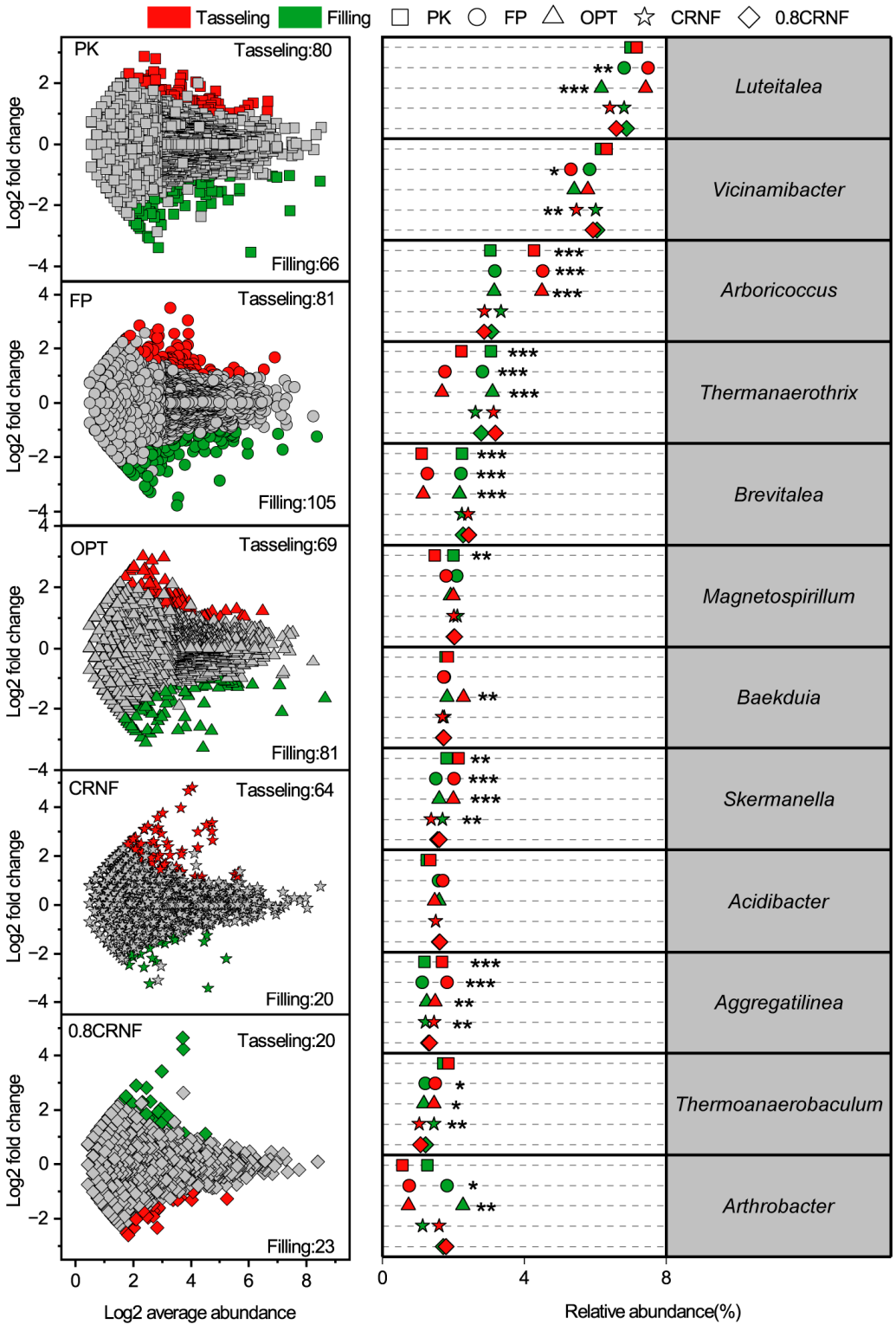

Figure 4. Effects of different fertilization treatments on bacterial composition at ASV and genus levels. *, **, and *** mark significant differences at $p < 0.05$, 0.01, and 0.001 between the tasseling and filling stages. PK: no nitrogen application; PK: no N fertilizer; FP: split application of urea at the farmer's practice rate; OPT: split application of urea at the optimal fertilizer rate; CRNF: one-time application of controlled-release nitrogen fertilizer; 0.8CRNF: one-time application of CRNF at a 20% reduced rate.

3.3. Nitrogen Function

Compared with the treatments with N fertilizer, PK treatment exhibited the highest urease activity (Figure 5), which was 2.27–6.26% higher at the tasseling stage, and 7.02–11.42% higher at the filling stage, respectively. The nitrate reductase activity varied among the different treatments and growth stages. At the tasseling stage, the nitrate reductase activity in the CRNF treatment exhibited a significant increase of 6.36% compared to the OPT treatment. In addition, nitrate reductase in the PK, FP, and NPK treatments increased significantly by 7.19%, 10.06%, and 10.59%, respectively, in the filling stage compared with the tasseling stage, but the changes were not significant in the CRNF treatment.

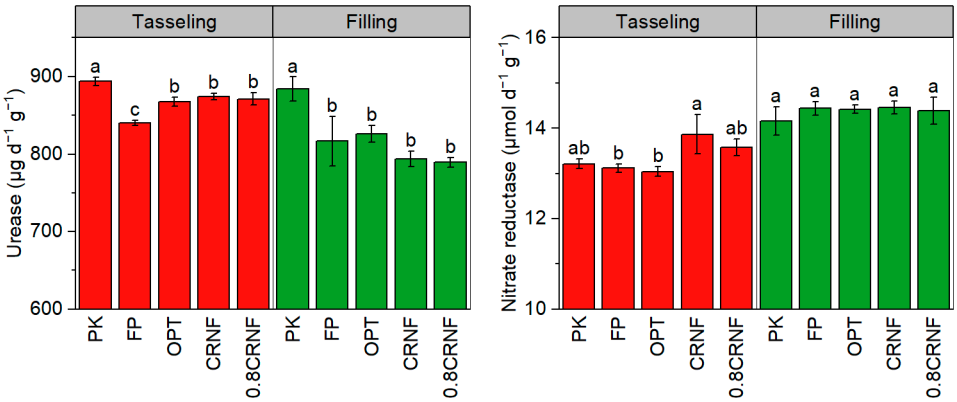

Figure 5. Soil enzyme activities under different fertilization treatments. Bars with different letters (shown above each) are significantly different ($p < 0.05$) according to Fisher's LSD test. PK: no N fertilizer; FP: split application of urea at the farmer's practice rate; OPT: split application of urea at the optimal fertilizer rate; CRNF: one-time application of controlled-release nitrogen fertilizer; 0.8CRNF: one-time application of CRNF at a 20% reduced rate.

PICRUSt2 was used to predict the abundance of key functional genes related to N-cycling (Figure 6). For the PK treatment, *ureC*, *gdh*, and *norB* were significantly enriched in the tasseling stage, while *amoA*, *amoB*, *amoC*, *nosZ*, and *nirK* were significantly enriched in the filling stage. For the FP and OPT treatment, *gdh*, and *hao* were significantly enriched in the tasseling stage, while *amoA*, *amoB*, *nirK*, *nosZ*, *narB*, and *nasA* were significantly enriched in the filling stage. For the CRNF treatment, *nirk* was significantly enriched in the tasseling stage, while *nifA* was significantly enriched in the filling stage. However, no significant changes in the N-cycling gene were detected in the 0.8CRNF treatment between the tasseling and filling stages. The above results indicated that the application of CRNF is beneficial to maintaining stable N-cycling genes.

Different N fertilizer application regimes significantly affected the soil N-cycling index (Figure 7). During the tasseling stage, the CRNF treatment showed the highest N-cycling index, increasing significantly by 44.02% compared to the PK treatment and by 30.88% compared to the OPT treatment. During the filling stage, there were no significant differences in the N-cycling index among the N fertilizer application treatments (FP, OPT, CRNF, and 0.8CRNF), but it increased significantly by 18.21%, 24.75%, 15.73%, and 20.89%, respectively, compared to the PK treatment. The results of the linear regression analysis showed that there is a significant correlation between the N-cycling index and the soil bacterial community structure under different fertilization treatments ($r = -0.61$ $p < 0.001$). The random forest model analysis found that *Arthrobacter* and *Thermoanaerobaculum* are closely related to the soil N-cycling index.

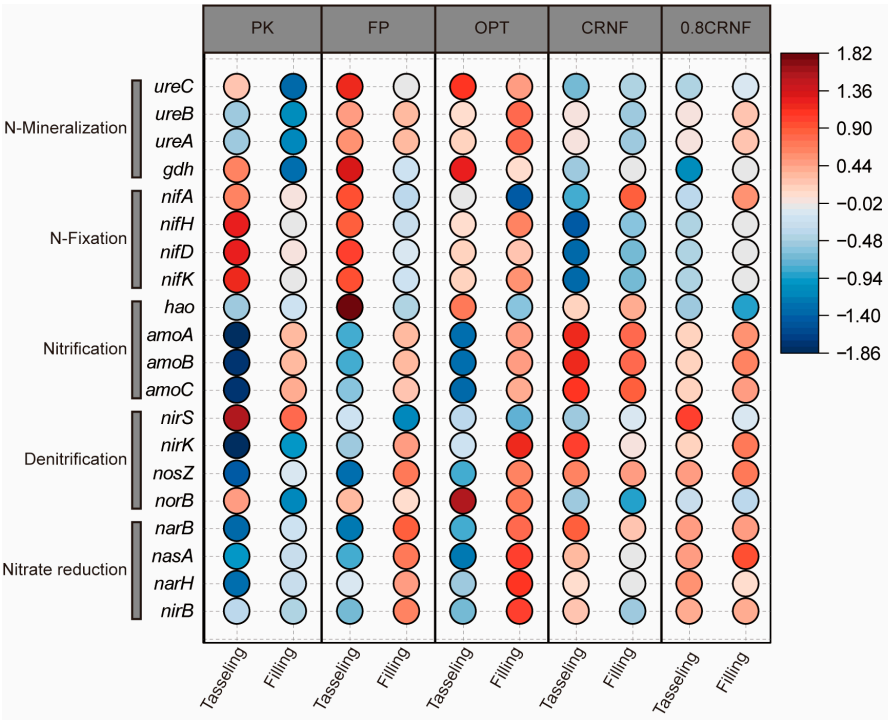

Figure 6. PICRUSt2 analysis predicted bacterial functional genes involved in nitrogen cycling under different fertilization treatments. PK: no N fertilizer; FP: split application of urea at the farmer's practice rate; OPT: split application of urea at the optimal fertilizer rate; CRNF: one-time application of controlled-release nitrogen fertilizer; 0.8CRNF: one-time application of CRNF at a 20% reduced rate.

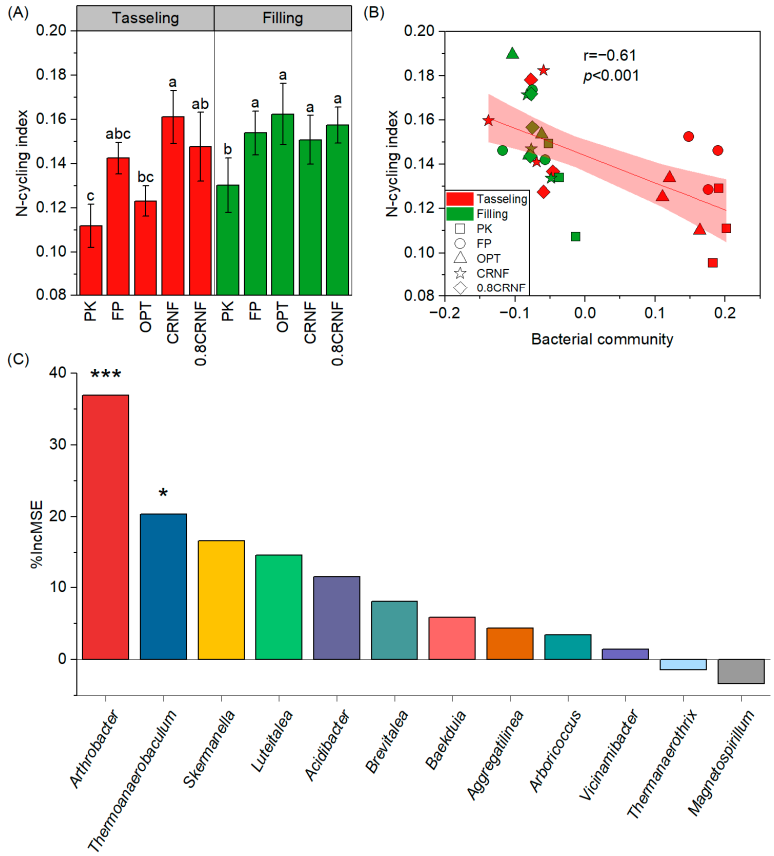

Figure 7. Soil N-cycling index under different fertilization treatments (**A**). Bars with different letters

(shown above each) are significantly different ($p < 0.05$) according to Fisher's LSD test. PK: no N fertilizer; FP: split application of urea at the farmer's practice rate; OPT: split application of urea at the optimal fertilizer rate; CRNF: one-time application of controlled-release nitrogen fertilizer; 0.8CRNF: one-time application of CRNF at a 20% reduced rate. The regression relationships between the soil N-cycling index and bacterial community (**B**). The bacterial community is represented by PCo1 in principal coordinates analysis. A random forest model showing the effects of the major bacterial genera on the soil N-cycling index (**C**). Significance levels of each predictor are indicated by * $p < 0.05$, and *** $p < 0.001$.

4. Discussion

4.1. Effects of CRNF on Soil Nitrogen Content

Traditional quick-release N fertilizers are susceptible to environmental factors such as moisture and temperature, which lead to dissolution, leaching, or volatilization, resulting in N loss [31]. Controlled-release nitrogen fertilizer (CRNF) can reduce these losses by releasing N slowly, thus maintaining more available N in the soil. In this study, we compared the effects of the one-time application CRNF and the split application of urea on soil available N content during maize growth stages. We found that the CRNF treatment significantly increased alkaline hydrolysis nitrogen (AN) content by 14.10% and 9.45% during the maize tasseling and filling stages, respectively, compared to the OPT treatment. This result agrees with previous studies that reported higher available N content in the soil after applying CRNF to maize [11], wheat [15], rice [32], and cotton [33], especially in the later stages of crop growth. This indicates that a single application of CRNF can meet the nutrient demands throughout the crop's growth cycle. Moreover, we found that the split application of urea (OPT treatment) resulted in unstable fluctuations in soil available N content. This was manifested by 39.67% higher NO_3^--N content in the tasseling stage, likely due to the conversion of NH_3^+-N to NO_3^--N after urea topdressing [34]. However, this advantage was diminished in the filling stage, as the OPT treatment exhibited a 13.99% decline in NO_3^--N content, possibly due to crop uptake and environmental losses [33]. A previous study also found that CRNF reduced soil NO_3^--N content during the maize spike formation stage but increased it after the flowering stage [35]. This may be due to the ability of CRNF to provide a steady supply of nutrients based on crop requirements, retain NO_3^--N for a longer time, and reduce losses [6,9]. Thus, CRNF has an advantage over urea in providing slow and sustained N release, which preserves high and stable N content in the soil.

4.2. Effects of CRNF on the Bacterial Community

Microbial diversity serves as a critical indicator for evaluating soil quality and fertility [36], yet recent meta-analyses have reported a 2.3% reduction in soil bacterial diversity due to N addition, especially in urea-fertilized croplands [37]. This phenomenon can be attributed to N-induced alterations in soil pH, with certain bacterial taxa exhibiting varying adaptability to pH conditions [38,39]. While CRNF application is also associated with decreased bacterial diversity [22,40], our study contradicts these trends, revealing a noteworthy increase in bacterial diversity post CRNF application. This positive outcome is likely due to the pH exceeding 8.5 in our experimental soil, with minimal pH variations induced by N fertilization. According to Geisseler and Scow [41], pH variations within the range of 6 to 8.5 and below 0.5 minimally impact microorganisms. Additionally, two meta-analyses suggested that the main factor influencing microbial communities was resource augmentation rather than soil acidification [19,42]. Aligning with this, our study establishes a strong positive correlation between bacterial diversity and AN, indicating that CRNF application augments soil AN, mitigating constraints associated with N deficiency in microbial growth. Therefore, CRNF has the potential to enhance soil bacterial diversity by providing a sustained N supply, particularly in alkaline flavor-aquic soil.

Our PCoA and RDA results suggest that long-term CRNF application leads to a stable soil bacterial community structure compared to urea. Microorganisms are very sensitive

to their environmental conditions and can quickly adjust to small changes [43]. N is an essential element for microbial growth and metabolism [44], so maintaining a balanced level of N in the soil allows microorganisms to adapt without suffering from excess or deficiency. CRNF provides a stable reservoir of available N, which facilitates the colonization of specific ecological niches by microbial communities, engendering a relatively stable community structure and sustaining competitive and cooperative relationships among microbial species [23,45]. CRNF can change the interaction between microbial species and increase the diversity of symbiotic modules, thereby reducing the risk of soil fertility degradation [21]. On the other hand, when soil available N content fluctuates, as in the case of urea application, the abundance of some species that use N as an energy source or electron acceptor also changes [19]. In addition, due to different survival strategies, an increase in available N content may promote the rapid growth of R-strategist eutrophic groups, while a decrease in available N content is beneficial to the survival of K-strategist oligotrophic species [46,47]. Therefore, the one-time application of CRNF can avoid fluctuations in available N caused by the split application of urea and help form a stable bacterial community structure.

Our different abundance analyses indicated that urea treatments (FP and OPT) affect more bacterial ASVs and genera between growth stages compared to CRNF treatments (CRNF and 0.8CRNF). This supports the argument that CRNF application prevents species succession induced by urea, fostering a stable bacterial community composition. At the genus level, a conspicuous alteration in the relative abundance of *Thermanaerothrix*, *Brevitalea*, *Luteitalea*, *Baekduia*, *Arthrobacter*, *Arboricoccus*, and *Thermoanaerobaculum* was observed in the OPT treatment between the growth stages. In contrast, such fluctuations were not evident in the CRNF treatment. *Thermanaerothrix* and *Brevitalea*, both characteristic K-strategist species [48,49], exhibited increased abundance with the reduction in soil N content after urea topdressing. *Luteitalea* and *Baekduia*, typical denitrifying bacteria [50,51], proliferated with higher NO_3^--N content in the FP and OPT treatments in the tasseling stage. Bacterial species engaged in nitrification and N fixation, such as *Arthrobacter* and *Arboricoccus*, showed sensitivity to N addition [52,53], with them also displaying significant alterations in OPT but not in CRNF treatments. Notably, there was a discernible augmentation of *Skermanella* and *Thermoanaerobaculum* in the tasseling stage under the urea application treatments. Conversely, a pronounced enrichment of these microbial species was observed in the filling stage when subjected to the CRNF treatment. *Skermanella* has been found to increase with N fertilizer application and is confirmed to be related to nitrate reduction and N fixation [54,55]. *Thermoanaerobaculum* has been demonstrated to play an essential role in decomposing organic carbon sources for denitrification microbial species [56]. These findings emphasize that alterations in N form and availability induced by urea application led to significant shifts in bacterial community composition involved in N-cycling, while CRNF application promotes the formation of a stable bacterial community structure and composition.

4.3. Effects of CRNF on Nitrogen Cycling Functions

Soil enzymes are important indicators for evaluating the functionality of N-cycling and transformation in soil [57,58]. Among these enzymes, urease is instrumental in catalyzing the hydrolysis of urea into carbon CO_2 and NH_3, thus influencing the levels of available N in the soil [59]. The application of N fertilizer can influence urease activity in different ways, depending on the type and amount of fertilizer, as well as the soil properties. In this study, we found that the long-term application of N fertilizer (either urea or controlled-release N fertilizer (CRNF)) resulted in a significant decrease in urease activity. This is contrary to most studies that have shown that N fertilizer can increase the substrate supply for urease-harboring bacterial species, thereby increasing urease activity [59,60]. However, the effect of N fertilizer application on urease also depends on the soil type. For example, urease activity was significantly increased in inceptisols, oxisols, and dystudepts in response to N addition but was significantly decreased in aridisols and showed no change in alfisols [59]. Moreover, a higher rate of application of N may either promote urease activity as an enzyme

substrate at the beginning or decrease its activity with subsequent high rates due to soil acidification and salinization since soil enzymes are strongly influenced by soil pH and saline conditions [60]. Another soil enzyme that is involved in N-cycling is nitrate reductase, which is a key denitrifying enzyme that reduces nitrate to nitrite [61,62]. Consistent with previous studies, we found that CRNF application significantly increased nitrate reductase activity [20]. Therefore, applying CRNF can slow down the conversion of nitrate into other forms, which can reduce N loss from the soil.

Soil N cycle is a complex biogeochemical process encompassing various N-transforming mechanisms, such as N-mineralization, N-fixation, nitrification, and denitrification [63]. The response of N-cycling function genes to N addition often exhibits variability contingent upon the form and duration of fertilizer [64,65]. For instance, the application of organic N fertilizers tends to exert a more pronounced influence than their inorganic counterparts [66]. In the present study, we discerned that the nitrate reductase activity and relative abundance of N-cycling functional genes (*gdh*, *hao*, *amoA*, *amoB*, *nirK*, *nosZ*, *narB*, and *nasA*) showed significant changes during the tasseling and filling stages in the FP and OPT treatments, as opposed to the CRNF and 0.8CRNF treatments. This result indicated that the application of CRNF is beneficial to sustained and stable N-cycling functions. N cycle transformation is closely related to existing soil N status [67]. The gradual and continuous release of nutrients by CRNF mitigates the risk of excessive N supply and transient concentration peaks associated with urea application, thereby preserving soil N content and existence form. This, in turn, fosters a stable transformation of N. Furthermore, the transformation of N unfolds with the active participation of soil microorganisms, and their utilization of N is intricately influenced by N supplementation [68]. Our linear regression analysis and random forest model unveiled a close correlation between soil N-cycling index and microbial community structure, as well as specific microbial species. The gradual release characteristic of CRNF aligns more harmoniously with the intricacies of the soil microbial ecosystem, thereby catalyzing a more equilibrated and organized N transformation orchestrated by microorganisms. This stable functionality in N cycle conversion contributes to the abatement of N loss, the enhancement of nutrient utilization efficiency, and the preservation of soil health.

5. Conclusions

Compared with common urea, the long-term application of controlled-release nitrogen fertilizer (CRNF) significantly increased soil alkaline hydrolysis N content and reduced the fluctuations in NO_3^--N content. This sustained and stable nitrogen supply significantly increased the bacterial diversity by 2.09–2.35%. Furthermore, long-term CRNF application engendered a more resilient bacterial community structure, forestalling alterations in species abundance induced by urea application. This stable microbial community structure and composition is conducive to the formation of robust N-cycling functions. Consequently, the long-term application of CRNF emerges as a promising strategy to enhance soil quality by optimizing nutrient supply and fortifying the soil microbial ecosystem, which presents an effective approach for promoting sustainable agriculture.

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