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Nitrogen Enrichment Regulates the Changes in Soil Aggregate-Associated Bacterial Community: Evidence from a Typical Temperate Forest

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Abstract: The nitrogen (N) enrichment induced by atmospheric N deposition affects both soil physicochemical properties and bacterial communities. However, how N enrichment affects soil aggregate-associated bacterial communities remains largely unclear. In this study, we conducted a two-year N addition experiment (four N levels: 0, 5, 10, and 20 g N m^{-2} $year^{-1}$, corresponding to normal N, low N, medium N, and high N, respectively) in a *Quercus liaotungensis* Koidz–dominated forest. The distribution, nutrient content, and bacterial community composition of the soil aggregates were measured under various N enrichment conditions. N enrichment changed the aggregate distribution, increased the content of nutrients in aggregates, and altered the aggregate-associated bacterial community composition. N enrichment reduced the complexity of the bacterial co-occurrence network and degraded the interactions between bacteria compared with those observed under the normal N level. Aggregate-associated bacterial community was determined to be primarily affected by N enrichment level but not by aggregate size. The litter properties are the key factors affecting the composition of bacteria in aggregates. These findings improve our understanding of aggregate-associated bacterial responses to N enrichment and the related influencing factors.

Keywords: N enrichment; natural forest; soil aggregate; litter; soil bacterial community

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1. Introduction

During the past few decades, atmospheric nitrogen (N) deposition has been steadily increasing owing to the widespread application of agricultural fertilizers and extensive burning of fossil fuels [1]. As one of the major regions contributing to escalating N deposition levels, China has exhibited a notably high growth rate in N deposition. For example, N deposition in China reached 21.1 kg N ha^{-1} $year^{-1}$ in the early 21st century, which is 60% higher than that observed in 1980 (13.2 kg N ha^{-1} $year^{-1}$) [2,3].

Soil microorganisms are vital to ecosystem functions and activities, such as nutrient cycling and soil organic matter decomposition [4,5]. As ubiquitous microorganisms in forest soils, bacteria play a crucial role in decomposing organic matter in forest ecosystems [6,7]. N deposition provides essential N for bacterial growth and can alter the environmental conditions of the soil, which may affect bacterial communities in the soil [8]. The response of bacterial communities to an increase in N deposition may seriously impact global carbon (C), N cycles, and climate change [9,10].

N deposition affects the activity and composition of bacterial communities in the soil [8,11]. These alterations may lead to an increase in the Shannon index and a decrease

in the Chao1 index of bacteria in forest ecosystems [9]. Specifically, N deposition may cause changes in the bacterial community by altering the relative abundance of bacteria. For example, a substantial increase in the relative abundance of copiotrophic taxa, such as Proteobacteria and Bacteroidetes, has been observed in soil with high N deposition [12,13]. Moreover, N deposition can alter the composition of microbial communities by increasing soil N availability [9]. N deposition may have no significant effect on bacterial alpha diversities, but it affects the relative abundance of some sensitive microbial genera [14]. Although various prior studies have evaluated the effects of N deposition on microorganisms, the specific effects of N enrichment on bacterial growth, function, and community composition in forest ecosystems remain unclear.

As the basic element of soil structure, soil aggregates provide a spatially heterogeneous microhabitat for soil microorganisms [15] and are one of the main factors regulating microbial community composition [16]. Within these microhabitats, differences in the quantities and properties of organic substrates may be the primary drivers of the corresponding differences observed in microbial communities [9]. The effect of N enrichment on soil aggregation varies depending on the ecosystem type and fertilization method [17]. N enrichment was found to change the aggregate distribution in different ecosystems; it either significantly improved aggregate stability or had no significant effect [17]. N enrichment also alters the elemental stoichiometry of soil aggregates, leading to changes in the soil bacterial community [18]. Another study demonstrated that differences in clay content can alter the bacterial community structure in soil aggregates of different sizes [19]. Notably, the niche heterogeneity in different aggregates is an essential reason for the variation in soil microbial community composition with aggregate particle size [20]. The soil aggregate distribution indirectly affects soil organic carbon (SOC) mineralization by regulating the community composition of bacteria and fungi [21,22]. Increases in soil aggregation may impede the microbially mediated breakdown of soil organic matter [17]. However, research on microbial communities within aggregates is scant despite their essential roles in soil biogeochemical cycling and SOC sequestration.

Although various studies have investigated the effects of N deposition on soil aggregates and their associated bacterial communities, how N enrichment specifically affects these soil aggregate-associated bacterial communities remains unclear. Prior research conducted in northeastern China revealed that aggregate size and fertilization significantly affect soil bacterial community structure and that fertilization has a more significant effect on bacteria than aggregate size [18]. Long-term N application can significantly increase the relative abundance of Proteobacteria and complicate the co-occurrence network of bacteria and fungi in aggregates [23]. However, another study suggested that the co-occurrence network can be simplified under N enrichment [24]. Additionally, a study on soil enzymes in aggregates from grasslands suggested that increasing N and water input results in a large redistribution of bacteria in soil particles of different sizes [25]. Nonetheless, further information on soil aggregate-associated bacterial communities in forest soils under N enrichment is required.

The Loess Plateau, China, the world's largest loess deposit in depth and area, is ecologically fragile and vulnerable to global environmental changes such as increasing N deposition [26]. *Quercus liaotungensis* Koidz is the dominant species in deciduous broadleaved forests in northern China [27]. In this study, we conducted simulated N addition experiments and in situ microbial cultivation of soil aggregates in a *Q. liaotungensis* Koidz natural forest on the Loess Plateau. The objectives were to (i) analyze the changes in soil aggregates and soil aggregate-associated bacteria under different N enrichment levels, (ii) determine whether N enrichment or aggregate size is the main factor affecting bacterial communities, and (iii) identify the main environmental factors affecting bacterial communities within soil aggregates. We hypothesized that an increase in N enrichment will affect the aggregate microhabitat by altering the physical and chemical properties of the soil, litter, and fine roots, thereby affecting soil aggregate-associated bacterial community composition.

2. Materials and Methods

2.1. Study Area

The study site is located in the Lianjiabian Forest Farm (35°03′–36°07′ N, 108°10′–109°18′ E) in Heshui Country, Gansu Province, China. This site has a typical hilly–gully loess topography, with an altitude of 1211–1453 m. With a mean annual temperature of 10 °C and a mean annual precipitation of 587 mm, this region is considered to have a mid-temperate continental monsoon climate [28]. Additionally, there are 112–140 frost-free days per year. According to the USDA Soil Taxonomical System, the soil is classified as Inceptisols, i.e., loess, formed from primitive or secondary loess parent materials [29]. In this region, the climax vegetation is *Q. liaotungensis* Koidz forest [30]. In pioneer forests, *Betula platyphylla Suk* and *Populus davidiana* Dode are the most predominant groups. The main shrub species in this area is *Hippophae rhamnoides*, and the main herbaceous species are *Bothriochloa ischaemum* and *Lespedeza dahurica* [30,31].

2.2. Experimental Design

Nitrogen deposition in Gansu Province is approximately 27.2 ± 6.7 kg N ha^{-1} $year^{-1}$ [32]. In September 2015, a 160-year-old *Q. liaotungensis* Koidz forest was selected as the study site. The experiment comprised four N enrichment levels in triplicate—a non-treated control sample with normal N (CK), low N (5 g N m^{-2} $year^{-1}$; LN), medium N (10 g N m^{-2} $year^{-1}$; MN), and high N (20 g N m^{-2} $year^{-1}$; HN) levels. Specifically, the N supplementation involved the addition of granular urea (46% N), which is commonly used in N addition experiments. The surface application of granular urea may cause ammonia (NH_3) volatilization, and the actual N input level may be lower than the target simulated level [33]. A laboratory experiment demonstrated that approximately 10%–40% of the added urea was lost in the form of NH_3 in Aridic Calciustolls soils. Therefore, when determining the rate of nitrogen application, the amount of nitrogen applied should be appropriately increased according to the actual nitrogen deposition.

We determined the amount of N that should be added to the treatment groups by referring to the estimated mean N deposition rate in northern China, the literature, and the loss of urea during the addition process [34,35]. The corresponding concentrations of urea were sprayed onto the plot surface in September of each year. The normal N plot received the same amount of water as the other groups. All 12 plots were subjected to the same climate, stand age, and terrain conditions. Each plot was 10 × 20 m in size, arranged in a randomized block design, and separated from the others by a 10 m wide buffer strip.

2.3. Sample Collection

Two years after the initial treatment with N, in September 2017, all samples were collected before the N addition of that year. In each plot, an area of 1 × 1 m was randomly selected, and all litter samples were collected. Next, a 9 cm diameter root auger was used to collect fine roots from a depth of 0–20 cm. We then placed the litter and clean fine root samples into an oven at 105 °C, where they were blanched for 30 min before drying to a constant weight at 60 °C; the sample weight was then recorded before the samples were ground to determine their nutrient content.

Three undisturbed soil samples from a depth of 0–20 cm (with the litter layer removed) were randomly collected from each plot and placed in aluminum boxes for aggregate analysis. These samples were then carefully transported back to the laboratory. Compared with the wet sieving method, the dry sieving method has less effect on the microbial community, so we used the dry sieving method to divide aggregates into silt–clay (<0.053 mm), microaggregates (0.053–0.25 mm), small macroaggregates (0.25–2 mm), and large macroaggregates (>2 mm) [36]. The existing studies on soil aggregate-associated microorganisms mostly adopted the method of isolating aggregates and cultivating them in the laboratory. Notably, soil environmental conditions (e.g., temperature, moisture) considerably change under laboratory conditions; accordingly, the composition of microorganisms cultured in the

laboratory might differ from that of in situ soil. To further elucidate the characteristics of microorganisms in soil aggregates, we evaluated soil aggregate-associated bacterial communities using in situ cultivation. After sieving, distilled water was sprayed at 50% of the field capacity. Next, aggregates of different sizes were placed into corresponding 300-mesh cloth bags and buried at a depth of 0–20 cm in the soil layer of the sample plot; this in situ culture was conducted for two weeks, at which point the microhabitats produced were considered similar to those of natural soil aggregates. After in situ culture, aggregate samples were taken out quickly and placed in an incubator with dry ice to maintain a temperature < 4 °C. These samples were then transported to the laboratory, stored at −80 °C, and analyzed to determine soil microbial community indices.

2.4. Physical and Chemical Analyses

The following methods were used to determine the physicochemical properties of soil. The carbon content of plants and soils was measured using the dichromate oxidation calorific method [37], and total nitrogen (TN) content was determined using the Kjeldahl method. Next, the contents of ammoniacal nitrogen (NH_4^+-N) and nitrate nitrogen (NO_3^--N) were measured using a continuous flow autoanalyzer (AutoAnalyzer 3, Bran and Luebbe, Hamburg, Germany).

2.5. DNA Extraction, Sequencing, and Data Processing

Soil DNA was extracted using the E. Z. N. A. Soil DNA Kit (Omega BioTek, Norcross, GA, USA). The concentrations and purity of DNA samples were determined using 1% agarose gel electrophoresis. The V4-V5 hypervariable regions of the bacterial 16S rRNA gene were amplified by PCR using the primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 909R (5′-CCCCGYCAATTCMTTTRAGT-3′). The PCR procedure used in this study was as follows: initial denaturation at 94 °C for 5 min; 27 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s and a final extension at 72 °C for 7 min before holding at 4 °C. Sequencing was performed using the MiSeq 2500 platform (Illumina, Inc., San Diego, CA, USA).

Raw sequences were analyzed using QIIME v.1.8.0, and UCLUST v.1.2.22 was used to detect chimeras. After quality filtering and eliminating of chimeric sequences, high-quality sequences with 97% similarity were clustered into the same operational taxonomic units (OTUs) using UPARSE [38]. The taxonomic identity of bacteria was ascertained using the Silva reference database (http://www.arb-silva.de (accessed on 10 March 2023)) and RDP classifier (http://rdp.cme.msu.edu/ (accessed on 11 March 2023)) [39].

Three soil samples under each treatment were sequenced to reduce the impact of random errors in the sequencing process. A total of 3,393,823 high-quality sequences were obtained from 36 samples (4 N treatments × 3 soil aggregates of different sizes × 3 replications). High-quality reads were clustered into 13,225 bacterial OTUs. The OTUs were classified into 399 genera, 295 families, 168 orders, 139 classes, and 51 phyla.

2.6. Statistical Analyses

Soil aggregate stability was evaluated according to the mean weight diameter (MWD) and geometric mean diameter (GMD) [40,41]. These factors were calculated using the following equations:

$$MWD = \sum_{i=1}^{n} w_i X_i \quad (1)$$

$$GMD = exp\left[\frac{\sum_{i=1}^{n}(w_i \ln X_i)}{\sum_{i=1}^{n} w_i}\right] \quad (2)$$

where w_i is the percentage of soil aggregates of the i-th size class; n is the number of size fractions; and X_i is the mean diameter of the i-th size class (mm).

To determine the significant differences in aggregate distribution, stability, nutrient content, and bacterial α diversity among multiple groups, one-way analysis of variance (ANOVA) was conducted. Tukey-HSD was performed to conduct multiple comparisons with statistical significance. Stepwise regression was used to determine the relationships between dominant bacterial phyla and environmental factors. All data were checked for normality and homogeneity with Shapiro–Wilk and Leven's tests, respectively, using the SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). Histograms of bacterial abundance were generated using Origin 2022b (OriginLab Corporation, Northampton, MA, USA) to analyze the differences in bacterial abundance.

To determine the overall variance in bacterial composition, similarities in the OTU composition of bacterial communities under different N treatments were visualized, applying nonmetric multidimensional scaling (NMDS) using "metaMDS" in the "vegan" R package, according to pairwise Bray–Curtis dissimilarities. Next, permutational multivariate analysis of variance (PERMANOVA) was used to determine whether the bacterial community composition differed significantly between the groups. Bacterial co-occurrence networks were then built based on Spearman's rank correlation to assess the effect of N enrichment on soil aggregate-associated bacterial communities. To reduce the complexity of the dataset and strengthen the validity of network analysis, only OTUs with a relative abundance > 0.05% were selected. The "psych" R package was used to calculate Spearman's rank correlations (p) between the retained OTUs. Only relatively high correlations ($r > 0.8$ and $p < 0.01$) were included in the network produced. Finally, independent nodes (degree zero) were removed. Network visualization and calculation of network topology attributes were performed using Gephi v.0.10.1. The OTUs were represented by network nodes, with the edges linking the two nodes representing positive or negative connections. Correlations between the 16s OTUs and environmental properties were identified using Mantel tests (Spearman method) based on the "linkET" package. The partial least-squares path model (PLS-PM) was applied to clarify both the indirect and direct impacts of environmental factors on bacterial community composition using the "PLS-PM" package in R 4.2.3.

3. Results

3.1. Effects of N Enrichment on Soil Aggregate Distribution and Nutrient Content

The proportion of different soil aggregate sizes varied, with large macroaggregates accounting for 59.56%–68.45% of the total soil aggregate, followed by small macroaggregates and macroaggregates. The proportion of silt–clay fraction was the lowest, accounting for 1.83%–3.55% of the total soil aggregate (Figure 1a). LN treatment increased the proportion of microaggregates by 88.77% compared with that in the soil with a normal N enrichment level ($p < 0.05$). However, N enrichment exhibited no observable effect on the proportions of the silt–clay fraction, small macroaggregates, and large macroaggregates. Moreover, N enrichment did not have a significant effect on the MWD of soil aggregates, but it did have a significant effect on the GMD (Figure 1b,c). Specifically, the GMD under the HN treatment was 36.82% higher than that under the LN treatment (Figure 1c; $p < 0.05$). Finally, at all N enrichment levels, the SOC, TN, and inorganic nitrogen (IN) contents in aggregates of all sizes were found to increase with increasing N enrichment levels (Figure 2).

3.2. Effects of N Enrichment on Soil Bacterial Communities across Aggregate Size Fractions

Proteobacteria, Acidobacteria, Planctomycetes, Actinobacteria, Gemmatimonadetes, Bacteroidetes, and Chloroflexi were the predominant bacterial phyla in the collected soil samples, with a relative abundance > 5% in all samples (Figure 3a). The N enrichment level had a significant positive effect on the abundance of Planctomycetes and Bacteroidetes ($p < 0.05$). Additionally, the abundance of Proteobacteria significantly increased ($p < 0.05$) in small macroaggregates under N enrichment. N enrichment tended to increase the richness and Shannon indices of bacteria in all aggregates (Figure 3b,c).

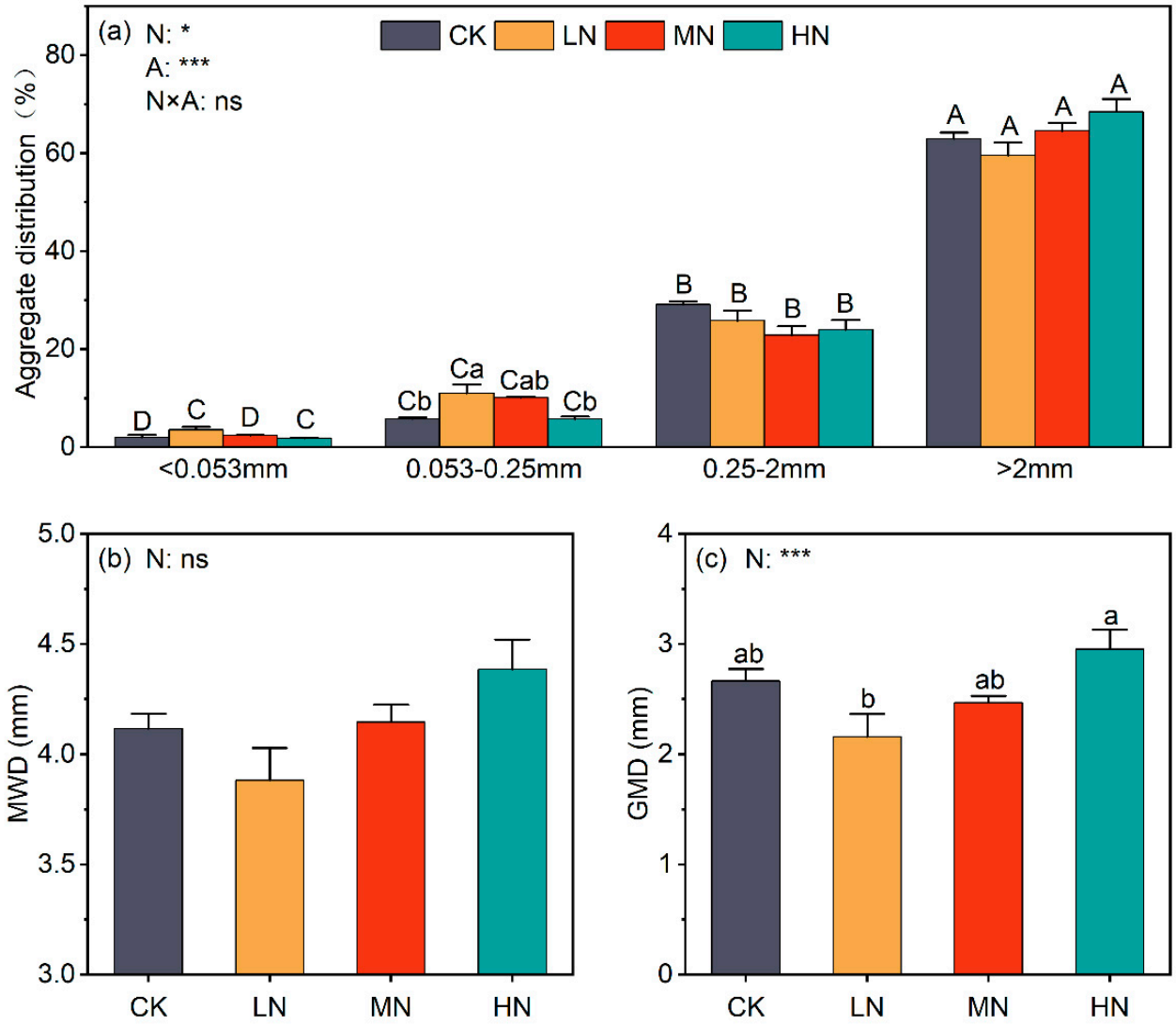

Figure 1. The soil aggregate distribution (**a**), MWD (**b**), and GMD (**c**) under different N enrichment levels. MWD, mean weight diameter. GMD, geometric mean diameter. Different capital letters indicate significant difference among aggregate sizes; different lowercase letters indicate significant difference among N enrichment levels ($p < 0.05$). Error bars indicate the standard error. * $p < 0.05$, *** $p < 0.001$, ns non-significant.

Soil bacterial communities in aggregates were clearly clustered at each N enrichment level (Figure 4) but were similar among the aggregate sizes. Significant differences were observed between the bacterial communities of any two N enrichment levels ($p < 0.01$), except in the soil with the normal N enrichment level and the LN level (Table S1). According to the classification of N levels, the data of bacterial community composition in different size aggregates were analyzed together to plot the co-occurrence network. The co-occurrence network also changed significantly with changes in N concentration (Figure 5). Moreover, the proportion of positive links in this co-occurrence network was higher than that of negative links. Additionally, compared with those in the soil with normal N enrichment levels, the number of edges, average degree, network density, and average clustering coefficient under N enrichment decreased in the co-occurrence network (Table S2).

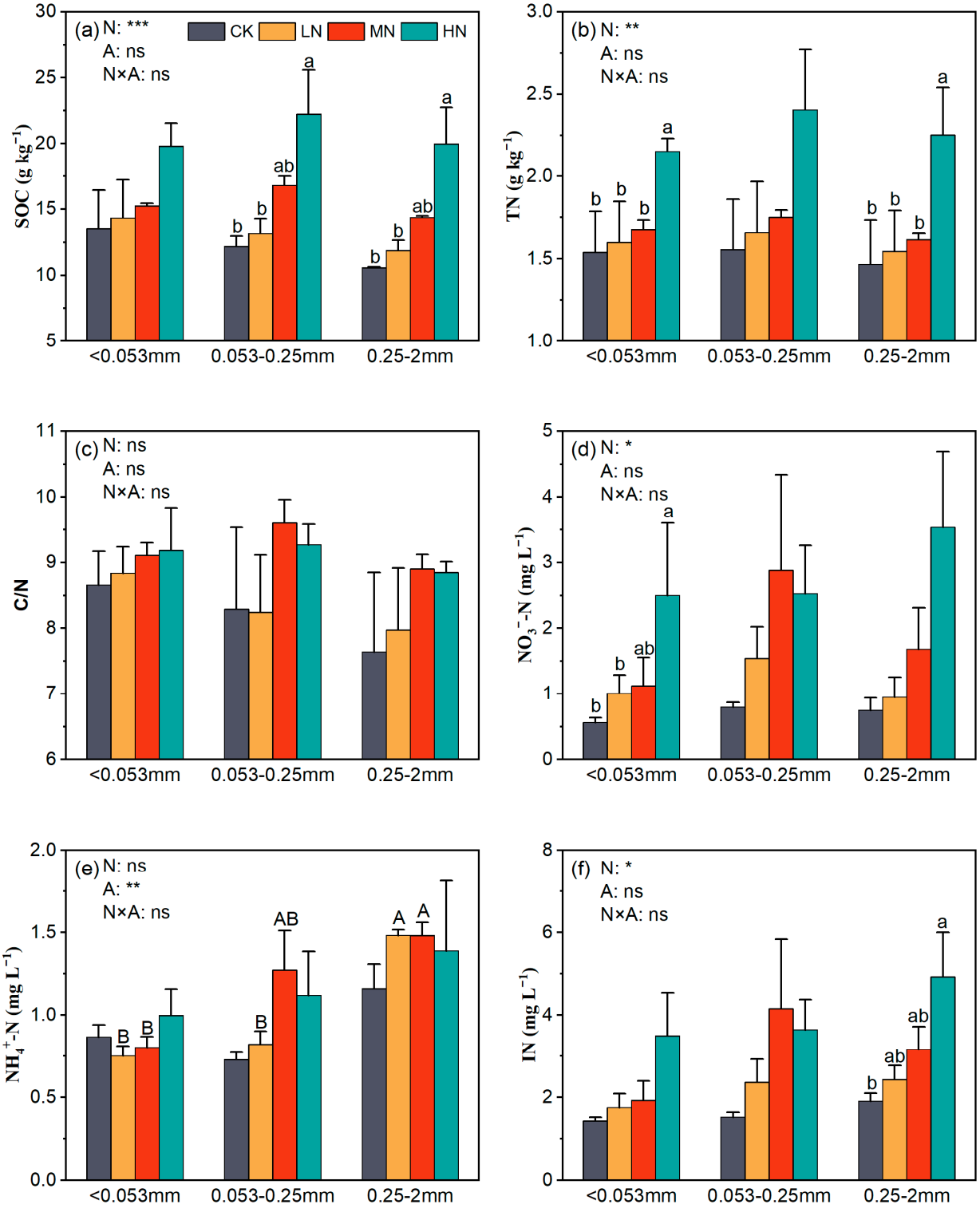

Figure 2. Distributions of SOC (**a**), TN (**b**), C/N (**c**), NO_3^--N (**d**), NH_4^+-N (**e**), and IN (**f**) in soil aggregates under different N enrichment levels. Different capital letters indicate significant difference among aggregate sizes; different lowercase letters indicate significant difference among N enrichment levels ($p < 0.05$). SOC, soil organic carbon. TN, total nitrogen. C/N, SOC/TN ratio. NO_3^--N, nitrate nitrogen. NH_4^+-N, ammoniacal nitrogen. IN, the sum of NO_3^--N and NH_4^+-N. Error bars indicate the standard error. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns non-significant.

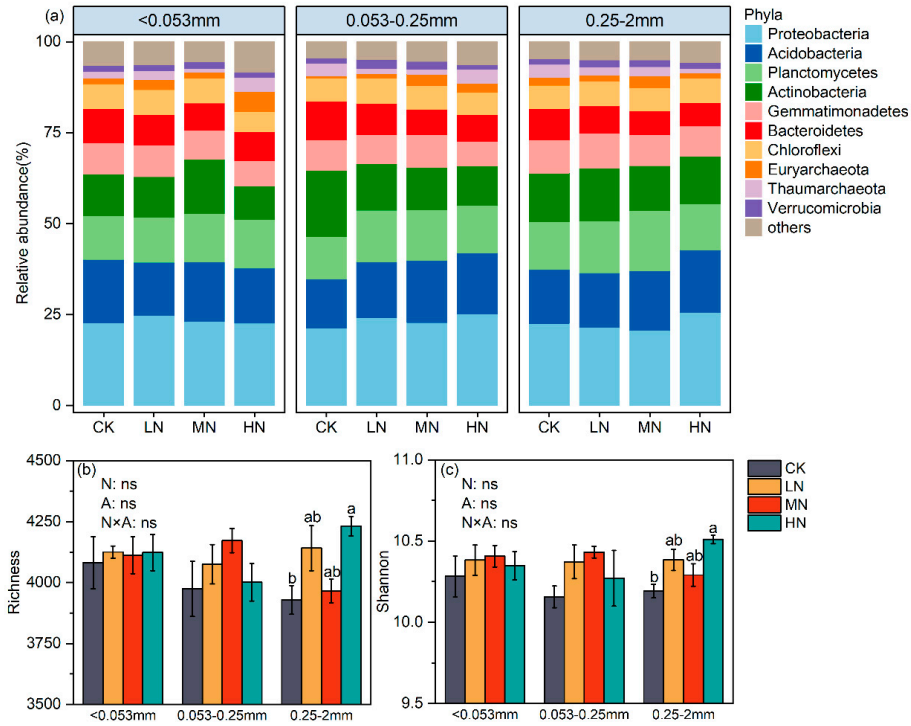

Figure 3. Relative abundance of the soil aggregate-associated bacterial communities at the phylum level under N enrichment (**a**), bacterial community richness (**b**), and Shannon (**c**) in soil aggregates under different N enrichment levels. Different lowercase letters indicate significant difference among N enrichment levels ($p < 0.05$). ns non-significant.

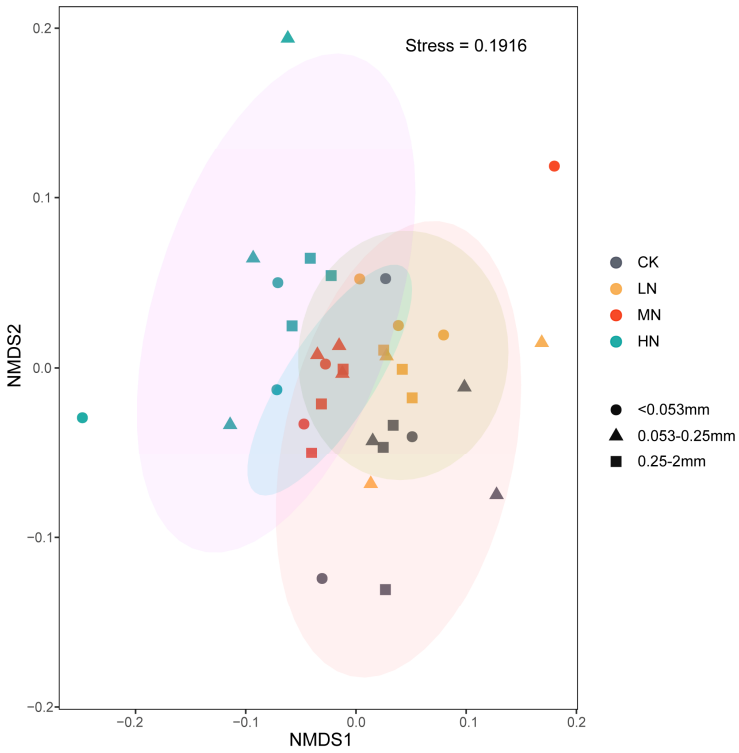

Figure 4. Nonmetric multidimensional scaling (NMDS) based on Bray–Curtis dissimilarity displays soil bacterial communities in aggregates of different sizes under different N enrichment levels.

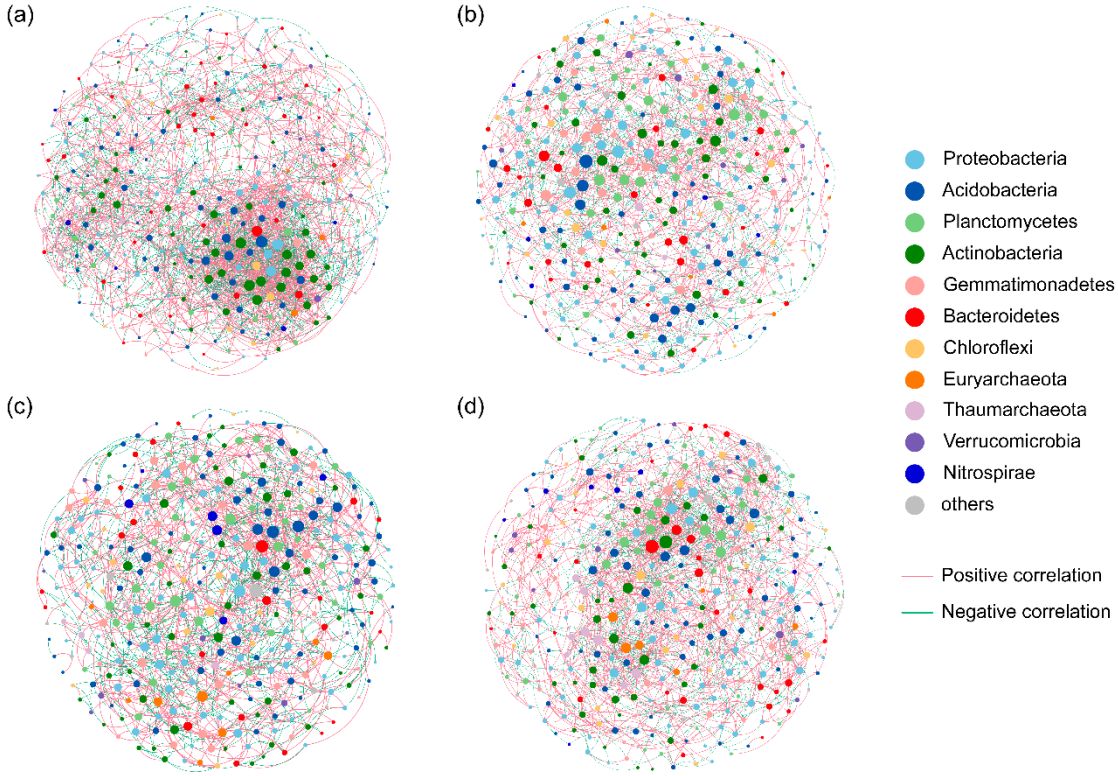

Figure 5. The networks visualize the co-occurrence patterns of soil aggregate-associated bacterial community under CK (**a**), LN (**b**), MN (**c**), and HN (**d**) treatments at operational taxonomic unit (OTU) level. Each node represents one OTU; the node size represents the degree and the node color represents the bacterial phylum to which it belongs. The edges are colored according to interaction types; positive correlations are labeled with red and negative correlations are green.

3.3. Relationship between Soil aggregate-Associated Bacteria and Environmental Factors under N Enrichment

SOC, TN, litter biomass (LB), and fine root biomass (RB) were significantly linked to bacterial community composition (Figure 6; $p < 0.05$); SOC and LB had the greatest influence on the bacterial communities in soil aggregates. In order to explore the environmental factors affecting the dominant bacteria, stepwise regression analysis was carried out, which further indicated that the abundance of Acidobacteria was positively correlated with RN; the primary environmental factors affecting the abundance of Planctomycetes were GMD and RN; the abundance of Actinobacteria was negatively correlated with SOC; the main factor affecting the abundance of Bacteroidetes was the litter carbon/nitrogen ratio (LC/N); the abundance of Chloroflexi was negatively correlated with TN (Table 1); and the abundance of Verrucomicrobia was negatively correlated with GMD.

Table 1. Stepwise regression of relative abundance of dominant bacteria at phylum level with environmental factors in soil aggregates.

Dependent Varibles	Regression Equation	R^2	*p*	n
Acidobacteria	Y = 0.113 + 0.029RN	0.121	<0.05	36
Planctomycetes	Y = 0.120 − 0.019GMD + 0.040RN	0.350	<0.01	36
Actinobacteria	Y = 0.174 − 0.003SOC	0.176	<0.05	36
Bacteroidetes	Y = 0.019 + 0.00022LC/N	0.308	<0.001	36
Chloroflexi	Y = 0.079 − 0.008TN	0.237	<0.05	36
Verrucomicrobia	Y = 0.032 − 0.006GMD	0.143	<0.05	36

Note: GMD, geometric mean diameter. SOC, soil organic carbon. TN, total nitrogen. LC/N, litter organic carbon: litter total nitrogen ratio. RN: fine root nitrogen.

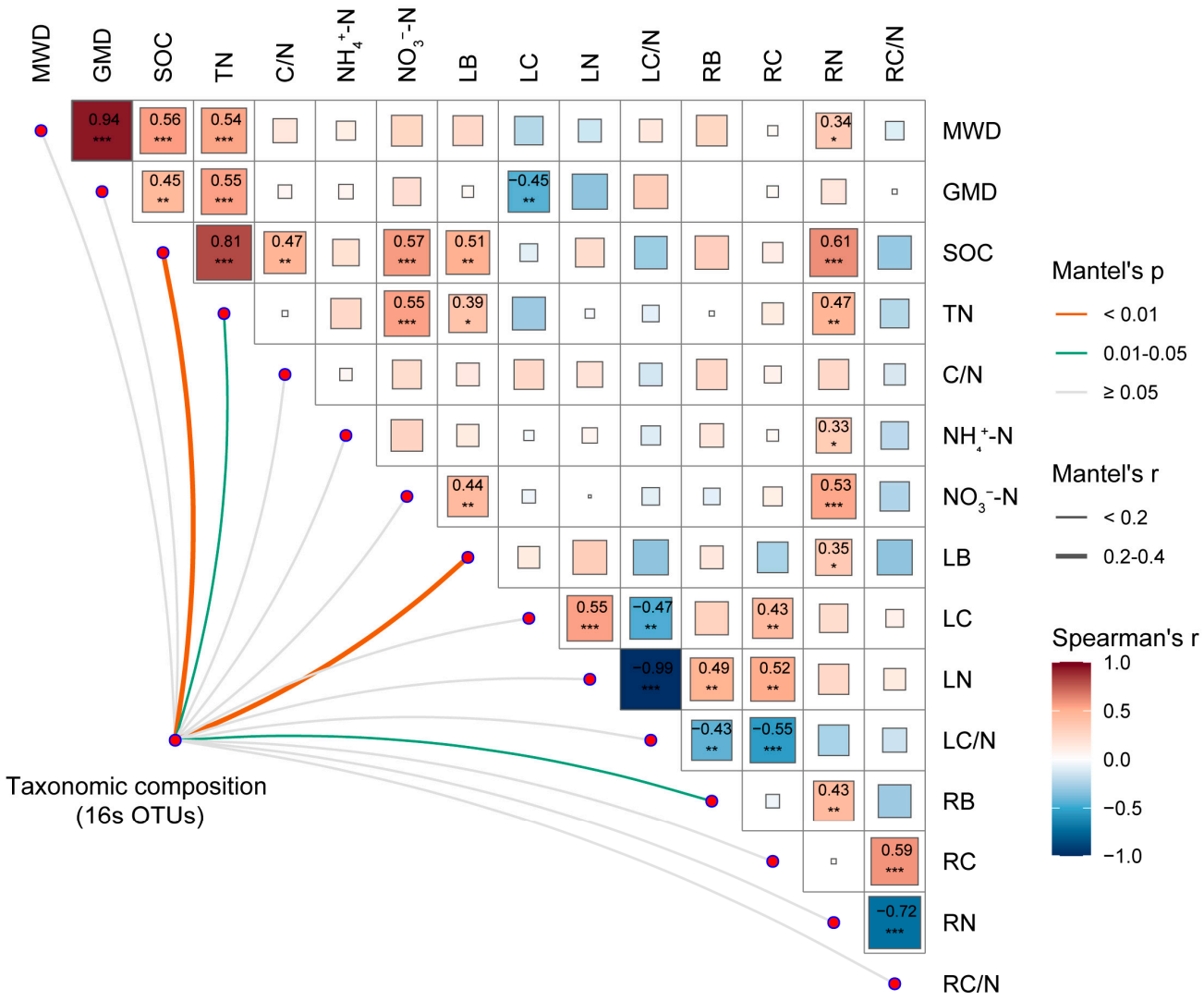

Figure 6. Environmental drivers of soil aggregate-associated bacterial community composition. Edge width corresponds to the Mantel's r statistic for the corresponding distance correlations, and edge color denotes the statistical significance based on 9999 permutations. MWD, mean weight diameter. GMD, geometric mean diameter. SOC, soil organic carbon. TN, total nitrogen. C/N, SOC/TN ratio. NO_3^--N, nitrate nitrogen. NH_4^+-N, ammoniacal nitrogen. IN, the sum of NO_3^--N and NH_4^+-N. LB, litter biomass. LC, litter organic carbon. LN, litter total nitrogen. LC/N, LC/LN ratio. RB, fine root biomass. RC, fine root organic carbon. RN, fine root total nitrogen. RC/N, RC/RN ratio. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

PLS-PM was used to disentangle the direct and indirect relationships between aggregated-associated bacterial community composition and environmental factors, and the explanation rates of variance were 60% (Figure 7). N enrichment had a significant positive effect on litter properties, and litter properties and SOC had significant positive effects on aggregated-associated bacterial community composition. The total standardized effects of litter properties, N enrichment, aggregate stability, SOC, root properties, and TN on soil aggregate-associated bacterial community composition were 0.56, 0.50, 0.40, 0.34, 0.14, and −0.30, respectively.

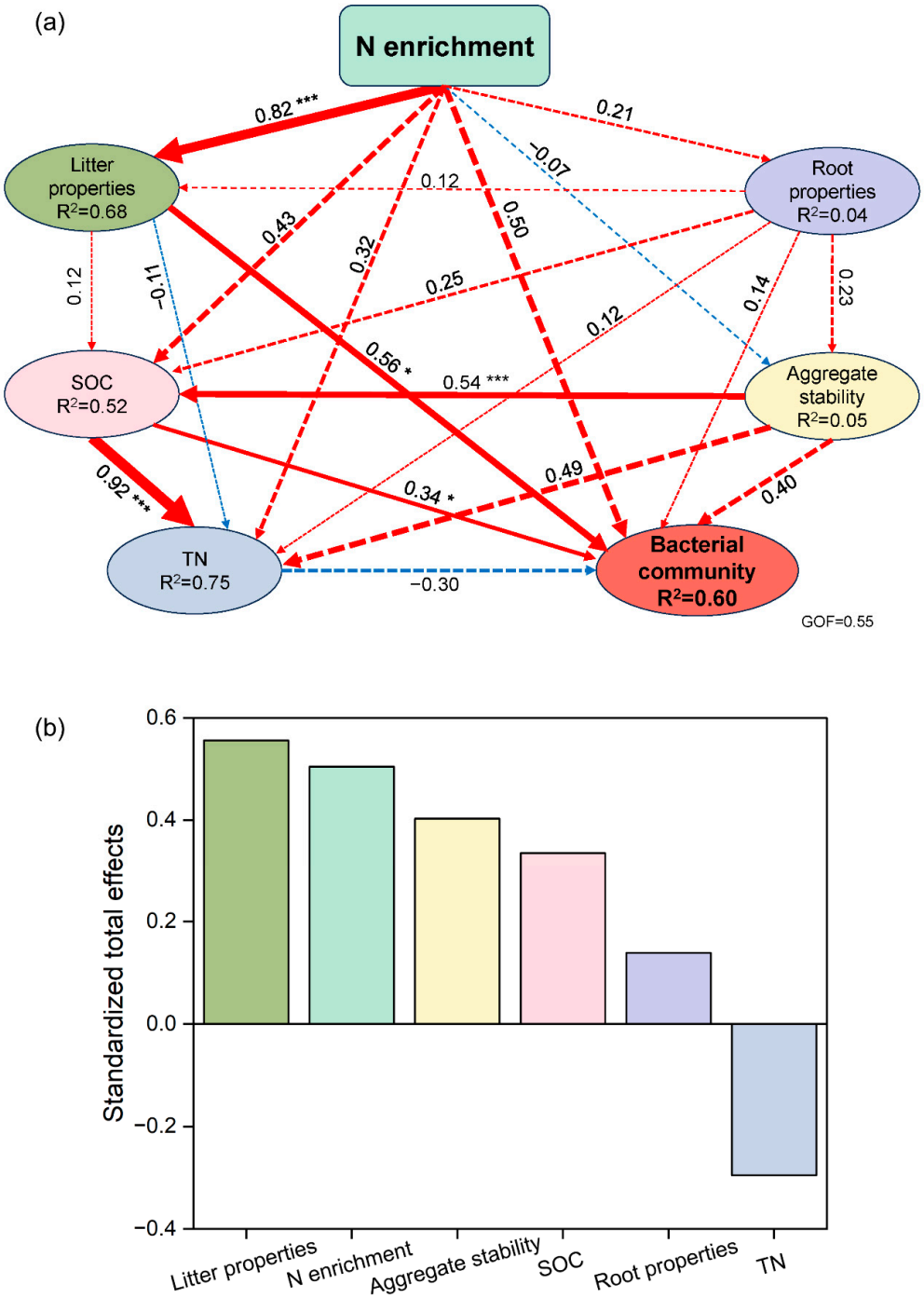

Figure 7. The partial least-squares path model (PLS-PM) evaluating the effects of environmental factors on soil aggregate-associated bacterial community composition under N enrichment (**a**) and standardized total effects of environmental variables derived from the PLS-PM (**b**). The red and blue lines indicate significantly positive and negative coefficients; the solid lines and dashed lines denote significant paths ($p < 0.05$) and non-significant paths ($p > 0.05$), respectively; the arrow weights are proportional to standardized coefficients. R^2 values represent the proportion of variance explained for each endogenous variable. Litter properties, litter biomass, and litter C/N ratio; root properties, root biomass, and root C/N ratio. SOC, soil organic carbon; TN, total nitrogen; Aggregate stability, geometric mean diameter, and NMDS1 and NMDS2 scores as used proxies for bacterial community composition. * $p < 0.05$, *** $p < 0.001$.

4. Discussion

4.1. Effects of N Enrichment on Soil Aggregate Stability and Soil Properties

N enrichment is an important factor influencing aggregate stability and nutrient content in the soil [42,43]. In the present study, the GMD under the HN level was significantly higher than that under the LN level (Figure 1c). This result may be explained by the increasing proportion of large macroaggregates within the soil [44]. For example, there was a greater proportion of large macroaggregates under the HN treatment than under the LN treatment; consequently, the GMD was significantly greater under the HN level. However, compared with that in the soil with normal N enrichment level, MWD was not significantly affected by any of the three N enrichment treatments (Figure 1b). One meta-analysis study demonstrated that N enrichment significantly enhances MWD in cropland systems but does not significantly affect MWD in forests and grasslands; this finding was attributed to the understanding that the dense roots of perennial plants in forests or grasslands may leave little space for root growth [45].

In the present study, N enrichment affected all the nutrient indices measured (Figure 2); SOC content, in particular, was affected by N enrichment, which is consistent with the results of previous studies [43,46]. Specifically, N enrichment enhanced the growth of LB, thereby increasing the carbon input to the soil (Figure S1). This indicates that N enrichment considerably contributes to SOC content. N enrichment is generally believed to promote SOC accumulation mainly via the following strategies: first, it enhances plant growth and litter accumulation, leading to increased SOC input; second, it inhibits the decomposition and mineralization of organic matter, promoting SOC accumulation [17]. However, how N deposition definitively affects SOC remains unclear. Different findings have been obtained depending on the soil type, plant community, and experimental setting [17,45]. This process depends on the balance between net primary production and the decomposition of soil organic matter [47].

The TN, NO_3^--N, and NH_4^+-N contents in all sized aggregates increased in N-enriched soil compared with the normal N enrichment level (Figure 2d–f). These results are consistent with those of previous studies [8,48]. Soil microorganisms can decompose organic N into NH_4^+-N via ammoniation; furthermore, ammonia or ammonium ions in the soil are eventually oxidized to NO_3^--N by nitrifying bacteria. The increased content of NH_4^+-N and NO_3^--N indicates that exogenous N input enhances N availability by promoting the nitrification and ammoniation of soil [49].

4.2. Effects of N Enrichment on Soil aggregate-Associated Bacterial Communities

In the present study, Proteobacteria and Acidobacteria were the dominant phyla across N treatments, which aligns with previous studies on temperate forest soils [50,51]. HN enrichment significantly increased the abundance of Proteobacteria in the small macroaggregates (Figure 3a). Proteobacteria are typical eutrophic bacteria that are sensitive to environmental changes. The greater the abundance of environmental resources, the better the survival and development of Proteobacteria [23]. In aggregates of different sizes, N enrichment altered bacterial abundance and enhanced Shannon diversity (Figure 3). Prior experimental studies and meta-analyses have also demonstrated that N enrichment has positive effects on bacterial diversity in forest ecosystems [13,52]. In the present study, N enrichment enhanced N availability for microorganisms by increasing the contents of TN, NO_3^--N, and NH_4^+-N (Figure 2). In addition, N enrichment increased litter quantity and RB (Figure S1). N enrichment has been shown to increase nutrient availability by promoting plant productivity, which indirectly increases bacterial abundance and diversity [53].

The impact of aggregate size on the bacterial community was negligible compared with the large impact of N addition on bacterial community composition. Similarly, a previous study indicated that fertilization and aggregate size fractions have different effects on bacterial community composition, with fertilization being the primary influencing factor [18]. The reason for this phenomenon may be that short-term N application has a greater effect on soil nutrients but has a little influence on aggregates. The present study

also established that the higher the N enrichment level, the greater the difference between the corresponding bacterial community composition at the N enrichment level and that at the normal N enrichment level (Figure 4, Table S1). This finding indicates that the soil aggregate-associated bacterial community composition can significantly change in response to N enrichment [11,54]. This was attributed to the increased N enrichment level enhancing the availability of soil nutrients (Figure 2).

The bacterial co-occurrence networks constructed in this study were primarily positively correlated (Figure 5), indicating that these bacteria act cooperatively [55]. The topological characteristics of bacterial co-occurrence networks were altered by N enrichment. Compared with that in the soil with a normal N enrichment level, N enrichment reduced the network density, average degree, and average clustering coefficient of the co-occurrence networks (Figure 5; Table S2); that is, the association between bacterial communities was reduced under N enrichment. Therefore, strengthened interactions between soil aggregate-associated bacteria may occur at the normal N enrichment level. According to the streamlining theory [55], the intensification of resource competition stimulates increased interactions between microbial groups. These microbial interactions were diminished under N enrichment. This result may be attributed to the abundance and accessibility of nutrients, which may result in the formation of a relatively simple network of bacterial communities. Notably, another study on the bacterial community in the rhizosphere of poplar saplings also demonstrated that the co-occurrence network could be decentralized and simplified by adequate N supply [24], which further confirms the results of this study.

4.3. Major Environmental Factors Affecting Soil aggregate-Associated Bacterial Communities under N Enrichment

N enrichment affects bacterial communities by altering soil physicochemical properties and aboveground plant communities [56]. In the present study, SOC, TN, LB, and RB significantly affected bacterial communities (Figure 6). These environmental variables were considered potential key factors that may affect soil aggregate-associated bacterial communities under N enrichment. The results of PLS-PM further indicate that compared with the effect of aggregates on bacteria, N enrichment has a greater effect on bacterial community composition (Figure 7). Litter property was a critical factor affecting the bacterial community composition in aggregates. In the present study, N enrichment increased soil C input by increasing LB and improving litter quality via an increase in N content and reduction in litter C/N (Figure S1). These results were supported by previous research [57,58]. Additionally, litter quality affects the relative abundance of specific taxa involved in the decomposition process, as does the diversity of bacterial communities [57]. Therefore, alterations in the structure of litter quality may affect bacterial community composition [59]. Specifically, high-quality litter is more easily used by bacterial communities than low-quality litter, which accelerates the turnover of bacterial biomass and the nutrient cycle [60,61].

In this study, it was found that aggregate stability significantly affected SOC content and further affected the composition of the bacterial community (Figure 7). Soil aggregates play a crucial role in soil organic carbon (SOC) turnover [23]. Aggregate stability is closely correlated with the relative abundance of bacteria [62]. The spatial heterogeneity of available resources of different aggregate sizes has a strong selection effect on the life strategy of bacteria, and bacterial secretions in turn affect the binding of substances in aggregates [63]. Changes in aggregate-associated SOC content have been significantly linked to bacterial abundance because the SOC content in soil particles influences the availability of nutrients and habitats [64]. N enrichment increases SOC and promotes microbial growth [65,66]. The effects of root properties on bacterial composition were found to likely be indirectly influenced by soil nutrients and other factors (Figure 7). Previous studies have established that N enrichment promotes the production of C-containing root exudates by stimulating the production of RB, which is conducive to SOC accumulation [65,66]. N enrichment indirectly affects microbial composition by altering the availability of C resources in the soil

environment. Plant roots also promote the formation of aggregate structures by producing substances that stabilize soil particles; these aggregates produce specific habitats for soil microorganisms, thereby affecting microbial composition [67]. As one of the primary nutrients in soil, TN affects the abundance and activity of microorganisms [18]; consequently, it is considered a key factor responsible for driving changes in bacterial communities [68]. N enrichment significantly increases the contents of SOC and TN, stimulates the growth and activity of microorganisms, and alters bacterial community structure [69]. In this study, TN had a negative effect on the bacterial community in aggregates, which may be because a high N content is unfavorable to the activities of some bacteria, such as Chloroflexi (Table 1).

Moreover, aggregate stability, soil properties, and plant properties were found to affect the dominant bacteria phyla in soil aggregates (Table 1). Specifically, aggregate stability and RN increased the abundance of Planctomycetes, which aligns with the understanding that Planctomycetes are often associated with plant roots [70]. Additionally, the abundance of Bacteroidetes was strongly affected by LC/N. As the LC/N ratio increases, litter quality deteriorates, and decomposition becomes more challenging; consequently, the relative abundance of Bacteroidetes increases. This can be attributed to the understanding that, regardless of the habitat, exogenous complex organic substances primarily consist of organic polysaccharides, and Bacteroidetes play an important role in the decomposition of these substances [71]. A negative association was observed between Chloroflexi and TN. This result was attributed to Chloroflexi being oligotrophic bacteria, which thrive in low-nutrient environments. By contrast, nutrient-rich environments caused by an increase in N are conducive to the growth of copiotrophic bacteria, which exhibit higher growth rates than slow-growing oligotrophic taxa [12].

The limitation of this study lies in the short N application time. To further improve the understanding of the changes in microorganisms in aggregates under N enrichment, it is necessary to extend the experiment time to observe the long-term effects of N enrichment in future studies.

5. Conclusions

N enrichment changed the distribution of silt–clay fractions and microaggregates, increased the nutrient content within these aggregates, and altered the aggregate-associated bacterial community composition. N enrichment treatment improved the availability of nutrients compared with a normal N enrichment level, thus reducing the resource competition among bacteria and reducing the complexity of the bacterial co-occurrence network. N enrichment has a larger impact on the composition of the bacterial community than aggregates do. The higher the N enrichment level, the greater the change in the bacterial community composition. Litter properties were key environmental factors affecting soil aggregate-associated bacterial communities. This study provides important data for improving our understanding of how N enrichment affects microorganisms at the soil aggregate level. In future studies, additional environmental parameters should be considered to comprehensively determine the factors that affect aggregate-associated bacterial communities.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f15010077/s1, Figure S1: distributions of LB (a), LC (c), LN (e), LC/N (g), RB (b), RC (d), RN (f), and RC/N (h) under different N enrichment levels. Table S1: permutational multivariate analysis of variance (PERMANOVA) of soil aggregate-associated bacterial communities under different N enrichment levels. Table S2: topological properties of soil aggregate-associated bacterial co-occurrence networks.

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