

Article

Nitrogen and Water Regulate the Soil Microbial Carbon Cycle in Wheat Fields Primarily via the Pentose Phosphate Pathway

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Abstract

To clarify how nitrogen (N) and water regulate the microbe mediated carbon (C) cycle in farmland, a 3-year experiment was conducted in a wheat–maize rotation at Jiaozhou Station, North China. Twelve treatments combined four drip irrigation regimes (T1: no irrigation; T2: 40 mm irrigation at flowering; T3: 40 mm irrigation at the grain filling stage; T4: both, 40 mm each) and three N levels (N0: 0 kgN·hm^{−2}; N1: 92 kgN·hm^{−2}; N2: 184 kgN·hm^{−2}). In this study, we measured wheat yield and biomass, soil organic carbon and nitrogen content, soil respiration, soil microbial community, and C-metabolic genes. The results showed that wheat yield increased with N, peaking at 8949.81 kg·hm^{−2} in the N2T3 treatment, while irrigation had no significant independent effect on yield but interacted with nitrogen fertilization: under identical nitrogen levels (N1, N2), yields in the T1 and T2 treatments were significantly lower than those in the T3/T4 treatments. The soil organic carbon content in N2 was significantly higher; the soil C/N ratio was highest in N2, and T3 resulted in a significantly higher C/N ratio than T1 under the same N level; total soil respiration in N0 was significant lower, and T4 had higher respiration than T2 under the same N level. N addition increased Actinobacteriota, Chloroflexi, Gemmatimonadetes, and Ascomycota, while decreasing Proteobacteria and Acidobacteriota. No reduction in fungal phylum was observed with nitrogen addition. N application significantly upregulated key enzymes in the pentose phosphate pathway (e.g., transketolase K00615, transaldolase K00616), while irrigation increased phosphoserine aminotransferase (K00831) abundance and decreased methylmalonyl-CoA mutase (K01848) abundance. N2T3 maintains high SOC content while achieving maximum yield, promoting soil fertility retention. Compared to T4, N2T3 also enhances water use efficiency. The N2T3 treatment (high N and grain filling stage irrigation) achieved the optimal balance between high wheat yield and SOC sequestration.

Keywords: carbon metabolism functional genes; microbial community structure; soil carbon cycle; water–nitrogen interaction; winter wheat



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1. Introduction

Soil is the largest carbon pool in terrestrial ecosystems, storing an estimated 1500–2400 Pg of carbon (C), and plays a critical role in regulating the global C cycle [1]. Within this cycle, soil microorganisms act as key mediators of soil C storage and govern the partitioning of plant-derived C between the soil organic carbon (SOC) pool and atmospheric CO₂ [2]. As such, they hold an irreplaceable role in responding to global climate change, sustaining ecosystem functions, and modulating carbon cycling processes. As the terrestrial ecosystem most heavily influenced by human activities, farmland ecosystems host soil

microorganisms that serve as sensitive and rapid indicators of soil quality changes. Various management practices—such as nitrogen fertilization and irrigation [3,4]—can modify soil carbon cycling by altering the distribution and interactions of microbial carbon-fixation genes and associated communities [5].

Nitrogen (N) fertilization is a key management strategy for maintaining soil health and crop productivity [6], and its impact on soil microorganisms is two-sided. An appropriate amount of N input can promote microbial growth by increasing the content of available nutrients [7] and increase microbial biomass in farmland [8]. However, long-term N fertilization has been shown to reduce microbial biomass [4], and excessive application can induce soil acidification [9] and cation imbalance [10]—thereby suppressing microbial nitrogen transformation efficiency and overall metabolic activity [11].

N addition may also shift microbial nutrient demand, leading to structural changes in soil microbial communities. Long-term N application has been found to increase fungal abundance while reducing fungal α -diversity; bacterial abundance, in contrast, often shows no significant response, but its diversity shows a trend of first increasing and then decreasing [12]. This difference is mainly due to the regulatory effect of pH [13]. At the functional level, nitrogen fertilizer has a significant impact on extracellular enzyme activity. Sufficient nitrogen supply seems to maintain soil microorganisms to produce more extracellular enzymes related to hydrolytic C acquisition [14], but high nitrogen can inhibit oxidase expression [14]. However, Hu's study showed that long-term nitrogen application did not significantly change the abundance of soil microbial carbon-fixing genes [11] but could indirectly affect carbon fixation function through nutrient regulation [15].

Water availability also profoundly influences microbe mediated carbon processes. As a key determinant of microbial activity, adequate soil moisture supports microbial physiological metabolism and facilitates population growth [16]. As an important agronomic measure to regulate farmland soil moisture, the regulatory effect of irrigation on the soil microbial carbon cycle is affected by various factors such as climatic conditions, soil moisture status, and irrigation methods. For example, in arid and semi-arid regions, irrigation can quickly relieve soil moisture limitations, enhance the diffusion efficiency of substances in the soil, activate enzymatic reactions, and facilitate the growth and reproduction of soil microorganisms [17], thereby accelerating organic matter decomposition and increasing short-term carbon mineralization and CO₂ emissions [18]. Conversely, excessive irrigation can cause soil hypoxia, inhibit microbial reproduction and growth, reduce biomass and activity, and delay the biogeochemical processes essential for soil quality [19]. Other studies indicate that increased water availability from precipitation may have positive or neutral effects on microbial diversity [20], whereas drought stress generally reduces microbial richness due to physiological constraints [21].

The interaction between nitrogen fertilizer and water has a more complex impact on soil microbial characteristics than either factor alone [22,23]. Water and nitrogen co-regulate microbial diversity through direct mechanisms (e.g., modulating microbial carbon use efficiency) [24] and indirect pathways (e.g., by altering soil physicochemical properties and plant productivity) [25,26]. Under sufficient moisture conditions, appropriate nitrogen inputs can promote microbial diversity by supporting growth; under water stress, however, nitrogen may inhibit microbial metabolism and reduce diversity [24]. In addition, there is a significant coupling relationship between the soil water use efficiency of microorganisms and nitrogen availability. A decrease in water use efficiency will exacerbate the negative effects of high nitrogen fertilization on microbial communities [27], whereas an appropriate increase in water supply can alleviate the adverse effects of excessive nitrogen application [4]; in contrast, increasing water supply has been shown to mitigate the adverse impacts of excessive nitrogen application [28].

Balanced water and nitrogen management can improve the soil environment, support microbial growth and reproduction, and optimize community structure, thereby contributing positively to soil fertility enhancement, soil carbon sequestration, and crop yield improvement. To better understand the mechanisms through which nitrogen and water influence the microbe-mediated carbon cycle in farmland soils, we conducted a study in a three-year wheat–maize rotation system in northern China. This research examines the effects of nitrogen and water amendments on wheat biomass, soil physicochemical properties, and the composition and functional profiles of soil microbial communities. The study aims to elucidate how nitrogen fertilization and soil moisture shape microbial community structure and carbon-related processes and to identify the key environmental drivers underlying microbial community dynamics under varying nitrogen and water conditions.

This research can reveal the micro-regulatory mechanisms of the carbon cycle, filling theoretical gaps; economically optimize water–nitrogen allocation, reducing agricultural production costs; environmentally support nitrogen reduction and carbon sequestration, alleviating non-point source pollution and the greenhouse effect.

2. Materials and Methods

2.1. Site and Samples

Soil samples were collected from the Jiaozhou Experimental Station of Qingdao Agricultural University. The experimental site is located in a warm-temperate monsoon climate zone (120°3' E, 36°15' N), characterized by concentrated precipitation, with an average annual precipitation of 637 mm and an average annual temperature ranging from 11 to 14 °C (Figure A1). The tested soil type is lime concretion black soil. The basic physicochemical properties of the soil are as follows: organic matter 12.65 g/kg, alkali-hydrolyzable nitrogen 69.60 mg/kg, available phosphorus 39.80 mg/kg, available potassium 110.80 mg/kg, and pH 7.68. This study focused on the most common winter wheat–summer maize rotation system in the region. Summer maize is sown in June and harvested in October, while winter wheat is sown in October and harvested in June the following year. The experiment was started at 2019 and lasted for a total of three years.

During the wheat growing season, twelve water and nitrogen management treatments were established based on local agronomic practices. Irrigation was conducted via drip tapes, with four different irrigation treatments set up in the experiment: no irrigation (T1), one irrigation event with 40 mm of water applied in the mid-April flowering period (T2), one irrigation event with 40 mm of water applied in the early May grain filling period (T3), two irrigation events with a total irrigation amount of 80 mm (40 mm of water applied in both the mid-April flowering period and the early May grain filling period, T4). Nitrogen application consisted of three levels: no nitrogen application (N0), low nitrogen application with 92 kgN·hm^{−2} (N1), high nitrogen application with 184 kgN·hm^{−2} (N2). Urea was used as the nitrogen source and applied entirely as basal fertilizer before wheat sowing. In addition, phosphorus (as P₂O₅) and potassium (as K₂O) fertilizers were uniformly applied to all plots at rates of 469 kg·hm^{−2} and 169 kg·hm^{−2}, respectively, both as basal fertilizers. No irrigation or fertilization was conducted during the maize growing seasons.

The tested soil samples were collected from the 0–20 cm soil layer on 24 May 2022. Fresh samples were brought back to the laboratory under refrigeration at 4 °C. A portion of the soil samples was stored at −80 °C for the determination of microbial community structure and function; another portion was stored at 4 °C for the determination of microbial biomass; the remaining samples were air-dried and passed through a 2 mm sieve, which were used for the analysis of soil physical and chemical properties such as soil organic carbon and total nitrogen. Soil water content was determined by the oven-drying method [29].

2.2. Determination of Wheat Yield and Biomass

Wheat plant samples were collected on the following dates in 2022: 24 April, 8 May, 15 May, 24 May, 1 June, and during the harvest period on 16 June. At each time, 10 wheat plants were randomly selected, their heights were measured, and each organ of the plants was placed in the oven at 105 °C for 30 min to inactivate the enzymes in wheat and then dried at 75 °C until constant weight was reached. After being ground with a plant grinder and sieved through a 2 mm sieve, the N content of the wheat was determined using an Italy VELP SCIENTIFICA NDA701 Dumas nitrogen analyzer, and the nitrogen accumulation of the wheat was calculated. The N accumulation of each organ was calculated by each organ dry weight multiplied by the N content. At harvest, wheat was collected from three fixed 1 m double rows per plot. Grain yield was estimated using the 1 m double-row sampling method.

2.3. Determination of Soil Physicochemical Properties and Respiration Rate

The content of SOC was determined by the dichromate oxidation method. The total nitrogen (TN) content in soil was measured using a Dumas nitrogen analyzer. Microbial biomass carbon and nitrogen were quantified by the chloroform fumigation–extraction method. For determining the initial physicochemical properties of soil, we used the alkali-leaching diffusion method for alkali-leachable nitrogen; the sodium bicarbonate extraction–molybdenum antimony colorimetric method for available phosphorus; the ammonium acetate extraction–flame photometric method for available potassium; and potentiometric titration for soil pH [29].

Soil respiration was monitored with the Li-8100 automated soil CO₂ flux system during the active growth period of wheat (27 November, 3 December, and 15 December in 2021; 24 April, 1 May, 8 May, 18 May, 22 May, 1 June, and 16 June in 2022).

2.4. Determination of Soil Microbial Community and Function

2.4.1. Determination of Soil Microbial Community Structure

Soil microbial DNA extraction was performed using the OMEGA kit E.Z.N.ATM Mag-Bind Soil DNA Kit. After detecting the DNA concentration via agarose gel electrophoresis, PCR amplification was conducted. The V3-V4 region of the bacterial 16S rRNA gene was amplified using the primer pair 341F-805R, and the fungal ITS sequences were amplified using the primer pair ITS1-ITS2. The amplified products were purified and recovered, followed by precise quantification. Sequencing was carried out using the Illumina MiSeq high-throughput sequencing platform [30].

2.4.2. Metagenomic Analysis of Carbon-Cycling Enzymes

Based on microbial community composition results, representative topsoil samples were selected for metagenomic sequencing. Total DNA was extracted again using the same OMEGA kit and sequenced on the Illumina HiSeq Xten platform. The amino acid sequences of the non-redundant gene set were aligned with the KEGG database using Diamond [31] (<https://github.com/bbuchfink/diamond>, (accessed on 2 January 2025) version 2.0.13) with the BLASTP parameter setting of an expected value (e-value $\leq 1 \times 10^{-5}$), to obtain the KEGG functions corresponding to the genes. Functional abundance was calculated based on the summed abundance of genes mapped to KEGG Orthology (KO), Pathway, Enzyme Commission (EC), and Module categories.

2.5. Statistical Analysis

Data processing and statistical analysis of environment and resource impact factors—including wheat biomass, nitrogen content, soil respiration, soil organic carbon, and total

nitrogen under different irrigation and nitrogen treatments—were performed primarily using Microsoft Excel 2019 and SPSS 25.0. To examine the interactive effects of water and nitrogen on key soil physicochemical properties, one-way and two-way ANOVA methods were used. And multiple comparison analysis was conducted using the least significant difference (LSD) method. The significance level of these tests was 0.05.

All microbial data analyses were conducted on the Majorbio Cloud Platform (<https://cloud.majorbio.com>). The mothur software (<http://www.mothur.org/wiki/Calculators>) was used to calculate alpha diversity indices including the Chao, Shannon, Simpson and Pielou_e indices. Shannon Index: Higher values indicate greater community diversity. Simpson Index: Lower values indicate less concentration of dominant species and greater community diversity. Chao 1 Index: Used to estimate community species richness; higher values represent greater species richness. Pielou's Evenness Index: Values closer to 1 indicate more even species distribution [32]. Group differences in alpha diversity were assessed using the Wilcoxon rank-sum test. MPermutational multivariate analysis of variance (PERMANOVA) was applied to evaluate the significance of structural differences in microbial communities across sample groups. Linear discriminant analysis effect size (LEfSe) was employed (<http://huttenhower.sph.harvard.edu/LEfSe>) to identify bacterial taxa showing significant abundance differences at the phylum level, using a logarithmic LDA score threshold of >2 and a significance level of $p < 0.05$. Non-redundant gene sets were functionally annotated against the KEGG database using Diamond software with BLASTP alignment (e-value $\leq 1 \times 10^{-5}$). This facilitated the investigation of how water and nitrogen regimes influenced the abundance of functional genes associated with soil carbon cycling.

3. Results

3.1. Wheat Biomass and Yield

Winter wheat yield and aboveground biomass dry weight increased significantly with increasing nitrogen application (Table 1). N application had significant effects on plant height, aboveground dry weight, and N accumulation of the stem. Wheat height and aboveground dry weight were greater in the N1 and N2 treatments than in the N0 treatment, while the N accumulation of the stem and leaf was significantly higher under N2 than under N0 and N1 across different growth stages (Figures A2–A4). Irrigation treatments had no significant effects on these parameters (Table 1, Figures A2–A4). However, under N1 application and N2 treatments, the yield of the T3 treatment was higher than that of the other irrigation treatments, while under N2 treatment, the yield of the T4 treatment was higher.

The effect of N treatments on soil TN content was not significant, but the average TN values under the N2 and N1 treatments were higher than that under N0 (Table 1). Irrigation treatments had significant effects on the soil TN content. Under the same application rate, the TN content in T3 and T4 was significantly lower than in T1, with T2 exhibiting intermediate values. N application significantly influenced SOC content. SOC under the N2 treatment was significantly higher than under N0 and N1 (Table 1). Irrigation treatments did not significantly affect SOC overall. Under the N1 treatment, the SOC of the T4 treatment was significantly lower than that of the other treatments; under the N1 treatment, the SOC of the T3 treatment was significantly higher than that of the T1 and T4 treatments, while the SOC of the T2 treatment was significantly lower than that of the T1 and T4 treatments. Under the N2 treatment, the SOC of the T2 and T3 treatments was significantly higher than that of the T4 treatment, whereas the SOC of the T1 treatment was significantly lower than that of the T4 treatment. The N2 treatment resulted in a significantly higher soil C/N ratio compared to both the N1 and N0 treatments, while no

significant difference was observed between the N1 and N0 treatments. Under the same N application level, the T3 treatment led to a significantly higher soil C/N ratio than the other treatment. Under N0 and N1 conditions, the T2 treatment exhibited the lowest soil C/N ratio; under N2, the T1 treatment showed the lowest C/N ratio.

Table 1. Wheat yield, aboveground biomass, and soil carbon–nitrogen indicators and respiration rates.

	Aboveground Biomass Dry Weight (g)	Yield (kg·hm ^{−2})	Soil Total Nitrogen (g·kg ^{−1})	Soil Organic Carbon (g·kg ^{−1})	C/N	Total Soil Respiration (μmol·m ^{−2})
N0T1	50.11 ± 2.40 abC	2575.93 ± 112.35 bC	0.80 ± 0.08 aA	0.65 ± 0.08 aB	0.78 ± 0.01 bB	18.66 ± 0.22 bB
N0T2	55.35 ± 1.12 aC	2766.28 ± 206.82 bC	0.71 ± 0.05 bA	0.52 ± 0.04 aB	0.55 ± 0.19 cB	21.27 ± 0.26 abB
N0T3	41.84 ± 1.84 bC	3415.78 ± 164.79 aC	0.60 ± 0.04 cA	0.68 ± 0.11 aB	1.09 ± 0.12 aB	19.90 ± 0.31 bB
N0T4	43.97 ± 0.24 bC	2733.95 ± 157.58 bC	0.50 ± 0.08 dA	0.39 ± 0.12 bB	0.65 ± 0.12 bcB	24.33 ± 0.32 aB
N1T1	64.63 ± 2.15 aB	4988.60 ± 289.27 bB	0.88 ± 0.15 aA	0.71 ± 0.07 bB	0.93 ± 0.15 bB	29.04 ± 0.41 bA
N1T2	54.32 ± 1.40 bB	5172.14 ± 326.45 bB	0.71 ± 0.05 bA	0.43 ± 0.04 cB	0.59 ± 0.02 cB	22.13 ± 0.29 cA
N1T3	57.75 ± 0.12 abB	6394.03 ± 443.97 aB	0.73 ± 0.07 bA	1.05 ± 0.13 aB	1.53 ± 0.02 aB	29.74 ± 0.45 abA
N1T4	63.94 ± 2.57 aB	6761.06 ± 401.88 aB	0.76 ± 0.07 bA	0.68 ± 0.15 bB	0.84 ± 0.15 bB	29.81 ± 0.42 aA
N2T1	78.37 ± 2.74 aA	7579.88 ± 413.52 bA	0.91 ± 0.07 aA	0.92 ± 0.07 cA	1.22 ± 0.16 cA	27.73 ± 0.38 bA
N2T2	80.20 ± 2.79 aA	7690.13 ± 463.53 bA	0.81 ± 0.08 bA	1.56 ± 0.09 aA	1.98 ± 0.10 bA	28.73 ± 0.32 bA
N2T3	80.90 ± 3.55 aA	8949.81 ± 358.07 aA	0.62 ± 0.05 cA	1.54 ± 0.07 aA	2.48 ± 0.04 aA	27.33 ± 0.44 bA
N2T4	69.81 ± 4.27 bA	8787.71 ± 282.47 aA	0.78 ± 0.08 bA	1.19 ± 0.02 bA	1.57 ± 0.14 cA	36.15 ± 0.56 aA

N0: N application rate of 0 kgN·hm^{−2}; N1: N application rate of 92 kg N·hm^{−2}; N2: N application rate of 184 kgN·hm^{−2}; T1: No irrigation; T2: 40 mm irrigation at flowering; T3: 40 mm irrigation at the grain filling stage; T4: 40 mm irrigation at both the flowering and grain filling stages. Different uppercase letters indicate significant statistical differences among nitrogen application treatments, while different lowercase letters indicate significant statistical differences among irrigation treatments under the same N application level. Aboveground biomass dry weight the average per plant.

N application significantly affected total soil respiration, with soil respiration under N2 and N1 being significantly higher than under N0 (Table 1, Figure A10). Under the same N treatment, the total soil respiration rate was highest in T4 and lowest in T2.

3.2. Soil Microbial Community Structure

The dominant phylum of Bacteria is Actinobacteria, followed by Proteobacteria, Acidobacteria, Chloroflexi, and Thaumarchaeota (Figure 1, Figure A5). The abundances of Actinobacteria, Chloroflexi, and Gemmatimonadetes show an increasing trend with the increase in nitrogen application, while those of Proteobacteria and Acidobacteria decrease with the increase in nitrogen application (Figure 1a). The abundance of Actinobacteria decreases with the increase in irrigation, while irrigation has no significant effect on the abundances of Proteobacteria, Acidobacteria, Chloroflexi, and Gemmatimonadetes (Figure 1b). The abundance of Actinobacteria in the N1T4 treatment is lower than that in the other treatments, but the abundance of Acidobacteria is higher than that in the other treatments; the abundance of Actinobacteria in the N2T4 treatment is higher than that in the other treatments, but the abundance of Acidobacteria is lower than that in the other treatments (Figure A5).

Among the fungi in Eukaryota, the dominant phyla are Ascomycota and Mucoromycota. The abundance of Ascomycota increases with the increase in nitrogen application and is the lowest in the T3 treatment (Figure 1c). Nitrogen application and irrigation treatments have no significant effect on the abundance of Mucoromycota.

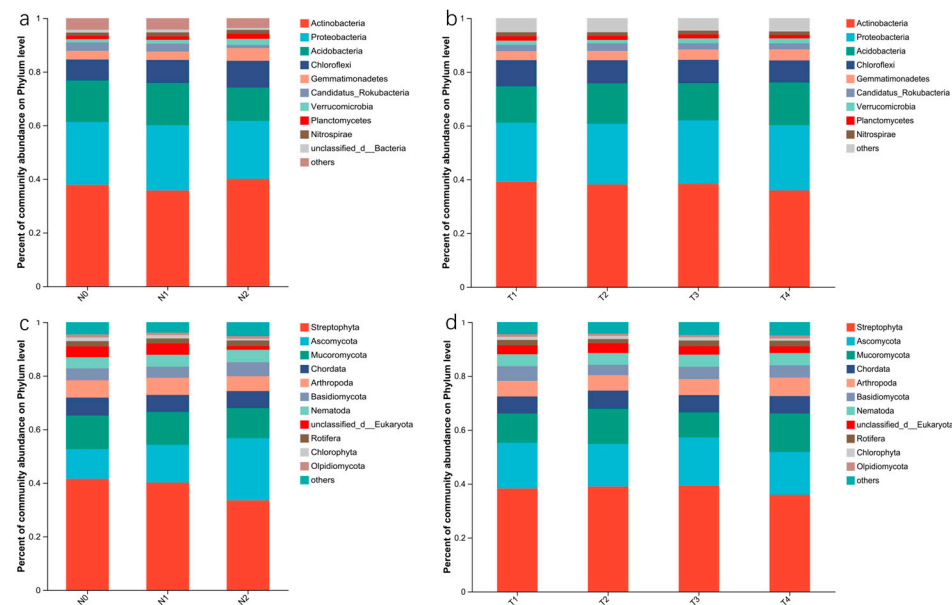


Figure 1. Phylum-level composition of bacteria community structure among nitrogen application treatments (a) and different irrigation treatments (b). Phylum-level composition of eukaryote community structure of among different nitrogen application treatments (c) and different irrigation treatments (d). N0: N application rate of 0 kgN·hm^{−2}; N1: N application rate of 92 kgN·hm^{−2}; N2: N application rate of 184 kgN·hm^{−2}; T1: No irrigation; T2: 40 mm irrigation at flowering; T3: 40 mm irrigation at the grain filling stage; T4: 40 mm irrigation at both the flowering and grain filling stages.

For the bacterial community, alpha diversity analysis revealed that the community richness was the highest in the N1T4 treatments and the lowest in the N1T3 treatment based on the Chao index (Table 2); however, based on the Shannon and Simpson indices, the N2T4 treatment had the lowest community diversity while the N1T4 treatment had the highest. Based on the Pielou index, the N2 treatment showed higher community evenness. No significant interactive effect was observed between irrigation and nitrogen treatments.

Table 2. Alpha diversity indices of bacteria and fungi under different treatments.

Sample	Bacteria				Fungi			
	Chao	Shannon	Simpson	Pielou	Chao	Shannon	Simpson	Pielou
N0T1	155	1.855	0.241	0.368	29	2.070	0.199	0.615
N0T2	154	1.857	0.235	0.369	29	2.049	0.214	0.609
N0T3	152	1.835	0.244	0.365	34	2.198	0.180	0.623
N0T4	157	1.842	0.236	0.364	31	2.143	0.204	0.624
N1T1	154	1.824	0.254	0.362	35	2.141	0.194	0.602
N1T2	156	1.836	0.243	0.364	31	2.007	0.215	0.584
N1T3	151	1.821	0.243	0.363	31	2.000	0.220	0.582
N1T4	158	1.986	0.193	0.392	34	2.188	0.167	0.621
N2T1	156	1.882	0.231	0.373	35	2.080	0.195	0.585
N2T2	155	1.872	0.234	0.371	35	2.112	0.189	0.594
N2T3	153	1.888	0.231	0.375	35	2.167	0.172	0.610
N2T4	157	1.752	0.275	0.347	35	2.084	0.182	0.586

N0: N application rate of 0 kgN·hm^{−2}; N1: N application rate of 92 kg N·hm^{−2}; N2: N application rate of 184 kgN·hm^{−2}; T1: No irrigation; T2: 40 mm irrigation at flowering; T3: 40 mm irrigation at the grain filling stage; T4: 40 mm irrigation at both the flowering and grain filling stages.

For the fungal community, alpha diversity analysis revealed that the community richness was generally higher in all samples of the N2 treatment and the lowest in the N0T1 and N0T2 treatments based on the Chao index; based on the Shannon and Simpson

indices, the N0T3 and N1T4 treatments had the highest community diversity while the N1T3 treatment had the lowest. Under the N0 treatment, the T3 and T4 irrigation treatments increased the richness and diversity of the fungal community compared with the T1 and T2 irrigation treatments. In contrast, under the same nitrogen-applied conditions, no consistent effect of soil moisture on fungal diversity was observed.

3.3. Significant Differences in Soil Microbial Enzymes Related to Carbon Metabolism

As shown in Figure 2, transketolase (K00615) was the most abundant enzyme among those associated with carbon metabolism and showed significant differences under different N application rates. The abundances of transketolase (K00615), transaldolase (K00616), glucose-6-phosphate isomerase (K01810), xylulose-5-phosphate/fructose-6-phosphate phosphoketolase (K01621), and 6-phosphogluconate dehydrogenase (K00033) increased with increasing nitrogen application rate—all of these enzymes are involved in the pentose phosphate pathway. However, the abundance of gluconolactonase (K01053), another enzyme in the pentose phosphate pathway, decreased with increasing nitrogen application.

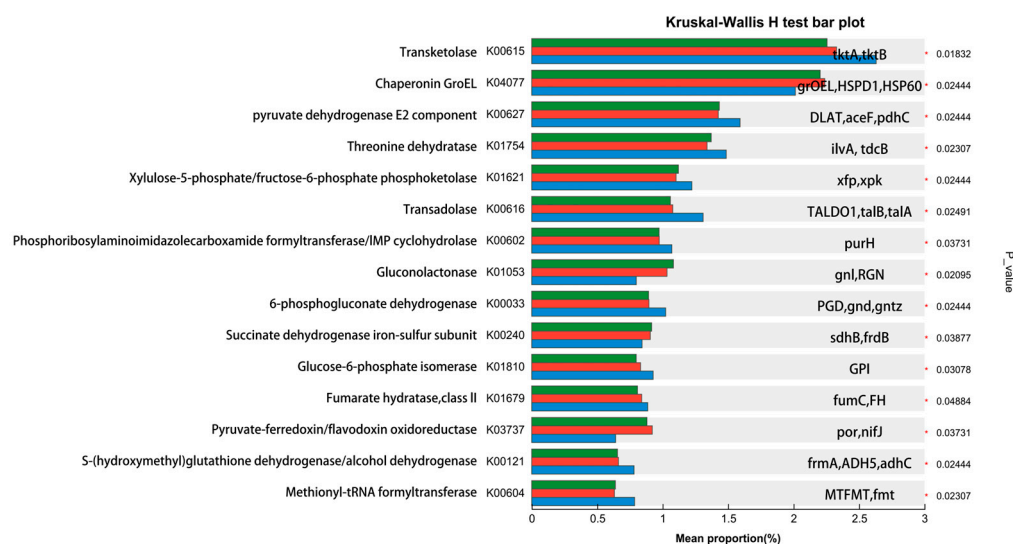


Figure 2. Differences in abundance of soil microbial enzymes related to carbon metabolism under different nitrogen application treatments. N0: N application rate of 0 kgN·hm^{−2}; N1: N application rate of 92 kgN·hm^{−2}; N2: N application rate of 184 kgN·hm^{−2}; T1: No irrigation; T2: 40 mm irrigation at flowering; T3: 40 mm irrigation at the grain filling stage; T4: 40 mm irrigation at both the flowering and grain filling stages.

Other enzymes whose abundances increased with increasing nitrogen application included pyruvate dehydrogenase E2 component (dihydrolipoyllysine residue acetyltransferase, K00627), threonine dehydratase (K01754), phosphoribosylaminoimidazolecarboxamide formyltransferase /IMP cyclohydrolase (K00602), fumarate hydratase class II (K01679), S-(hydroxymethyl) glutathione dehydrogenase/alcohol dehydrogenase (K00121), and methionyl-tRNA formyltransferase (K00604).

Enzymes whose abundances decreased with increasing nitrogen application included the chaperone GroEL (K04077), succinate dehydrogenase iron-sulfur subunit (K00240), and pyruvate ferredoxin/flavodoxin oxidoreductase (K03737).

Under different irrigation treatments, the abundance of phosphoserine aminotransferase (K00831) increased significantly with an increasing irrigation amount, while the abundance of methylmalonyl-CoA mutase (K01848) decreased with an increasing irrigation amount (Figure 3).

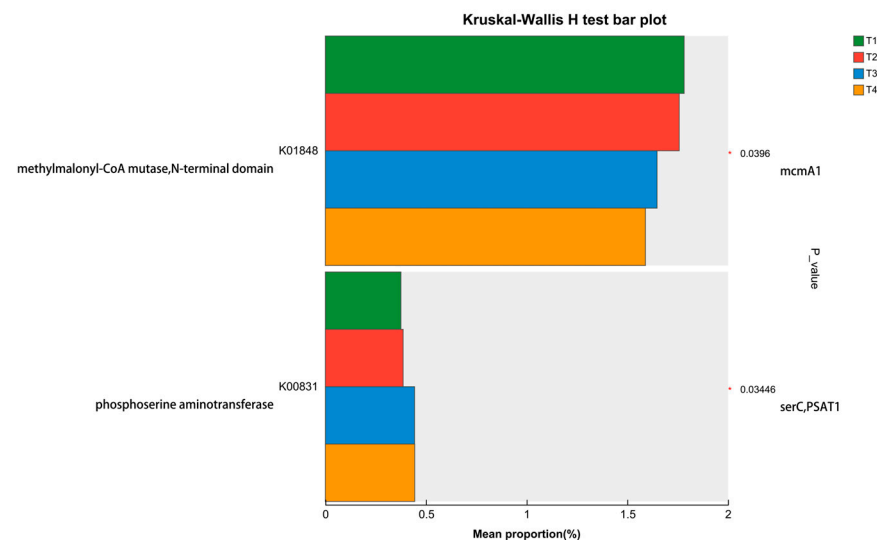


Figure 3. Differences in abundance of soil microbial enzymes related to carbon metabolism under different irrigation treatments. N0: N application rate of 0 kgN·hm^{−2}; N1: N application rate of 92 kgN·hm^{−2}; N2: N application rate of 184 kgN·hm^{−2}; T1: No irrigation; T2: 40 mm irrigation at flowering; T3: 40 mm irrigation at the grain filling stage; T4: 40 mm irrigation at both the flowering and grain filling stages.

3.4. Environmental Factors Affecting Soil Microbial Carbon Processes

As illustrated in Figure 4, methanol dehydrogenase (cytochrome c) subunit 1 (K14028) showed a positive correlation with soil moisture, N application, and soil respiration. 3-hydroxybutyryl-CoA dehydratase (K17865) also showed a positive correlation with soil moisture. Pyruvate carboxylase (K01958) showed a negative correlation with soil nitrogen. 6-phosphogluconolactonase (K01057) showed a positive correlation with soil organic carbon, grain nitrogen, and aboveground biomass. Methionyl-tRNA formyltransferase (K00604) showed a positive correlation with soil organic carbon, grain nitrogen, aboveground biomass, and soil nitrogen, but a negative correlation with soil moisture. 2-oxoglutarate/2-oxoacid ferredoxin oxidoreductase subunit beta (K00175) showed a negative correlation with wheat grain weight. Acetyl-CoA C-acetyltransferase (K00626) showed a positive correlation with grain weight but a negative correlation with aboveground biomass and soil nitrogen.

The abundances of methanol dehydrogenase (cytochrome c) subunit 1 (K14028) and 3-hydroxybutyryl-CoA dehydratase (K17865) in the N1 and N2 treatments were significantly higher than those in the N0 treatment; the abundances of 6-phosphogluconolactonase (K01057) and methionyl-tRNA formyltransferase (K00604) in the N0 treatment were significantly higher than those in the N1 and N2 treatments.

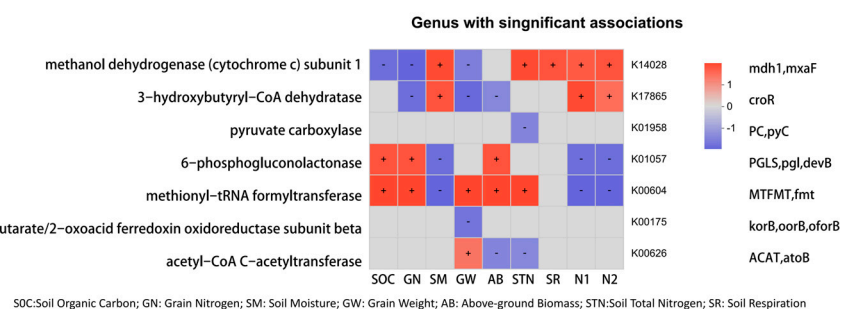


Figure 4. Heatmap of relationships between environmental factors and soil microbial carbon metabolism functions. The depth of red color blocks indicates the significance of positive correlations: a darker shade represents a larger p -value and a more significant positive correlation. The

depth of blue color blocks indicates the significance of negative correlations: a darker shade represents a smaller p -value and a more significant negative correlation. The color depth reflects the magnitude of the significance metric, specifically the adjusted p -value (i.e., q -value). (Significance threshold: q -value < 0.25.) N1: N application rate of 92 kgN·hm^{−2}; N2: N application rate of 184 kgN·hm^{−2}.

4. Discussion

4.1. Effects of Nitrogen Application

Increased N application can improve wheat yield [33], increase litter and root biomass [33], and promote soil carbon input. Meanwhile, N application supplements soil nitrogen, which is an essential element for microbial growth; thus, nitrogen content affects microbial growth. In nutrient-limited ecosystems, this can stimulate soil microbial growth and improve functional diversity [34]. However, continuous application of excessive inorganic nitrogen fertilizer can have negative effects on microbial community structure, possibly increasing some taxa while inhibiting others. This may be because excessive nitrogen supply reduces soil organic matter input and nitrogen availability, leading to a decline in microbial functional diversity.

In this study, although nitrogen application did not exert a significant effect on the overall microbial community structure, the bacterial community under the N1 treatment exhibited higher diversity and evenness while the diversity under the N2 treatment was lower than that under N1 (Table 2). The reduction in bacterial diversity may be attributed to a threshold effect of nitrogen fertilization on microbial structure and activity [35]. Beyond this threshold, the diversity of certain bacterial taxa may be suppressed. Additionally, previous studies have suggested that the decline in bacterial diversity could result from the inability of some taxa in the soil microbial community to tolerate nitrogen deposition [36] or from biological activities such as competition for nutrient sources and the production of inhibitory compounds [37]. Regarding the fungal community, the richness in the no-nitrogen treatment was generally lower than that in the nitrogen-applied treatments, while the evenness was higher in the absence of nitrogen application (Table 2). This pattern may be explained by the fact that under nitrogen addition, microbial taxa with rapid growth rates that rely on more readily decomposable carbon sources are more likely to increase in abundance—which may be attributed to the promotion of certain dominant taxa and a concomitant reduction in the diversity of functionally similar taxa. This can lead to certain fast-growing fungal taxa becoming dominant, while those adapted to lower nutrient conditions or characterized by slower growth may decline [38], ultimately resulting in reduced overall diversity [39,40].

In this study, the relative abundances of Actinobacteriota, Chloroflexi, Gemmatimonadetes, and Ascomycota increased with increasing N application rate, while those of Proteobacteria and Acidobacteriota decreased (Figure 1). Acidobacteriota, as key decomposers of soil organic matter, exhibited a decline in relative abundance under increased nitrogen application which promoted wheat yield (Table 1), plant height, nitrogen accumulation, and aboveground biomass (Figures A2–A4) and enhanced the input of labile organic carbon. Proteobacteria, which have nitrogen-fixing capacity, decreased with increasing N application. Additionally, the high relative abundance of Actinobacteriota in farmland soil may be due to the frequent application of herbicides, insecticides, and other agents in farmland, leading to the selective enrichment of Actinobacteriota with drug resistance [41], and Actinobacteriota exhibit high tolerance to salt, heat, and drought, while Proteobacteria play an important role in the cycling of nutrients such as carbon, nitrogen, and phosphorus—thus, these two phyla became the dominant bacterial taxa in the study area.

The abundance of methanol dehydrogenase (cytochrome c) subunit 1 (K14028) and 3-hydroxybutyryl-CoA dehydratase (K17865) in the nitrogen-free treatment was significantly lower than that in the nitrogen application treatment (Figure 4). Methanol dehydrogenase (cytochrome c) subunit 1 (K14028) is widely present in methanotrophs of the phylum Proteobacteria [42]; methanotrophs can convert methane into methanol [43], and nitrogen addition can indirectly increase the abundance or metabolic efficiency of methanogens by enhancing root exudates [44]. The abundance of 3-hydroxybutyryl-CoA dehydratase (K17865) is high in hypoxic environments [45], and the pathway of carbon dioxide fixation by this enzyme has the highest energy utilization efficiency [46] which may also be the reason for the positive correlation between this enzyme and soil moisture.

In the nitrogen-free treatment, the abundances of 6-phosphogluconolactonase (K01057) and methionyl-tRNA formyltransferase (K00604) were significantly higher than those in the nitrogen application treatment. 6-phosphogluconolactonase (K01057) is involved in the metabolic bypass producing pentose phosphate and NADPH from G6P. The disadvantages of this extracellular shunt are that it is more energy-intensive, partially beyond the scope of metabolic regulation, and easily replaced by competing cells [47]. For methionyl-tRNA formyltransferase (K00604), under malnutrition conditions, Gcn2 kinase enhances the cytoplasmic localization of Fmt1 formyltransferase and may also increase its enzymatic activity [48]. Therefore, the abundances of these two enzymes decrease instead after the increase in nitrogen application.

In this study, the actinomycetes phylum exhibits a strong correlation with transketolase (K00615), and both increase with nitrogen application (Figure A6). The differences in soil microbial carbon metabolism function under different nitrogen application treatments were mainly related to enzymes in the pentose phosphate pathway, including increased abundances of transketolase (K00615), transaldolase (K00616), glucose-6-phosphate isomerase (K01810), xylulose-5-phosphate/fructose-6-phosphate phosphoketolase (K01621), and 6-phosphogluconate dehydrogenase (K00033). As shown in Figure 5, in the oxidative phase of the pentose phosphate pathway glucose-6-phosphate is decomposed into 6-phosphogluconolactone by 6-phosphogluconate dehydrogenase (K00033), with the simultaneous production of NADPH; 6-phosphogluconolactone is catalyzed by gluconolactonase (K01053) to form 6-phosphogluconic acid; and 6-phosphogluconic acid is further catalyzed by 6-phosphogluconate dehydrogenase (K00033) to generate ribulose-5-phosphate and NADPH [49]. Meanwhile, xylulose-5-phosphate/fructose-6-phosphate phosphoketolase (K01621) decomposes glucose-6-phosphate into carbon dioxide, ribulose-5-phosphate, and two molecules of NADPH. The large amount of NADPH produced in these processes serves as an important reducing power, participating in the synthesis of biomolecules such as fatty acids, cholesterol, amino acids, and nucleotides, as well as in antioxidant defense and cellular metabolic regulation—thus affecting cell proliferation, metabolic reprogramming, and DNA synthesis [50]. This suggests that nitrogen application increases the activity of enzymes in the pentose phosphate pathway, which not only stimulates the catabolism and resynthesis of plant residues input into soil by microorganisms but also may promote the proliferation of microbial communities, thereby increasing soil microbial biomass in nitrogen application treatments (Figure A7).

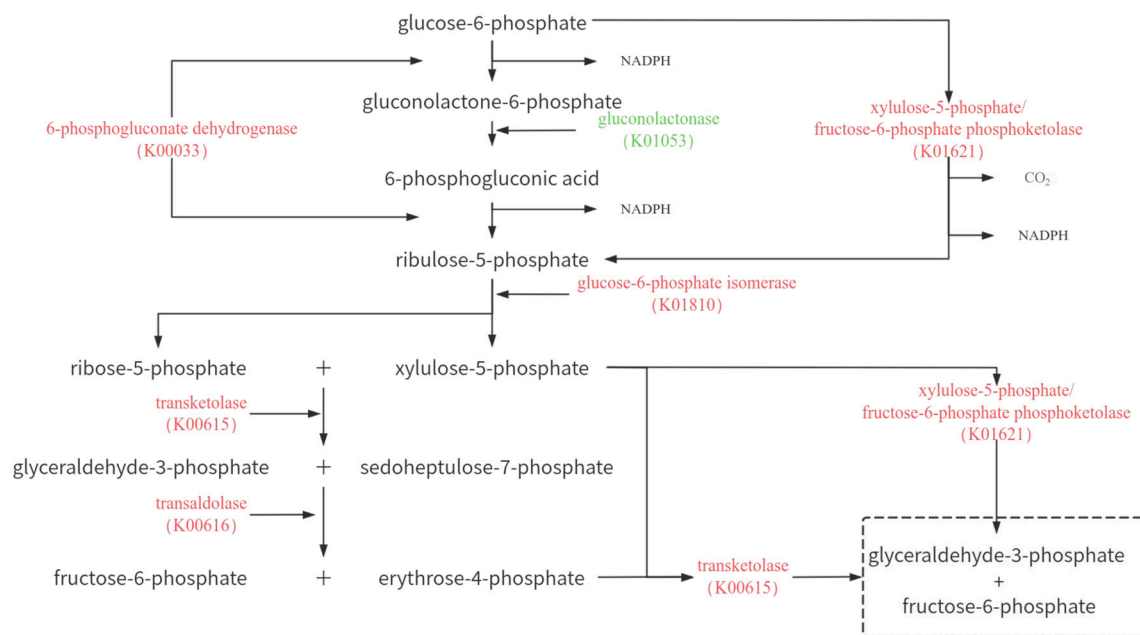


Figure 5. Framework diagram of the pentose phosphate pathway. The red markers indicate enzymes that increase with nitrogen application, while the green markers indicate enzymes that decrease with nitrogen application.

Meanwhile, the activation of the pentose phosphate pathway leads to the excessive accumulation of ribose-5-phosphate (Figure 5). The excess ribose-5-phosphate enters the non-oxidative phase and is decomposed into xylulose-5-phosphate and ribose-5-phosphate under the action of glucose-6-phosphate isomerase (K01810); these two products are further converted into glyceraldehyde-3-phosphate and sedoheptulose-7-phosphate under the catalysis of transketolase (K00615). Furthermore, transaldolase (K00616) transfers the three-carbon unit from sedoheptulose-7-phosphate to glyceraldehyde-3-phosphate, generating fructose-6-phosphate (which can participate in glycolysis) and erythrose-4-phosphate. In addition, xylulose-5-phosphate/fructose-6-phosphate phosphoketolase (K01621) decomposes xylulose-5-phosphate into glyceraldehyde-3-phosphate; simultaneously, erythrose-4-phosphate and xylulose-5-phosphate can be decomposed into glyceraldehyde-3-phosphate and fructose-6-phosphate under the catalysis of transketolase (K00615). Both products participate in glycolysis, thereby increasing glycolytic flux, consuming the carbohydrate pool, and meeting the energy and intermediate material requirements for the biosynthesis of other metabolites [51]. Su [52] found that N application level had a significant effect on the metabolic intensity and metabolic carbon source richness of soil microbial communities; with increasing nitrogen application level, the metabolic carbon source richness of soil microorganisms showed a trend of first increasing and then decreasing which is consistent with the data of K00240 and K03737 in this experiment.

Guo [53] reported that N application stimulates crop growth and the increased crop residues can be converted into microbial biomass through soil microbial metabolism, which then forms stable soil organic carbon as a byproduct of anabolism. And Shen [54] pointed out that increased soil nitrogen content promotes microbial utilization of labile carbon sources such as alkoxy carbon and carbonyl carbon in carbohydrates and amino acids. In the microbial community structure associated with transketolase (K00615), it was found that with the increase in nitrogen application, the abundance of Thermoleophilia under the phylum Actinobacteria also increased accordingly (Figure A5). Therefore, N application stimulates microorganisms to convert labile carbon (alkoxy and carbonyl carbon) in carbohydrates and amino acids into energy (ATP), reducing power (NADH), or biosynthetic

precursors through pathways such as glycolysis, the pentose phosphate pathway (PPP), and the TCA cycle to adapt to different environments. Similarly, in our results, as the N application rate increased, higher wheat yield, biomass, and other physiological indicators indicated higher soil carbon input (Table 1, Figures A2–A4), which stimulated soil microbial biomass (Figure A7) and increased the activation of the pentose phosphate pathway and glycolytic flux, consuming the carbohydrate pool (Figures 2 and 5). This could also be proved by the higher soil C/N value under N2 (Table 1), which often represented higher amounts of degraded soil organic matter [55].

In this study, the soil total nitrogen (TN) content showed no significant differences among the different nitrogen treatments (Table 1). This may be attributed to the fact that moderate nitrogen addition promoted crop growth and stimulated microbial activity, thereby enhancing the uptake and utilization of soil nitrogen, ultimately resulting in no net change in TN content [6–8]. This could also be contributed by the strengthening N lost through microbial nitrification metabolism pathways, which was evidenced by the abundances of enzymes related to nitrate reduction, denitrification, complete nitrification, and anaerobic ammonium oxidation pathways in soil microorganisms increasing significantly with an increasing N application rate (Figure A8).

Nitrogen fertilizer application directly regulates soil nitrogen availability, modulates crop growth and carbon input, and influences soil processes across three levels: microbial community structure, key microbial community composition, and carbon metabolism functional genes. The direct effects of nitrogen application and the increased input of readily decomposable carbon driven by crop biomass expansion promote the abundance of Actinobacteria and Chloroflexi, while reducing the abundance of Proteobacteria (nitrogen-fixing) and Acidobacteria (organic matter decomposition) (Figure 1). At the carbon metabolism level, nitrogen application significantly increased gene abundances associated with methane conversion and anaerobic carbon fixation (Figure 4), activated key enzymes in the pentose phosphate pathway, and generated substantial NADPH (Figure 5), thereby regulating microbial metabolism and biomass. In summary, optimal nitrogen application enhances soil carbon–nitrogen synergistic cycling by regulating microbial communities and carbon metabolism functions, thereby influencing soil processes such as organic matter decomposition and nutrient cycling.

4.2. Interactive Effects of Different Irrigation and Water Treatments

Previous studies have shown that under the interactive effect of water and nitrogen, nitrogen fertilizer can alleviate the negative effect of insufficient soil moisture on microbial growth; the highest soil microbial biomass is achieved under moderate moisture conditions with sufficient nitrogen supply [56]. In our study, irrigation had no significant effect on wheat yield (Table 1), plant height, nitrogen accumulation, and aboveground biomass (Figures A2–A4). This may be attributed to the abundant underground water in the study area, resulting in an insignificant effect of irrigation (Figure A9). Nevertheless, N application and irrigation had an interactive effect on yield, manifested as the yields of the T1 and T2 treatments being significantly lower than those of the T3 and T4 treatments under the same nitrogen application treatment (Table 1). This might be because soil moisture limits wheat nitrogen uptake under water-deficient conditions [57].

Overall, some long-term field studies have found that irrigation treatments had no significant effect on soil microbial community structure [58]; this may be due to the fact that local microbial communities have sufficient time to recover and adapt from humidity changes [59]. These inconsistent findings highlight the necessity of studying the direct response of microorganisms to environmental changes. However, the abundance of Proteobacteria (a phylum of bacteria) increases with the increase in irrigation. Some studies

have pointed out that Proteobacteria, which affect the bacterial community, are Gram-negative bacteria and highly susceptible to environmental disturbances and irrigation water [60].

This is because increased soil moisture is beneficial to crop growth and metabolism, thereby promoting the secretion of some rhizodeposits. Proteobacteria can synthesize carbohydrates and proteins from crop secretions [61]. Under no-nitrogen conditions, irrigation treatments increased indicators such as the richness and diversity of the fungal community. This indicated that under no N treatment, compared to prokaryotes, the fungal community was more sensitive to soil water content [62,63].

In this study, only the abundance of phosphoserine aminotransferase (K00831) increased significantly with an increasing irrigation amount. This may be because under sufficient moisture conditions, the demand for serine by plants and soil microorganisms increases (e.g., for photorespiration repair and antioxidant synthesis), thereby activating the expression of phosphoserine aminotransferase (K00831) in the serine synthesis pathway. In contrast, the abundance of methylmalonyl-CoA mutase (K01848) decreased with an increasing irrigation amount. This enzyme is mainly distributed in mitochondria and functions to catalyze the isomerization of methylmalonyl-CoA to succinyl-CoA (which can enter the TCA cycle for metabolism). The enzyme represented by K01848 is a component of the metabolic pathways in aerobic organisms, but its inherent catalytic mechanism requires it to function in a low-oxygen microenvironment [64]. The reason for the lack of significant differences in other functions may be as follows: irrigation did not exert a significant disturbance of the metabolic environment of microorganisms, and microorganisms were able to rapidly adjust their metabolic strategies within their adaptive range to maintain balance which ultimately led to no significant difference in their carbon metabolism function.

Overall, under the same nitrogen application level, the T4 treatment (two irrigations) resulted in a greater increase in soil respiration (soil carbon output) compared to the T3 treatment (single irrigation at the grain filling stage) (Table 1, Figure A10), but had a smaller promoting effect on wheat growth (soil carbon input) (Table 1). In contrast, the T3 treatment led to a greater enhancement in wheat growth (soil carbon input) compared to the T2 treatment (single irrigation at the jointing stage) (Table 1), while the difference in soil respiration (soil carbon output) between these two treatments was not significant (Table 1). Consequently, the highest soil organic carbon (SOC) content was observed under the T3 treatment under the same N addition level. Under the same irrigation level, high nitrogen application (N2) was more beneficial for wheat growth compared to low nitrogen (N1) (Table 1), although no significant difference was observed in soil respiration between the two nitrogen levels (Table 1). Thus, high nitrogen application was more conducive to SOC accumulation. Taking these results together, the N2T3 treatment offered the best cost–benefit balance in terms of achieving both high wheat yield and enhanced SOC storage.

5. Conclusions

This study revealed the significant regulatory effect of different nitrogen application treatments on the pentose phosphate pathway in soil microbial carbon metabolism. With increasing nitrogen application rate, the abundance of key enzymes in the pentose phosphate pathway showed a significant upward trend. This phenomenon may be attributed to nitrogen application enhancing the metabolic activity of soil microorganisms, thereby increasing the demand for NADPH and ultimately promoting the cycling of the pentose phosphate pathway. The results indicated that nitrogen application not only altered soil physicochemical properties but also indirectly regulated the microbial community structure and its functional characteristics by influencing plant growth.

Based on metagenomic analysis, this study systematically clarified the dynamic changes in the abundance of soil microbial carbon metabolism functional genes under different nitrogen application and irrigation treatments and explored the underlying regulatory mechanisms. It should be noted that although this study provided important data on the abundance of soil microbial carbon metabolism functional genes, there remains uncertainty regarding the response of microbial diversity to fertilization practices. This variability may be determined by the combined effects of multiple complex factors, including soil type, fertilizer source, and environmental conditions. Additionally, the dynamic changes in microbial community structure and function under different irrigation treatments were not fully captured; further in-depth research in this area is required in the future.

Author Contributions: Q.M.: Writing—original draft preparation, software, investigation, data curation, writing—review and editing. B.W.: Investigation, data curation, supervision. Q.F.: Methodology, validation, formal analysis, supervision. Z.Z.: Data curation, supervision. Y.C.: Data curation, supervision. X.S.: Conceptualization, methodology, resources, writing—review and editing, visualization, supervision, project administration. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data that support the findings of this study are available from the authors upon reasonable request.

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Conflicts of Interest: The authors declare no conflicts of interest.

Appendix A

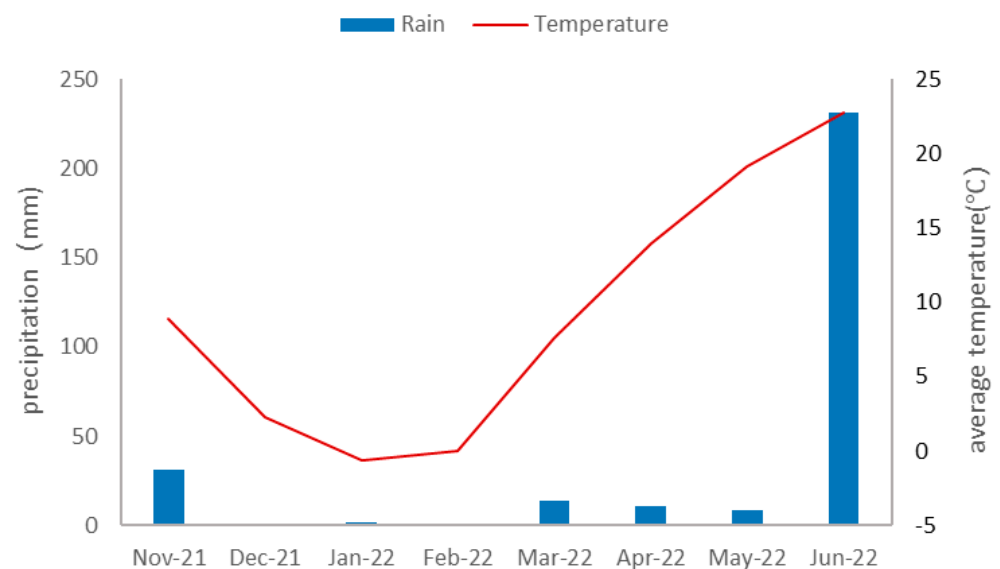


Figure A1. Monthly precipitation and monthly average temperature during wheat growth period. Precipitation refers to the total rainfall for the month.

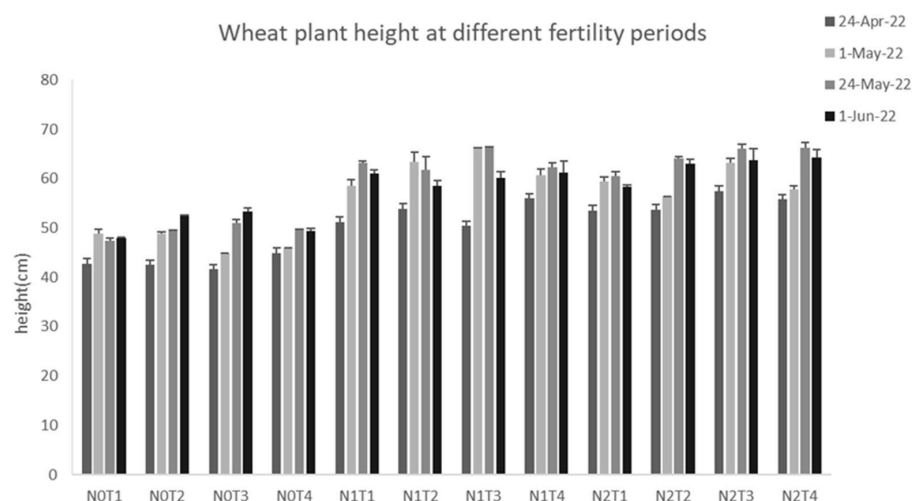


Figure A2. Wheat plant height under different treatments and on different dates. N0: N application rate of $0 \text{ kgN} \cdot \text{hm}^{-2}$; N1: N application rate of $92 \text{ kgN} \cdot \text{hm}^{-2}$; N2: N application rate of $184 \text{ kgN} \cdot \text{hm}^{-2}$; T1: No irrigation; T2: 40 mm irrigation at flowering; T3: 40 mm irrigation at the grain filling stage); T4: 40 mm irrigation at both the flowering and grain filling stages. Data represent the mean values for each treatment, with error bars indicating standard errors.

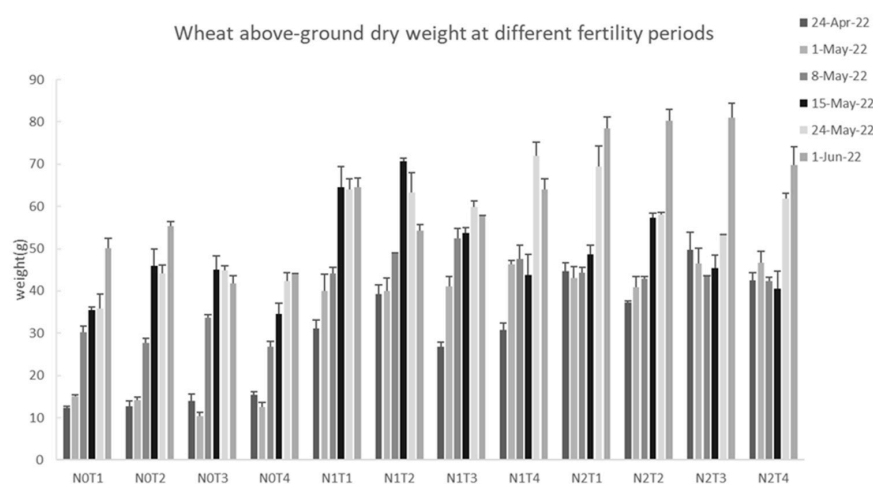


Figure A3. Aboveground dry weight of wheat under different treatments and on different dates. Data represent the mean values for each treatment, with error bars indicating standard errors.

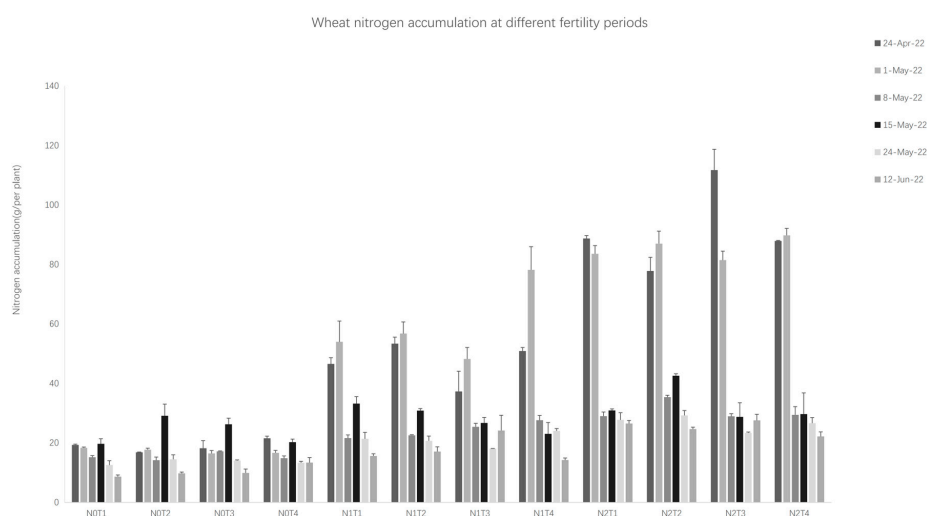


Figure A4. Nitrogen accumulation in wheat stems under different treatments and on different dates. Data represent the mean values for each treatment, with error bars indicating standard errors.

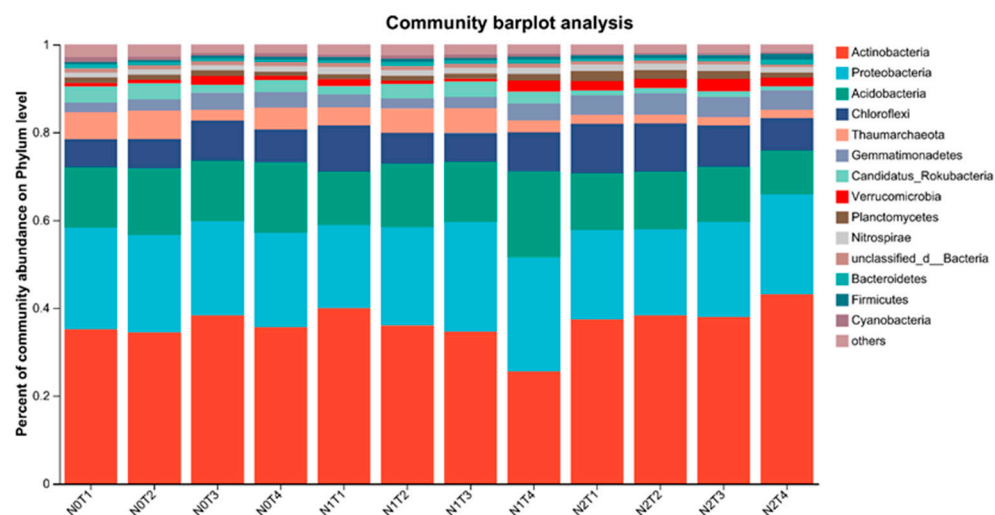


Figure A5. Bar chart of soil microbial community at the phylum level under different treatments.

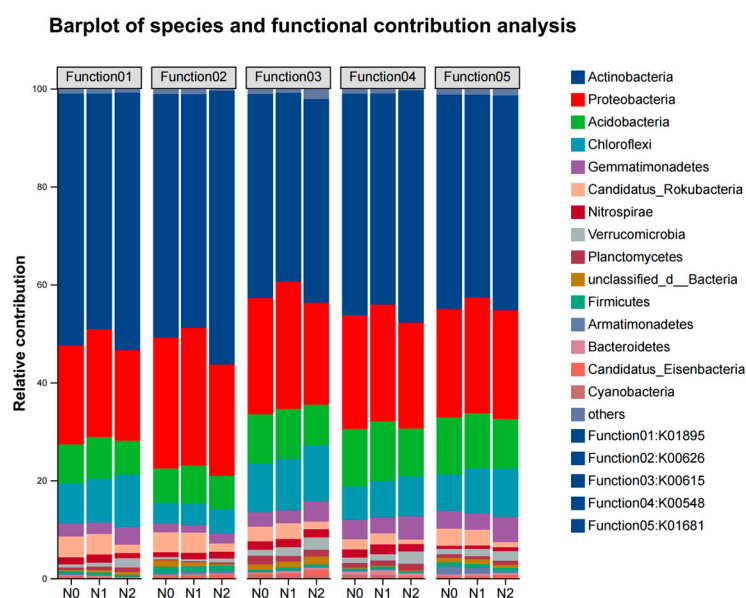


Figure A6. Bar plot of species and functional contribution analysis.

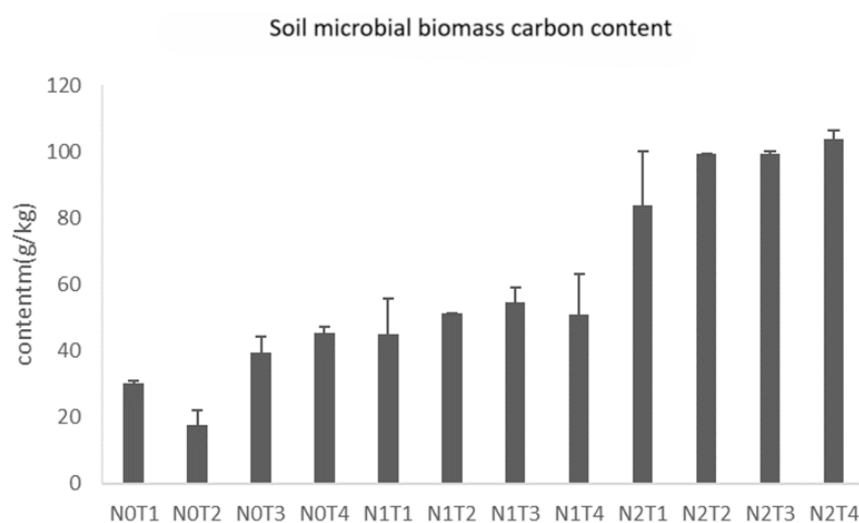


Figure A7. Soil microbial biomass carbon content under different treatments. Data represent the mean values for each treatment, with error bars indicating standard errors.

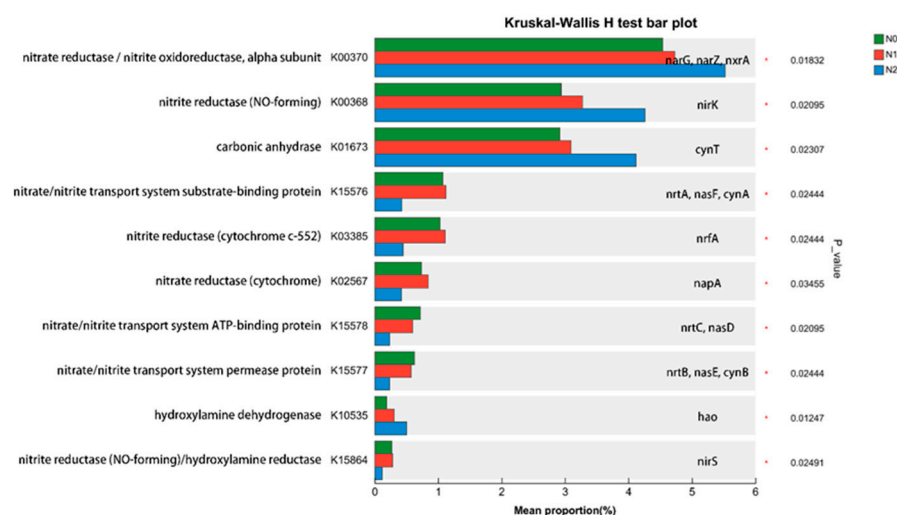


Figure A8. Differences in the abundance of soil microbial nitrogen metabolism-related enzymes under different nitrogen application treatments. * indicates a significant difference ($p_value < 0.05$).

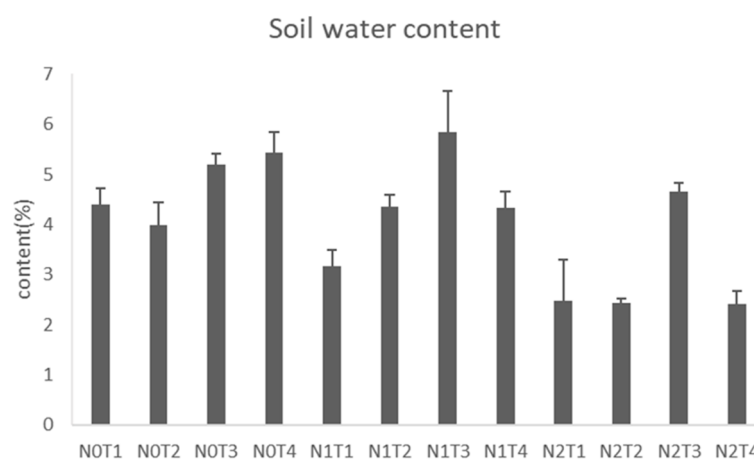


Figure A9. Soil water content under different treatments. Data represent the mean values for each treatment, with error bars indicating standard errors.

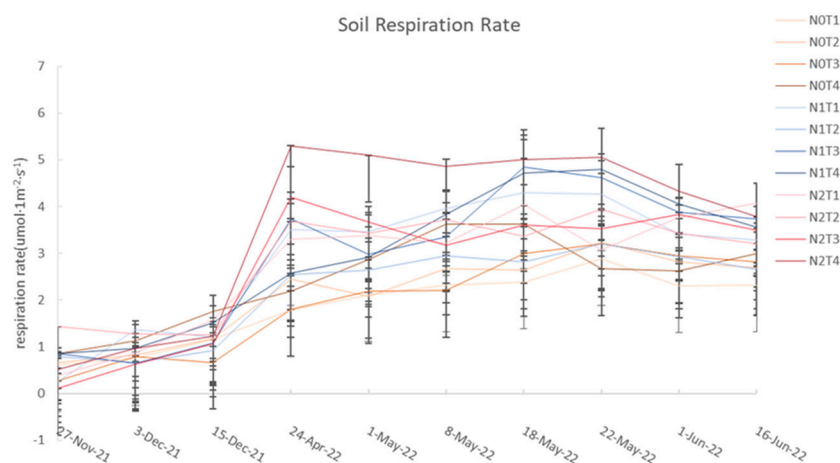


Figure A10. Soil respiration rate under different treatments and on different dates. Data represent the mean values for each treatment, with error bars indicating standard errors.

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